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THE F_0F_1-ATPase OF *ESCHERICHIA COLI:* IDENTIFICATION OF THE TRANSMEMBRANOUS PROTON PORE

by

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Statement

All the experimental work detailed in this thesis was performed by the author unless otherwise stated. Unless otherwise stated this thesis is all my own work,

Bob Lightowlers

PREFACE

An initial note is made regarding the nomenclature of both strains and plasmids used in this thesis. When characterising specific mutant strains, properties of the mutant are compared to both 'coupled' and 'uncoupled' control strains. These control and mutant strains are essentially isogenic but differ in the gene-product of interest. Thus, the coupled control strain synthesises normal copies of the gene-product of interest and is capable of oxidative phosphorylation, whilst the uncoupled control strain either does not synthesise any of the specific gene-product or synthesises a truncated gene product and is incapable of coupling oxidative phosphorylation. These strains are referred to in the text either with the suffix *unc*$^{+}$ or unc^{-} denoting the coupled or uncoupled control strains respectively, or with a suffix detailing both the relevant genotype of the parent strain and number of the plasmid carried in the strain.

The construction of plasmids carrying different base substitutions is detailed in each chapter. The gene containing the specific substitution is alloted an *unc* allele number. For ease of reference, strains carrying plasmids with base substitutions are identified by a strain number and a suffix referring to the amino acid substitution. The suffix utilises the single letter code for amino acids with the first letter and the following number referring to the amino acid in the primary sequence of the normal polypeptide followed by a letter representing the residue which has replaced the usual amino acid. For example, R210Q represents the replacement of an arginine residue at position 210 by a glutamine. The single letter amino acid code is given in the list of abbreviations.

Apologies are made for any inconvenience the reader experiences with this nomenclature and the reader is informed that a full strain and plasmid list detailing relevant genotypes is shown in Tables 2.1 and 2.2.

LIST OF ABBREVIATIONS USED

ATP	Adenosine 5'-triphosphate
ADP	Adenosine 5'-diphosphate
ATPase	Adenosine triphoaphatase
Pi	Inorganic phosphate
E.C.	Enzyme Commission
ECF_oF_1-ATPase	*Escherichia coli* F_oF_1-ATPase
MF_oF_1-ATPase	Mitochondrial F_oF_1-ATPase
CF_oF_1-ATPase	Chloroplast F_oF_1-ATPase
OSCP	Oligomycin sensitivity conferring protein
EDTA	Ethylenediaminetetra-acetic acid
NADH	Reduced nicotinamide adenine dinucleotide(DPN)
ΔpH	pH gradient across membrane
Δ	Electrical potential across the membrane
CCCP	Carbonylcyanide *m*-chlorophylhydrazone
DCCD	N, N'-dicyclohexylcarbodi-imide
EACA	ε-amino-n-caproic acid
PAB	*p*-aminobenzamidine
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid
Tes	2-((2-hydroxy-1,1-bis(hydroxymethyl)ethyl) amino) ethanesulphonic acid
EGTA	ethyleneglycol-bis-(2-amino ethylether)N-N'-tetra-acetic acid
Tris	Tris(hydroxymethyl)aminoethane
2,3-DHB	2, 3-dihydroxybenzoic acid
DTT	D, L-1,4-dithiothreitol
Bisacrylamide	N, N'-methylene bisacrylamide
SDS	Sodium dodecyl sulphate
Nonidet NP-40	Nonidet NP-40; octyl phenoxy-polyethoxy-ethano containing 7-9 mols/mol of ethoxy linkages
TEMED	N, N, N', N', -tetramethylethylenediamine
UV	Ultraviolet
kb	Kilobase pairs
PEG 6000	Poly(ethylene glycol) average M_r 6000
dGTP	2'-deoxyguanosine 5'-triphosphate
didGTP	2'-3'-dideoxyguanosine 5'-triphosphate
dATP	2'-deoxyadenosine 5'-triphosphate
didATP	2'-3'-dideoxyadenosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
didTTP	2'-3'-dideoxythymidine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
didCTP	2'-3'-dideoxycytidine 5'-triphosphate

ABBREVIATIONS FOR AMINO ACIDS

AMINO ACID	THREE LETTER ABBREVIATION	ONE LETTER SYMBOL
Alanine	Ala	A
Arginine	Arg	R
Asparginine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Finally, I hesitate before recognising the contribution of my parents as their encouragement, altruism and loyalty has been of such magnitude that I feel inadequate in attempting to thank them for it.

ABSTRACT

This thesis investigates the energy-transducing F_oF_1-adenosine triphosphatase (ATPase, E.C. 3.6.1.3) and attempts to delineated the residues responsible for facilitating proton flux through the F_o-moiety of the complex. A model for the mechanism of ATP synthesis was proposed by Cox *et al.* (1984, 1986). The model described putative secondary structures for each of the three F_o-sector polypeptides and highlighted an amphipathic intramembranous helix, helix IV, in subunit-*a* which was highly conserved in F_oF_1-ATPase subunit-*a* analogues from all species sequenced. It was proposed that protons flowing down a respiration-driven pH gradient could drive the endergonic synthesis of ATP at the F_1-sector by fuelling a rotation of an asymmetric inner core of minor subunits -γ, δ and ε, within a static hexamer of -α and -β subunits cycling three active sites through three successive conformers exhibiting differing binding affinities for substrate and product based on the proposal of Cross (1981). This rotation is affected by the minor subunits, bound to the cytoplasmic helices of the F_o-sector subunit-*b* dimer. The N-terminal helices of the subunit-*b* dimer are located within the membrane and with the five putative intramembranous helices of subunit-*a* constitute the rotator. The rotation of this inner core within the membrane is predicted to occur within an outer static ring of *c*-subunits mediated by a proton flux through successive residues on one helical face of the subunit-*a* amphipathic helix IV and residue Asp-61 of subunit-*c*.

This model was challenged by a combined biochemical and genetic approach. Specific amino acids implicated as integral members of the F_o-mediated proton pore were substituted by other residues with different physico-chemical properties. The resultant strain was then analysed for changes in growth and membrane-enzymic properties with the data obtained from each mutant strain being used to update the model and to elucidate those residues

involved in proton translocation. Mutant strains carrying the R210Q, H245L and E219Q substitutions in subunit-*a* were unable to grow on succinate as sole carbon source and had growth yields similar to the uncoupled control strains. The properties of membranes from these strains were essentially identical, with a lack of F_O-mediated proton translocation, proton impermeable membranes even after removal of the F_1-ATPase and ATPase activities resistant to the inhibitor DCCD. Furthermore, in these mutant strains, the F_1-ATPase activities were inhibited by approximately 50% when bound to the membrane. Several amino acid substitutions in subunit-*a* did not affect the coupling of oxidative phosphorylation and the implications with regard to the secondary structure of subunit-*a* are discussed.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

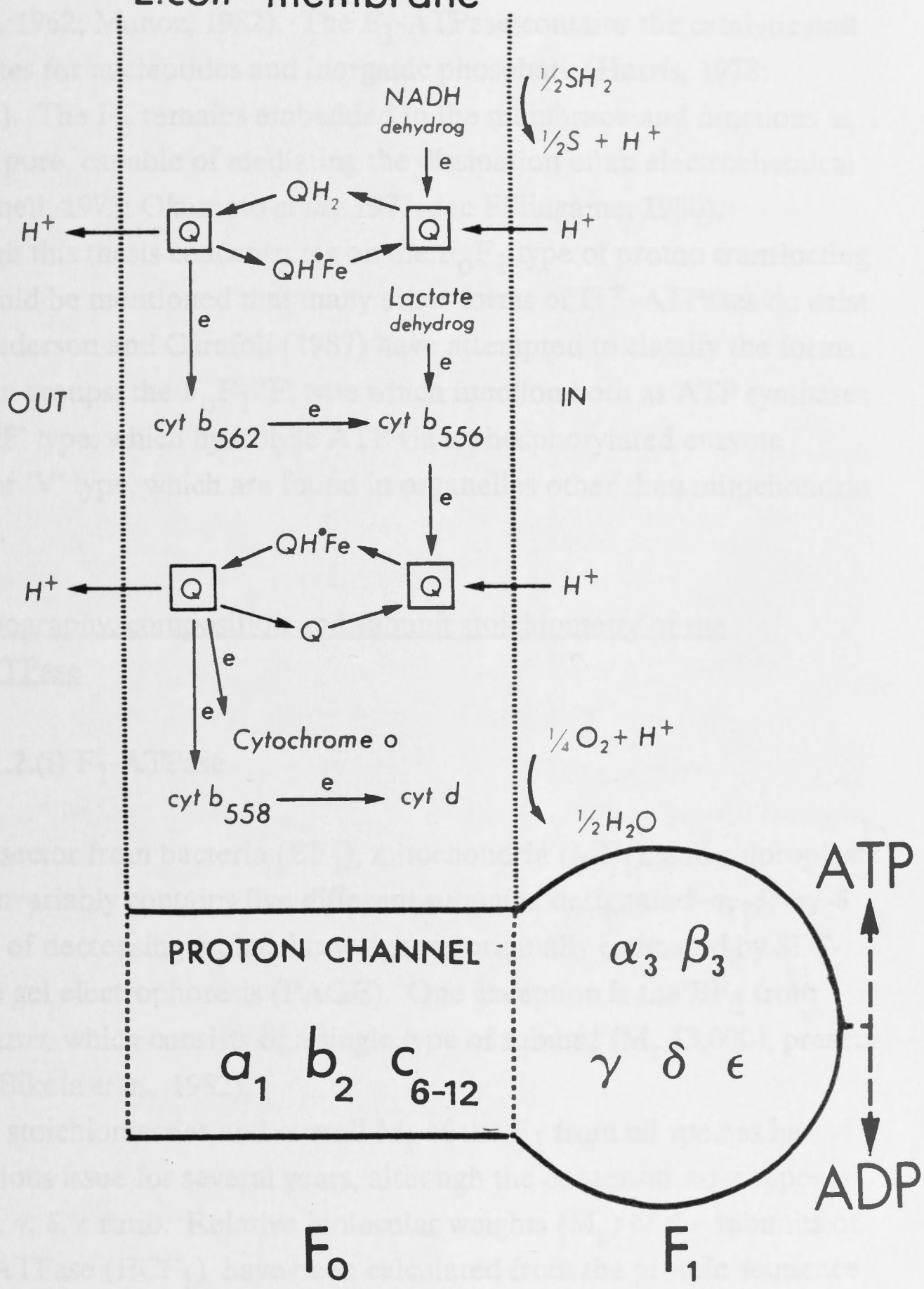
Adenosine triphosphate (ATP) is utilised either directly or indirectly as an energy source in almost all biological systems. All cell types are forced, by the impermeability of their membranes to the nucleotide, to recycle ATP from its constituents, adenosine diphosphate (ADP) and inorganic phosphate (Pi). This necessity for recycling is illustrated by Erinska and Wilson (1978), stating that an average 70 Kg adult at rest may use more than half their body weight per day in ATP, a figure which may rise to almost one tonne if the adult is involved in vigorous exercise. Oxidative phosphorylation, the mechanism whereby synthesis of ATP is coupled to the transfer of electrons from reduced coenzymes such as NADH or $FADH_2$ to oxygen, is primarily responsible for the recycling of ADP and Pi to produce ATP. The ATPase is shown in relation to the electron transfer chain in Fig 1.1.

The mechanism of oxidative or photophosphorylation is recognised as one of the great unsolved biochemical problems. By the 1930s, the concept of a respiratory chain, a complex of redox carriers, had been established largely due to the efforts of Keilin working on the cytochrome system in yeast and heart muscle cells (Keilin, 1929). However, it was not until the late 1940s that interest turned to the coupling of this redox process to ADP phosphorylation (Friedkin and Lehninger, 1948; see also Arnon *et al.,* 1954). Two papers published in 1960, (Pullman *et al.,* 1960; Penefsky *et al.*, 1960) described the purification and properties of a dinitrophenol-stimulated Adenosine triphosphatase(ATPase) from beef heart submitochondrial particles, and highlighted the possibility that the enzyme responsible for hydrolysing ATP was the same enzyme that coupled respiration to ADP phosphorylation.

Membrane-bound, energy-transducing ATPases have since been isolated from bacterial, inner mitochondrial, and chloroplast thylakoid membranes from diverse species (Senior and Wise, 1983; Godinot and DiPietro, 1986; Walker *et al.,* 1987). These adenosine triphosphatases, designated F_0-F_1, catalyse the reversible hydrolysis of the β-γ phosphoryl bond in ATP and are the terminal (complex V (Hatefi, 1985)) enzymes in oxidative phosphorylation. In anaerobic bacteria, the F_0F_1-ATPase functions as an electrogenic pump to generate an electrochemical proton gradient by hydrolysing ATP, to drive the uptake of nutrients which occur via a symport mechanism with protons, mediated by specific transport proteins in the membrane (Simoni and Postma, 1975).

The F_0F_1-ATPase complex is readily dissociable into an aqueous multimer, F_1 and an intramembranous multimer, F_0 (see below). Separation of

Fig 1.1. The F_oF_1-ATPase of *E. coli* shown in relation to the electron transport chain (Downie and Cox, 1978), emphasising the coupling role of the enzyme complex. The periplasm is depicted on the left of the membrane, with the cytoplasm on the right.



ELECTRON TRANSPORT CHAIN AND $F_1 - F_o$ ATPase

the two multimers in many species can be achieved by washing their isolated membranes in EDTA, or low ionic strength buffer containing EDTA (Ishikawa and Lehninger, 1962; Munoz, 1982). The F_1-ATPase contains the catalytic unit with binding sites for nucleotides and inorganic phosphate (Harris, 1978; Penefsky, 1977). The F_o remains embedded in the membrane and functions as a passive proton pore, capable of mediating the dissipation of an electrochemical gradient (Mitchell, 1973; Okamoto *et al.,* 1977; see Fillingame, 1980).

Although this thesis concentrates on the F_oF_1 type of proton translocting ATPase, it should be mentioned that many other forms of H^+-ATPases do exist and recently Pederson and Carafoli (1987) have attempted to classify the forms in to three main groups; the F_oF_1 'F' type which function both as ATP synthases and ATPases, 'P' type, which hydrolyse ATP via a phosphorylated enzyme intermediate, or 'V' type, which are found in organelles other than mitochondria or chloroplasts.

1.2. Topography, composition and subunit stoichiometry of the F_oF_1-ATPase

1.2.(i) F_1-ATPase

The F_1 sector from bacteria (BF_1), mitochondria (MF_1), and chloroplasts (CF_1) almost invariably contains five different subunits, designated -α, -β, -γ, -δ and -ε in order of decreasing molecular weight as originally estimated by SDS-polyacrylamide gel electrophoresis (PAGE). One exception is the BF_1 from *Lactobacillus casei,* which consists of a single type of subunit (M_r 43,000), present as a hexamer (Biketa *et al.,* 1982).

Subunit stoichiometries and overall M_r of the F_1 from all species has been a contentious issue for several years, although the consensus now appears to be an α_3, β_3, γ, δ, ε ratio. Relative molecular weights (M_r) of the subunits of the *E. coli* F_1-ATPase (ECF_1) have been calculated from the protein sequence to be 55,282, 50,286, 31,559, 19,328 and 15,051 (Walker *et al.*, 1984), thus predicting an M_r for the total complex of 382,642. The first report of an α_3, β_3 ratio utilised the growth of *E. coli* on media containing [U-^{14}C]-labeled amino-acids. This radiolabel was incorporated into the ECF_1 which was then purified and individual subunits separated by gel electrophoresis. The subunits were then removed from the gel and a ratio determined after counting the radiolabel incorporated and correcting the ratios for the differences in subunit molecular weight (Bragg and Hou, 1975). That the ECF_1 complex contained 3 β-subunits was evidenced by modification studies with the ATPase inhibitor N-N'-

dicyclohexylcarbodiimide(DCCD). DCCD chemically modifies the β-subunit by binding covalently to a single glutamic acid residue, altering the isoelectric point of the protein (Satre *et al.*, 1982). Only one mole of DCCD bound per mole of ECF_1 is sufficient to totally inactivate the enzymes hydrolytic activity (Satre *et al.*, 1980). Satre *et al.* (1982), after treatment of the ECF_1 with radiolabeled DCCD, were able to separate the modified and nonmodified β-subunits, showing that, at full inactivation of the enzyme, only 30-35% of the β-subunits were modified. Reconstitution studies by Dunn and Futai (1980), mixing isolated ECF_1 subunits, highlighted an optimal level of ATPase activity with an α_3, β_3, γ complex, and were able to recouple the F_oF_1-ATPase on the addition of δ and ε to one copy per complex. In summary, it appears well established that ECF_1 has a subunit stoichiometry of α_3, β_3, γ, δ, ε. The apparently anomalous observation of Vogel and Steinhart (1976) that the ECF_1 had subunit stoichiometries of α_2, β_2, γ_2, $(\delta_{1\text{-}2})$, ε_2, based on the composition of fragments of F_1 obtained from polyethylene glycol precipitation, followed by ion exchange chromatography, have now been largely discarded.

Similar [U-^{14}C]-labeling studies have been attempted on several bacterial species to elucidate the subunit stoichiometries of their respective BF_1's (Godinot and Dipietro, 1986). An α_3, β_3 stoichiometry was reported for purified BF_1 from the thermophile *PS3* (Kagawa *et al.*, 1976), the anaerobe *Streptococcus faecalis* (Abrams *et al.*, 1976) and by Huberman *et al.* (1979), who showed an overall ratio of α_3, β_3, γ, δ, ε_3 for the BF_1 from the Gram positive *Micrococcus Lysodeikticus*. Yoshida *et al* (1978), using the [^{14}C]-labeled sulphydryl modifying agent DTNB (Ellmans reagent; 5',5' Dithiobis-(2-nitrobenzoic acid)) showed that purified BF_1 from the thermophile *PS3* contains three readily titratable sulphydryl groups. The only F_1 subunit containing a cysteine residue is the α, and as there is only one cysteine residue per subunit, three α-subunits must be present per molecule of F_1.

The subunit stoichiometry for MF_1 is not fully resolved although there are five subunits of similar M_r to those found in ECF_1. The most favoured stoichiometry is α_3, β_3, γ, δ, ε. However, the case for an α_2, β_2 stoichiometry was presented by Baird and Hammes (1977) on analysis of bisimidate cross-linked products after two dimensional gel electrophoresis, and on the basis of fluorescence enhancement on the binding of aurovertin to bovine MF_1, leading to the conclusion that there are two aurovertin binding sites (Muller *et al.*, 1977). Earlier work by Senior (1975) had indicated a potential α_2, $\beta_?$, γ_2, $\delta_?$, ε_2 stoichiometry after labeling beef heart MF_1 with the tritiated sulphydryl

modifying agent N-ethylmaleimide.

More recent studies by Walker *et al.* (1985), have utilised a different sulphydryl titration method using labeled iodoacetate to show an α_3, β_3, γ, δ, ε stoichiometry in bovine MF_1. MF_1 purified from the yeast *Sacchromyces carlsbergensis* grown in tritiated leucine was shown to contain α_3, β_3, γ, and δ (Stutterheim Iet al., 1981). Further evidence for the α_3, β_3 composition came from the incorporation of ^{14}C-FSBA (*p*-fluorosulfonyl[^{14}C]benzoyl-5'adenosine) into beef heart MF_1 (Esch and Allison, 1979) and, as suggested by Senior and Wise (1983), the extensive amino acid homology between the β-subunits from CF_1, MF_1, and BF_1 and their similar molecular weights, suggest these complexes will share similar stoichiometries. Walker *et al.*,((1985) highlights a strong sequence homology between MF_1 and ECF_1 subunits -α, -β, and -γ. However, the MF_1 δ is not homologous to its bacterial counterpart, bearing a stronger comparison to the *E. coli* ε-subunit. MF_1 ε has no known bacterial homologue (For ECF_1 subunit-δ homologue see below).

As mentioned above, CF_1 consists of five distinct subunits. Experimental data obtained from growing pea seedlings in [$^{14}CO_2$] led Nelson (1976) to suggest a stoichiometry of α_2, β_2, γ, $\delta_{1\text{-}2}$, ε_2. More recent data has largely discounted these results. Sàss and Schmidt (1982) grew the broad bean *Vicia fabia* in an atmosphere of $^{14}CO_2$. The distribution of label in subunits separated by gel electrophoresis showed a clear α_3, β_3, γ, δ, ε arrangement. Similar results were noted by Merchant *et al.* (1982) working with *Chlamydomonas reinhardi* grown in [2-^{14}C] acetate. An α_3, β_3, γ, δ, ε stoichiometry is now widely accepted for CF_1.

Electronmicroscopic studies using negative staining have shown ECF_1, MF_1, and CF_1 to be spherical and membrane extrinsic, with a diameter of approximately 9-10 nm (Amzel and Pedersen, 1983). Image enhancement has indicated the structure is hexagonal. Recent immunoelectron microscopic data summarised by Godinot and DiPietro (1986)(and see Akey *et al.*, 1986), suggest an alternating sequence of 3α- and 3β-subunits, arranged on two separate plains both in BF_1 and CF_1. Interestingly, X-ray diffraction patterns determined to 0.9nm resolution from crystals of rat liver MF_1 (Amzel and Pedersen, 1978) suggest a structure where all α- and β-subunits are not equivalent, judged by the lack of a three-fold symmetry. This apparent asymmetry was explained by suggesting that two α,β pairs would be equivalent, whilst the third was juxtaposed with the minor subunits (see Amzel and Pedersen, 1983). A recent observation by Vignais *et al.* (1984), using a high resolution scanning electron microscope also highlighted an asymmetry in ECF_1 which was lacking subunit-δ. Gogol *et al.* (1987), using a new method of specimen preparation, have visualised by electron

microscopy a 4.5 nm 'stalk' connecting the ECF_1 with the membrane-embedded ECF_0-moiety. This stalk is approximately 2 nm wide, large enough to accommodate four or five α-helices. The authors also state that the ECF_1 does not appear to exhibit any hexagonal or 'pseudohexagonal' structure.

1.2.(ii) F_0-sector

The membrane-intrinsic, proton-translocating F_0 multimer in ECF_0 is comprised of three subunits designated *-a*,- *b*, and *-c* (sometimes chi, psi or omega (Fillingame ,1981)). Relative molecular weights for the three subunits of *E. coli* F_0 have been calculated as *a*: 30,285, *b*: 17,230, and *c*: 8,264 from the amino acid composition derived from the published nucleotide sequence (Walker *et al.*, 1984).

Hydrophobic affinity chromatography has enabled relatively pure preparations of F_0 to be isolated (Schneider and Altendorf, 1982). However, exact subunit stoichiometries for the F_0-sectors of the F_0F_1-ATPases from many species are unresolved. Nielsen *et al.* (1981), had reported a stoichiometry of a_1b_1 for ECF_0 based on incorporation of radiolabel. However, the ratio a_1b_2.now appears to be accepted after cross-linking studies using dithiobis(succinimidyl)proprionate (Hermolin *et al.*, 1983), diazoniumbenzenesulphonate (Aris and Simoni, 1983) and azidonitrophenylfluoride (Sebald *et al.*, 1982). Intergenic complementation between two abnormal *b*-subunits constitutes genetic evidence that at least two *b*-subunits are present per ATPase complex (Jans *et al.*, 1985).

The number of copies of subunit-*c*, the proteolipid DCCD-binding protein, per molecule of ECF_0 has been calculated at anything between 5-15 (von Meyenberg *et al.*, 193; Foster and Fillingame, 1982; Nielsen et al (1981)). Foster and Fillingame (1982), working with *E. coli* grown in medium containing [^{35}S]-sulphate and [U-^{14}C]-glucose, estimated an incorporation of 10±1 *c*-subunits per complex. A recent survey of the literature showed the stoichiometry a_1, b_2, $c_{10\pm1}$ to be the most favoured, although the matter remains unresolved (Senior and Wise (1983)). The subunit composition of several other bacterial F_0 complexes have been analysed (Futai and Kanazawa, 1983). Three types of subunit are present in *S.faecalis* with M_r 27,000, 15,000 and 6,000 respectively, with the smallest being present as an oligomer equivalent to the DCCD binding protein (Leimgruber *et al.*, 1981). Originally, a similar pattern of three subunits was categorised for the thermophile *PS3*, although the largest component, designated band 4, M_r 19,000, was considerably smaller than the ECF_0 subunit-*a* (Sone *et al.*, 1977). Reconstitution studies showed a 50% level of proton

conductance and normal levels of F_1 binding in the absence of the larger subunit. The authors suggested the subunit was not part of the normal F_o complex, but was similar to the mitochondrial OSCP on the basis of its size and strongly negative charge (Sone *et al.*, 1978; see below). Although accepted as consisting of three different forms of subunits in most BF_o, the composition of the thermophilic F_o-sector remains unresolved.

Both subunit composition and stoichiometry of mitochondrial F_o are not known, but appear to be more complex than the bacterial system. (Godinot and Di Pietro, 1986; Houstek *et al.*, 1982; Montecucco, *et al.*, 1983; Walker *et al.*, 1987). Claims of active MF_o-F_1 complexes from rat liver containing only nine different forms of subunits have been made (McEnery and Pedersen, 1986), whereas the most recent studies on the bovine heart complex suggest 13 types of subunits (Walker *et al.*, 1987).

MF_o does contain a DCCD-binding protein homologous to the ECF_o subunit-*c*. This subunit, designated subunit *9*, binds one molecule of DCCD at a glutamic acid residue totally conserved in MF_o proteolipid subunits sequenced from many diverse species and present as an aspartate in *E. coli* subunit-*c* (see Sebald and Hoppe, 1981). The observation that DCCD binds specifically to a single residue in the F_o (Sebald *et al.*, 1980), has enabled researchers to estimate the DCCD-binding proteolipid stoichiometry from the F_o of several different sources to be approximately six (Sebald *et al.*, 1979 MF_o from S.cerevisiae; Kiehl and Hatefi, 1980 MF_o from beef heart). A second peptide present in MF_o, subunit *6*, is homologous to the largest ECF_o component, subunit-*a* (Walker *et al.*, 1984; see below). A third, small proteolipid, component, has been categorised in the yeast *S.cerevisiae* (Hadikusomo *et al.*, 1984; Velours *et al.*, 1984), and designated subunit *8*, and in bovine heart MF_o (Fearnley and Walker, 1986) designated *A6L*. No convincing homologue for the ECF_o subunit-*b* has been reported from MF_o, although recent characterisation of the MF_o from beef heart (Walker *et al.*, 1987) highlighted two intrinsic subunits, -*b* (M_r 24,500) and -*d* (M_r 18,500) (apparently analogous to subunits F_o*1* and F_o*2* from pig heart (Montecucco, 1983). Subunit-*b* shows no primary structure homology to its ECF_o counterpart, but does share a similar hydropathy profile. The functional importance of subunits -*b* and -δ from MF_o has not been shown although their intramembranous position was confirmed by labeling with photoactivatable lipid soluble probes.

MF_o-F_1, in addition to the subunits already mentioned, also contains four loosely associated subunits designated *OSCP* (Oligomycin Sensitivity Conferring Protein), the natural ATPase inhibitor protein, IF_1, coupling factor F_B, and F_6 (Vignais and Satre, 1984; Sanadi, 1984). *OSCP* from beef heart mitochondria is

present in two copies per complex (Penin *et al.,* 1985), has been sequenced and has a M_r of approximately 21,000 (Grinkevich *et al.*, 1982; Ovchinnikov *et al.,* 1984a). Together with coupling factor F_6 it is believed to constitute the stalk joining the MF_1 and MF_o (see Hatefi, 1985). Walker *et al.* (1982, 1984), maintain the OSCP is homologous to ECF_1 subunit-δ, as the two share extensive sequence homology and also appear to function in similar fashions. ECF_1 can be prepared which lacks δ (Futai *et al.,* 1974) and, although the majority of preparative methods for MF_1 do not include *OSCP*, both rat liver and beef heart MF_1 can be prepared as a six subunit complex (Fisker et al., 1981). Partial tryptic digests of the ECF_1 α-subunit and the MF_1 α-subunit prevent binding of the δ-subunit and *OSCP* respectively on reconstituting the complex. ATP hydrolysis is unaffected in both reconstituted complexes, and, although MF_1, unlike the ECF_1 still retains its F_o binding capacity, synthesis of ATP can no longer be coupled to proton translocation (Dunn *et al.,* 1980; Hundal *et al.,* 1984). Ovchinnikov *et al.*(1984b), found a region of homology between *OSCP* and ECF_o *b*-subunit, and with the ADP/ATP carrier protein. A functional similarity between ECF_o subunit-*b* and *OSCP* from beef heart mitochondria has been claimed by Joshi *et al.* (1986) after trypsin-cleaved *OSCP* in F_1-depleted complex exhibited increased passive proton permeability on reconstitution with untreated MF_1, similar to the affect of trypsin-cleaved *b*-subunit. Both *OSCP* and subunit-*b* are protected from proteolysis in F_1 bound complexes. However, unlike the protease treated ECF_o unable to bind native F_1 (Perlin *et al,* 1983), MF_1 could bind F_o containing trypsin treated *OSCP*. Walker *et al.* (1985), also report partial homology of *OSCP* with the bovine γ.

Coupling factor F_6, (Fessenden-Roden, 1972) has been sequenced and has an M_r of 9,000. Several partial homologies have been noted between this factor and bovine *OSCP*, bovine inhibitor protein, bovine δ, and *E. coli* ε (Walker *et al.*, 1985). The inhibitor protein, IF_1, M_r 9000-10000, first isolated by Pullman and Monroy (1963), has been sequenced from two sources, bovine heart and yeast (see Hatefi, 1985; see Pullman, 1986) in which it is present in a single copy (Godinot and Dipietro, 1986). Although no significant homology has been found between this protein and *E. coli* ε, they both appear to be functionally similar. Coupling factor F_B, purified from beef heart mitochondria is present in one copy per complex. It has not been sequenced although amino acid analysis and electrophoretic migration indicate an apparent M_r of 15,000 (Sanadi, 1982). Walker *et al.* (1987), have purified a functional MF_o-F_1 complex from beef heart containing 13 subunits. However, the authors report a lack of F_B in their preparations.

Chloroplast CF_0 is similar to the bacterial sector with subunit *I* equivalent to subunit-*b* (Bird *et al.*, 1985), subunit *III* equivalent to the proteolipid DCCD-binding protein (Sebald and Hoppe, 1981) and subunit *IV* similar to subunit-*a* (Cozens *et al.*, 1986). The genes encoding these three peptides cover a 4 kb region of spinach chloroplast chromosome, acting as a single transcriptional unit (Hennig and Hermann, 1986). However, there is a nuclear encoded extra subunit in the CF_0, subunit *II*, which has no apparent *E. coli* homologue (Hennig and Hermann, (1986)). Subunit stoichiometries have not been fully defined, although early experiments with [^{14}C]-DCCD incorporation indicated that the proteolipid subunit *III* was present in six copies per complex (Sigrist-Nelson *et al.*, 1982 for CF_0 from lettuce)

In summary, Table 1.1 attempts to correlate known and potential homologies between bacterial, mitochondrial and chloroplast F_0F_1-ATPase subunits. *E. coli* F_0F_1-ATPase is comprised of eight different forms of subunit with subunit stoichiometries of α_3, β_3, γ, δ, ε for F_1 and a_1, b_2, $c_{6\text{-}12}$ for F_0. Mitochondrial F_0F_1-ATPase is believed to consist of at least 12 different types of subunit with MF_1 subunit stoichiometries of α_3, β_3, γ, δ, ε. Reported subunit composition of MF_0 and associated subunits differ between species. However, the majority of MF_0 contain subunit *6* (one copy), the DCCD-binding subunit, subunit *9* (possibly six copies) in the F_0 sector, and four loosely associated subunits, *OSCP* (2 copies), *IF1* (one copy), coupling factor F_6 and coupling factor F_B (one copy). In addition, bovine MF_0 contains subunits *-b*, *-d*, and *A6L*, whilst *S. cerevisiae* MF_0 contains subunit *8*. Chloroplast F_0F_1-ATPase is comprised of eight or nine different types of subunits with similar subunit composition and homology in the F_1 to ECF_1. The F_0 component consists of three or four subunits designated CF_0I, (*II*), *III* and *IV*. Subunit *III*, the proteolipid DCCD-binding protein is present in six copies, whereas the stoichiometries of the remaining CF_0 subunits is unknown.

1.3. Biochemical and genetic characterisation of the transcriptional unit encoding the *E. coli* F_0F_1-ATPase: the *unc* operon

The first mutant strain of *E. coli* unable to couple the synthesis of ATP to electron transport was described by Butlin *et al.* in 1971. This mutant designated *uncA401*, was unable to grow utilising a nonfermentable carbon source such as succinate. The term *unc* has been used to signify a mutant which is 'uncoupled' in oxidative phosphorylation. Several other groups have isolated mutants with defective ATPase complexes which they describe as *bcf* (Bacterial Coupling Factor (Rosen *et al.*, 1978)), *atp* (Atpase Translocating Protons (von Meyenberg

TABLE 1.1

EQUIVALENT SUBUNITS OF F_o-F_1 ATPase SUBUNITS IN BACTERIA, BOVINE MITOCHONDRIA, AND CHLOROPLASTS (Modified from Walker *et al* (1987)).

Bacteria *(E.coli)*	Bovine mitochondria	Chloroplasts	References
α	α	α	1-4
β	β	β	1–5
γ	γ	γ[a]	1,2
δ	OSCP	δ[a]	1,6
ε	δ	ε	1–3,7
-	ε	-	1
a	ATPase-6	CFo IV	1,8-10,19
b	(b)[b]	CFo I (II)[c]	1,11,12,19
c	ATPase-9	CFo III	1,13,14,19
-	d	-[d]	12
-	IF_1	-[d]	15,19
-	F6[e]	-[d]	16,17
-	A6L	-[d]	18,8
-	F_B	-[d]	20

[a] These subunits have not been sequenced.

[b] Little experimental evidence exists to support the potential homology between the bacterial *b* subunit and mitochondrial *b* subunit. (12).

[c] The homologue of CFo II is not indisputably the *b* subunit in E.coli(19).

[d] It is unlikely after consideration of polyacrylamide gel analysis (Westhoff et al (1985)) that any similar subunits are present in chloroplasts.

[e] Partial homologies have been noted between F6 and several other ATPase components (Walker et al(1985);see section 1.2.(ii)).

References: 1. Walker *et al* (1984); 2. Walker *et al* (1985); 3. Howe *et al* (1985); 4. Deno *et al* (1983); 5. Krebbers *et al* (1982); 6. Ovchinnikov *et al* (1984); 7. Zurawski *et al.* (1982); 8. Anderson *et al* (1982); 9. Velours *et al* (1984); 10. Cozens *et al* (1986); 11. Bird *et al* (1985); 12. Walker *et al* (1987); 13. Sebald and Hoppe (1981); 14. Howe *et al* (1982); 15. Frangione *et al* (1981); 16. Fang *et al* (1984); 17. Grinkevich *et al* (1984); 18. Fearnley and Walker (1986); 19. Hennig and Herrmann (1986); 20. Sanadi (1982) .

and Hasan, 1980) and *pap* (Proton translocating Atpase Protein (Kanazawa *et al.,* 1981a). The *unc* genes were initially located by cotransduction frequencies with the *ilv* locus at around 74 minutes on the *E. coli* chromosome (Butlin *et al.*, 1971), but after updating the linkage map the *unc* genes are now located at 83.7 minutes, between *AsnA* and *GlmS* (Bachmann, 1983)(Fig 1.2.).

1.3.(i) Gene-polypeptide relationships

Originally, mutants sharing the classic *unc* phenotype could be separated into two classes on the basis of reconstitution studies (Cox *et al.,* 1973). Reconstitution of both oxidative phosphorylation and an ATP-dependent transhydrogenase activity were obtained with a combination of 'Tris-wash' containing the F_1-ATPase from a membrane preparation of a mutant strain designated *uncB* with the membranes from a mutant strain designated *uncA.* After demonstrating the dominance of normal *unc* genes in merodiploid strains with chromosomal *unc* mutations and F plasmids with normal *unc* genes, (Gibson *et al.,* 1977a) a system whereby chromosomal *unc* mutations were transferred to F plasmids by recombination repair facilitated the definition of four complementation groups designated *uncA, B, C,* and *D* (Gibson *et al.,* 1977b; Cox *et al.,* 1978).

Gibson *et al.* (1978), showed the *unc* genes constituted an operon after analysing complementation patterns with partial diploid strains carrying chromosomal bacteriophage Mu insertions in different *unc* genes. On the basis of Mu insertion terminating RNA transcripts from operons (transcriptional polarity)(Daniell and Abelsen, 1973) the gene order was deduced as *uncBADC* with *uncB* being promoter proximal.

The first clear gene-polypeptide relationship was that of the β-subunit to *uncD*. 2-Dimensional gel electrophoresis highlighted an altered isoelectric point for the β-subunit from strains carrying the *uncD409* mutation (Fayle *et al.,* 1978). This technique was also used (Senior *et al.,* 1979) to confirm the observations of Dunn (1978) and Kanazawa *et al.* (1978) whose reconstitution studies with *uncA* mutants suggested the gene product was synthesised from the *uncA* gene. Further clarification of gene-polypeptide relationships was achieved after the successful cloning of the *unc* genes into a series of plasmids. Two plasmids were constructed which contained either the promoter proximal region of the *unc* operon coding for *unc* genes *B, E,* and *A* (pAN51), or the promoter distal region coding for the *uncD* and *uncC* genes (pAN36). A third plasmid containing all the *unc* structural genes was formed after cleaving both plasmids pAN36 and pAN51 with endonuclease *Hin*d III and then religating with T4 DNA ligase(pAN45;see

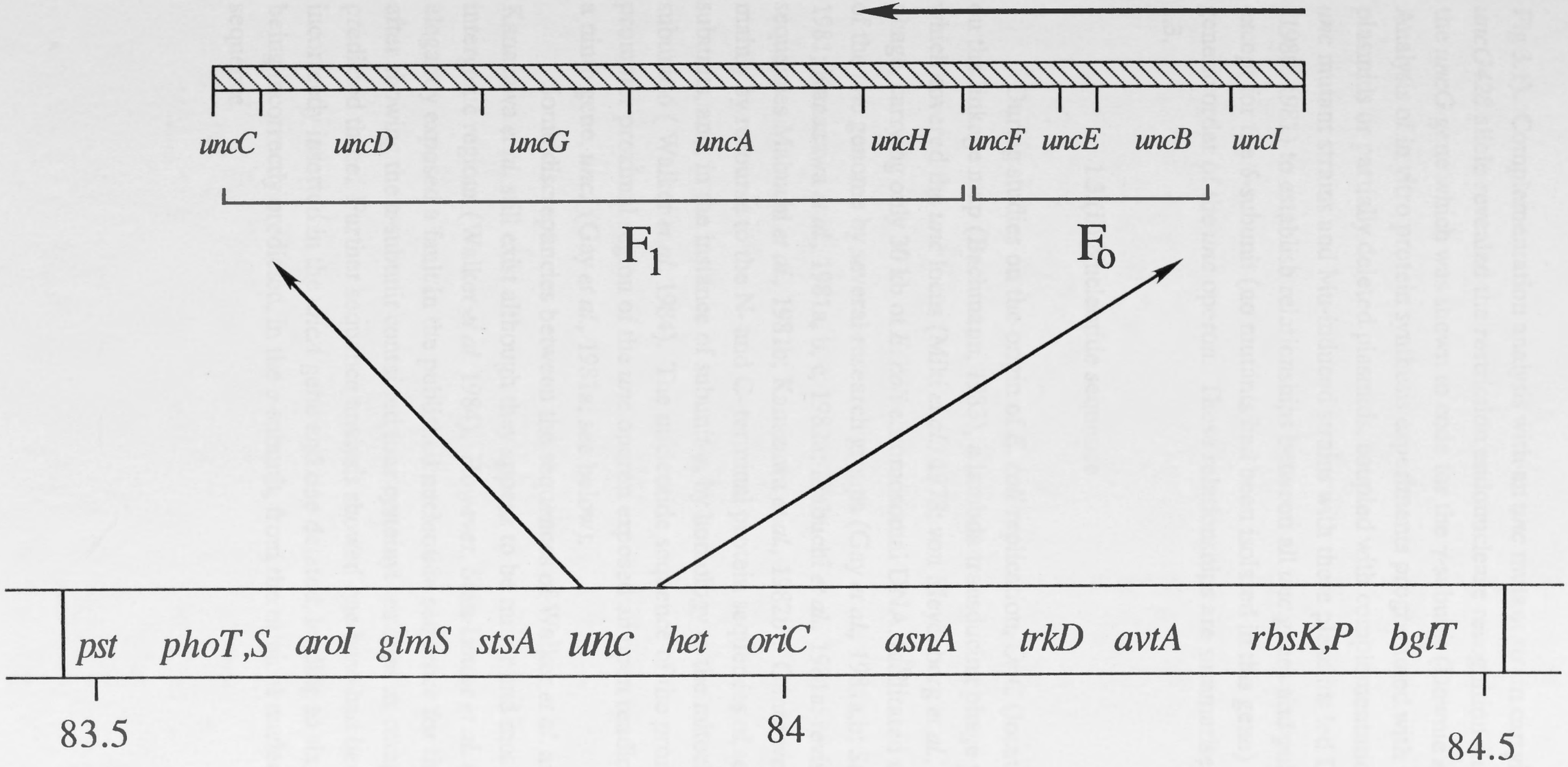


Fig 1.2 Location on the E. coli chromosome and gene order of the unc operon. Relative map position and neighbouring gene abbreviations given as per Bachmann (1983).

Fig 3.1). Complementation analysis with an *unc* mutant strain carrying the *uncG428* allele revealed the restriction endonuclease recognition site lay within the *uncG* gene which was shown to code for the γ-subunit (Downie *et al.,* 1980). Analysis of in vitro protein synthesis experiments programmed with the three plasmids or partially deleted plasmids, coupled with complementation analysis of *unc* mutant strains and Mu-induced strains with these plasmids led Downie *et al.* (1980, 1981) to establish relationships between all *unc* genes and polypeptides except for the δ-subunit (no mutants had been isolated in this gene) and also the genetic order of the *unc* operon. These relationships are summarised in figure 1.3.

1.3.(ii) Nucleotide sequence

During studies on the origin of *E. coli* replication, *Ori*C (located at 84 min on the linkage map (Bachmann, 1983), a lambda transducing phage was isolated which covered the *unc* locus (Miki *et al.,* 1978; von Meyenberg *et al.,* 1978). This phage, carrying only 30 kb of *E. coli* chromosomal DNA, facilitated sequencing of the *unc* genome by several research groups (Gay *et al.,* 1981a,b; Saraste *et al.,* 1981; Kanazawa *et al.,* 1981a, b, c, 1982a; Mabuchi *et al.,* 1981a; revised sequences Mabuchi *et al.,* 1981b; Kanazawa *et al.,* 1982b). Genes were identified mainly by recourse to the N- and C- terminal protein sequences of several of the subunits, and, in the instance of subunit-*a*, by homology to the mitochondrial subunit-*6* (Walker *et al.* 1984). The nucleotide sequence of the promoter and promoter proximal region of the *unc* operon exposed an open reading frame for a ninth gene, *uncI* (Gay *et al.,* 1981a; see below).

Some discrepancies between the sequences of Walker *et al.* and Kanazawa *et al.* still exist although they appear to be minor and mostly in intergenic regions (Walker *et al.* 1984). However, Stan-Lotter *et al.* (1986) elegantly exposed a fault in the published nucleotide sequence for the *uncA* gene after showing the α-subunit contained four cysteinyl residues as compared to the predicted three. Further sequence analysis showed one base had been incorrectly inserted in the *uncA* gene and one deleted, leading to six residues being incorrectly predicted, in the α-subunit, from the original nucleotide sequence.

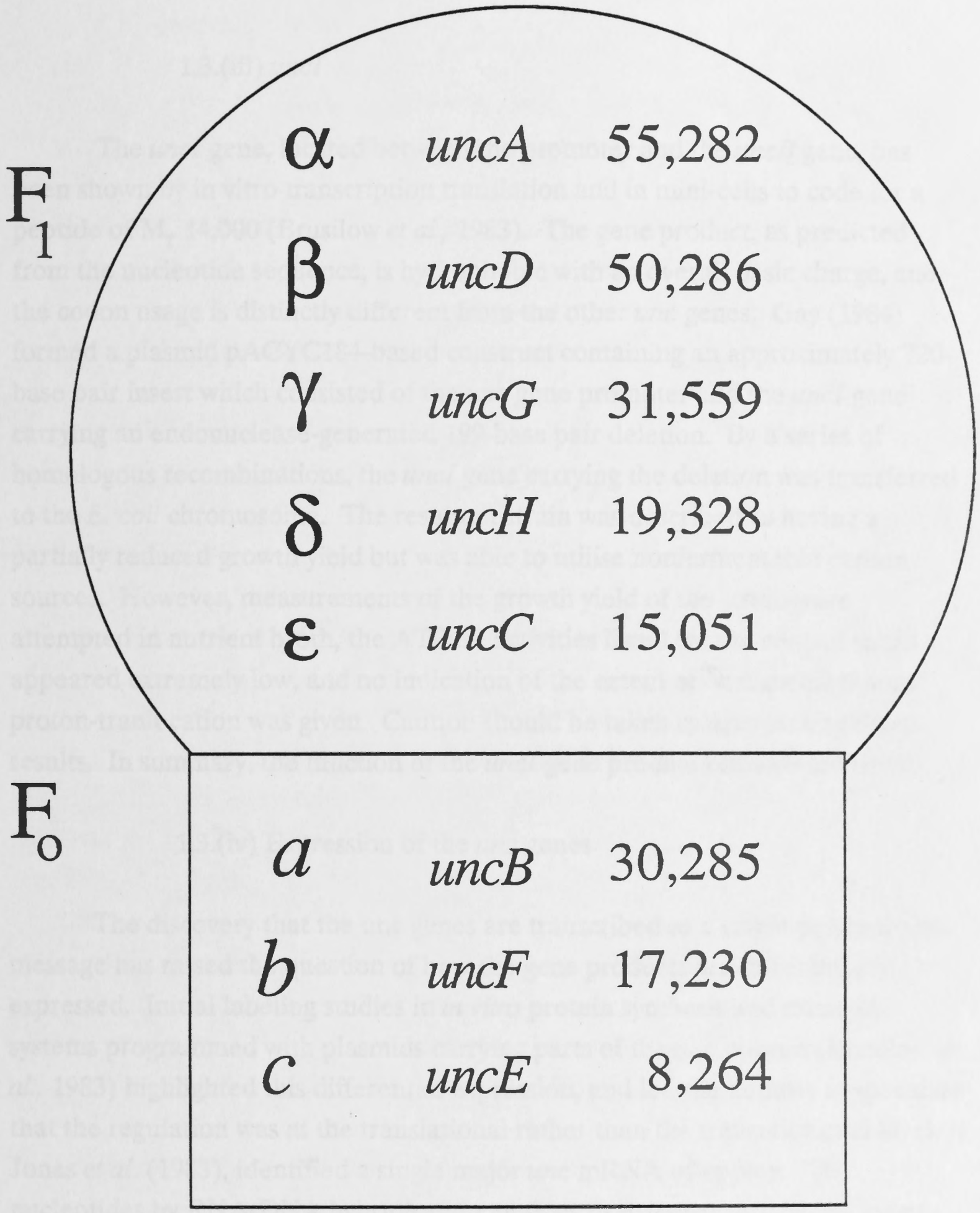


Fig 1.3. Gene-polypeptide relationships of the unc operon encoding the subunits of the FoF1-ATPase of E. coli. Assignments were made after the experiments detailed in section 1.3.(i). Relative molecular weights of each of the subunits are calculated from the primary amino acid sequence (Walker et al., 1984).

1.3.(iii) *uncI*

The *uncI* gene, located between the promoter and the *uncB* gene, has been shown by in vitro transcription translation and in mini-cells to code for a peptide of M_r 14,000 (Brusilow *et al.,* 1983). The gene product, as predicted from the nucleotide sequence, is hydrophobic with an overall basic charge, and the codon usage is distinctly different from the other *unc* genes. Gay (1984) formed a plasmid pACYC184-based construct containing an approximately 720-base pair insert which consisted of the *unc* gene promoter and the *uncI* gene carrying an endonuclease-generated 199-base pair deletion. By a series of homologous recombinations, the *uncI* gene carrying the deletion was transferred to the *E. coli* chromosome. The resultant strain was described as having a partially reduced growth yield but was able to utilise nonfermentable carbon sources. However, measurements of the growth yield of the strain were attempted in nutrient broth, the ATPase activities listed for the control strain appeared extremely low, and no indication of the extent of transmembranous proton-tranlocation was given. Caution should be taken in interpreting these results. In summary, the function of the *uncI* gene product remains unknown.

1.3.(iv) Expression of the *unc* genes

The discovery that the unc genes are transcribed as a single polycistronic message has raised the question of how the gene products are differentially expressed. Initial labeling studies in *in vitro* protein synthesis and minicell systems programmed with plasmids carrying parts of the *unc* operon (Brusilow *et al.,* 1983) highlighted this differential expression, and led the authors to speculate that the regulation was at the translational rather than the transcriptional level. Jones *et al.* (1983), identified a single major *unc* mRNA of approx. 7000 nucleotides by RNA-DNA hybridisation analysis (a message sufficiently long to transcribe all nine *unc* genes), and a minor mRNA species of 5-6 kb, the role of which remains unclear. Both transcripts appeared stable and were not readily degraded. No difference in *unc* mRNA levels were noted if the cells were grown anaerobically, and the authors concluded that control of differential expression of the *unc* genes under aerobic or anaerobic conditions was not at the transcriptional level. By poisoning transcription initiation with rifampicin and pulse-labeling cells over a time course, von Meyenberg *et al.* (1984a), demonstrated there is no differential degradation of *unc* mRNA supporting the concept that regulation of expression is at the translational level.

One potential way of enforcing 'uncoordinacy' of expression is by regulation during peptide elongation. A strong correlation between the relative abundance of tRNAs and common codons (Ikemura, 1981) has been used by Walker *et al.* (1984) to suggest that the presence of rare codons within a gene may cause a retardation of peptide synthesis (see also Talkad *et al.*, 1976). Brusilow *et al.* (1983) also tested the effects of increased tRNA on expression, concluding the regulation was not significantly affected by the presence of rare codons. However, analysis of the *unc* operon exposes a higher level of rare codons in the genes which are expressed at comparatively low levels (*unc*F, H, G,; see Walker *et al.*, 1984). McCarthy, (1987), argues that the rate of translation is rarely rate limiting, and it is translational initiation which is the limiting factor. Only when initiation efficiency is artificially improved or where genes carrying rare codons are in multicopies in the cell are tRNA pools likely to be drained (Pedersen, S. 1984).

Recently, attention has focussed on the intergenic regions of the *unc* operon and their potential role in differential regulation (Ferguson, 1984). McCarthy (1987), uses the term TIR to represent the translational initiation region, which includes the translational start codon and parts of the intergenic region, the Shine-Dalgarno site complementary to the 3' terminus of the 16S rRNA, and the N-terminal coding region for the peptide. Local secondary structure in this region has been implicated in differential expression regulation, possibly by rendering the initiation region more inaccessible to the initiation complex. Klionsky *et al.* (1986) artificially introduced two single base changes into the TIR for *uncF*, generating 2-3 fold increases in subunit-*b* synthesis, after predicting the changes would destabilise a potential stem-loop structure in the intergenic region. This structure was presumed to block the ribosome binding site, the removal of which leads to a higher efficiency of translational initiation.

The *uncE* TIR is predicted to be unlikely to form any stable secondary structure, leading to an efficient initiation of translation. McCarthy *et al.* (1985), cloned the *unc* genes *uncE, uncF* and *uncH i*ndividually under the *tac* fusion promoter and in front of the *galK* gene, which functioned as a built in reference gene. Expression of the *unc* gene products, estimated by the incorporation of [^{35}S]-methionine both *in vivo*, and *in vitro*, illustrated rates of synthesis of subunit-*c* of approximatelysix times subunit- *b* and 18 times subunit -δ. By progressively shortening the intergenic region upstream from *uncE*, the maximal expression was shown to be dependent upon a sequence stretching more than 20 nucleotides upstream from the Shine-Dalgarno site. The *unc*E TIR has been artificially introduced upstream from other weakly expressed *unc* genes,(McCarthy, 1987), human cDNA coding for interleukin 2 (IL-2) and

interferon β (INFβ) (McCarthy *et al.,* 1986) and has been used to construct a high efficiency translation vector in tandem with the two strong lambda promoters, P_L and P_R (Schauder *et al.,* 1987). In each case, increases of approximately ten fold of translation of the protein have been achieved, with INFβ and IL-2 being produced at between 10-30% of the total cell protein in *E. coli.*

Weak translational coupling between *uncE* and *uncF* and also *uncF* and *uncH* have been noted (McCarthy, 1987). The author suggests this coupling is affected by ribosomes from the upstream cistron aiding in initiation of translation at the downstream cistron possibly by breaking any intermediate secondary structure which could interfere with the initiation process. No coupling occurs between *uncA* and *uncG*, even when the α-subunit is produced to 50% of total cell protein. Translational efficiency of *uncC* is higher than the other unc genes whose gene products are present in a single copy per complex, with the *uncC* TIR predicted to form no substantial secondary structure. Cox *et al.* (1987), have isolated an *uncC* mutant containing an altered Shine-Dalgarno sequence which reduces the amount of membrane-bound ATPase activity by about 70%. A partial revertant strain carries a second mutation causing elongation of the β-subunit by translating through most of the *uncC* TIR. The authors conclude that elongation leads to an increase in translational coupling between *uncD* and *uncC*. Intriguingly, two further partial revertants carrying additional mutations at positions -18 and -19 upstream from the *uncC* translational start codon show increases in the formation of both membrane bound ε and β, suggesting that as well as translational coupling where the levels of translation of β can affect the translational levels of ε downstream, affects within the *uncC* TIR which increase ε translation can be somehow transmitted upstream to affect β translation.

Overexpression of the *unc* genes (von Meyenberg *et al.,* 1984b)), has been achieved by cloning the genes under the strong phage lambda promoter, P_R, into a high copy number plasmid, with levels reaching around 20% of total cell protein. However, this overproduction leads to marked inhibition of growth and cellular division, with the appearance of membrane cisternae, intracellular vesicles and inclusion bodies which are believed to carry the excess complexes. Further investigation (von Meyenbeg *et al.*, 1985) showed the deleterious affects on growth were primarily due to subunit-*a* acting as a protonophore and effectively dissipating the proton electrochemical gradient on induction of the operon.

1.4 Theories of oxidative phosphorylation

1.4.(i) Introduction.

The precedent established for glycolysis of the esterification of inorganic phosphate to form an acyl phosphate intermediate before being transferred to ADP in the formation of ATP (Warburg and Christian, 1939), led Lipmann (1943) to propose a high energy phosphorylated compound as an intermediate of respiratory-linked phosphorylation. However, as respiration was later shown to occur in the absence of inorganic phosphate (Keilin and Hartree, 1947), which was in contrast to glycolysis, and that oxidative phosphorylation could be uncoupled by agents such as 2,4 dinitrophenol (Lardy and Wellman, 1952), it was proposed that either as well as, or instead of, a high-energy phosphate intermediate, there must also exist an independent high-energy source (Slater, 1953). This source was depicted as involving chemically modified electron-transfer catalysts linked to a ligand by a covalent high energy bond; the chemical hypothesis. After an extensive search no such intermediate was isolated and the hypothesis was abandoned.

In 1961, R.J.P. Williams (1961) and P. Mitchell (1961) independently proposed that protons may be the 'high energy source' responsible for coupling electron transfer to the endergonic synthesis of ATP. Mitchells 'chemiosmotic theory' envisaged the production of a proton electrochemical gradient, delta μ_H+, across a sealed proton impermeable membrane when electrons are transferred along a series of components embedded in the membrane. Equilibration of the two bulk phases occurs through the proton permeable F_o (Mitchell, 1974, 1976, 1977), which he described as an aqueous well. This electrochemically driven flux provided both the energy to drive, and the protons to participate in ATP synthesis at the active site of the F_1 moiety via the overall reactions:

$$A_{red} + B_{ox} \longrightarrow A_{ox} + B_{red} + \Delta\tilde{\mu}_H+$$

$$ADP + P_i + \Delta\tilde{\mu}_H+ \longrightarrow ATP + H_2O$$

The proton electrochemical gradient, $\Delta\mu_H+$, or Δp, is comprised of the pH gradient, pH, and the membrane potential, $\Delta\psi$ such that;

$$\Delta p = \Delta\psi - 2.303\frac{RT}{F}\Delta pH$$

The hypothesis is experimentally verifiable and since its conception, has been rigorously explored. Jagendorf and Uribe (1966), prepared chloroplasts in acidic media, keeping the preparation in darkness. After rapidly transferring the acid-loaded chloroplasts to an alkaline bath in the dark, production of ATP was shown both enzymatically with luciferase, and by the incorporation of radiolabeled [^{32}P] from inorganic phosphate into ATP (Jagendorf and Uribe, 1966). Reid *et al.* (1966), pre-incubated intact rat liver mitochondria in an alkaline environment in the presence of a high K^+ ion concentration and valinomycin. Sudden transfer of the mitochondria to a high concentration of trichloroacetic acid resulted in the incorporation of [^{32}P] inorganic phosphate into ATP. The rate of incorporation was also shown to be dependent on the duration of the 'acid bath'. After reconstituting phospholipids with partially purified bacteriorhodopsin, Racker and Stockenius (1974), were able to measure proton flux into the lipid vesicles on their illumination. By further incorporating purified mitochondrial F_oF_1-ATPase complexes into the membrane vesicles, ATP synthesis could be coupled to the formation of the bacteriorhodopsin-generated proton flux, a synthesis evidenced by the hexokinase-catalysed trapping of incorporated [^{32}P] as glucose-[^{32}P]-phosphate. Similar experiments involving acid/alkali 'jumps' and the concomitant production of ATP have also since been described in protoplasts and membrane vesicles of several bacteria (Sone *et al.* 1977; Tsuchiya, 1977; Tsuchiya and Rosen, 1976). Witt *et al.* (1976) managed to couple the synthesis of ATP in chloroplasts to the generation of a membrane potential via an externally applied electrical field. Although direct measurements of proton/hydroxide conductance and permeability through phospholipid bilayers have highlighted a permeability several orders of magnitude higher than alkali or halide ion permeabilities at pH 7, this permeability is not believed to be significantly high to markedly affect chemiosmosis (Deamer and Nichols, 1983; Gutknecht, 1987). Results from the above experiments suggest that Mitchells' hypothesis may be a valid one.

However, although he points out that in general terms the delocalised chemiosmotic theory has resolved the problem of energy transduction in oxidative and photophosphorylation, Ferguson (1985) has collated experimental data from many sources which cannot be reconciled with Mitchells' hypothesis and that he claims have been widely ignored, and cast doubt on the theory providing a complete description of energy coupled phosphorylation. Perhaps one of the most difficult observations to explain in terms consistent with the chemiosmotic hypothesis is the ability of some alkalophilic bacteria, such as *Bacillus alkalophilus*, to couple oxidative phosphorylation in an external medium

of pH 11. The internal pH of the bacteria has been measured as pH 9, with a membrane potential estimated to be 135 mV, far too low to drive ATP synthesis against such a large pH gradient (Guffanti *et al.*, 1978). Several strains of uncoupler-resistant bacterial mutants have been isolated. One strain of *Bacillus megaterium*, was able to grow in high concentrations of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)(Decker and Lang, 1977). Guffanti *et al.* (1983), using vesicles prepared from the uncoupler resistant strain, were unable to measure any appreciable ATP synthesis on the production of a potassium diffusion potential across the vesicle membrane, showing the mutant strain was incapable of coupling phosphorylation to an electrochemical gradient. Similar uncoupler-resistant mutant strains of *E. coli* have also been isolated (Date *et al.*, 1980; Ito *et al.*, 1983).

Recently, experimentation has focussed on the possibility of protons remaining within the membrane or on the surface of the membrane without appreciable diffusion. This 'localised' hypothesis was originally proposed by Williams in 1961, who suggested that any proton driven process would be more effective if the proton gradients and ATP synthesis were more localised and under a form of diffusion control between the source and the F_oF_1-ATPase (Williams, 1961; see Williams, 1987). This theory originally precluded any role of bulk phase protons from being involved in ATP synthesis, but has more recently been revised to accommodate the irrefutable data outlined above (see Williams, 1978a). Evidence has been presented for the existence of a proton-diffusion barrier at the outer surface of the thylakoid membrane, by using the hydrophilic pH-indicating dye, cresol red (Junge and Aüslander, 1973: Aüslander and Junge, 1974). The rate of proton uptake was shown to be shielded by a proton diffusion barrier which could be removed either by sand-grinding the chloroplasts or by treatment with digitonin. van Walraven *et al.* (1984), working with proteoliposomes from the cyanobacteria *Synechococcus 6716* reconstituted with native carotenoids and F_oF_1-ATPase complexes, used the characteristic intramembranous carotenoid absorbance shift on protonation, along with the fluorescent dyes cresol red and neutral red to monitor proton diffusion after the production of a pH gradient generated by ATP hydrolysis. The primary response to the gradient was the carotenoid absorbance shift, indicating the immediate effect was felt either within the membrane or at the membrane interface, with a distinct lag period before the protons reached the aqueous dye, cresol red. The authors suggested the bulk phase acted mainly as an "energy buffer".

A recent series of experiments on the effects of membrane permeant buffers have shown that both localised and bulk phase protons are able to drive ATP synthesis. Mitchells' chemiosmotic hypothesis predicts that any lag period

experienced in chloroplast ATP synthesis after illumination will be proportional to the time taken to build up a sufficient transmembranous electrochemical gradient, and thus, to the buffering capacity of the bulk phases. Ort *et al.* (1976), originally maintained that the lag period before ATP synthesis in chloroplasts in response to illumination was independent of any permeant buffer, whilst other groups showed a dependence in keeping with the delocalised theory (Davenport and McCarthy, 1980; Vinkler*et al.*, 1980). This apparent contradiction was resolved by Dilley and Beard (1986), who showed the bulk phase, chemiosmotic, response was dependent on the chloroplasts being suspended in buffer containing a high concentratiom of K^+ ions.

Prats *et al.* (1985), had also noted a considerable K^+ effect on the translocation of protons along an artificial phospholipid monolayer. Using fluorescent probes covalently attached to the phospholipid headgroups, Teissie *et al.* (1985), had originally shown that proton diffusion along the monolayer was twenty times faster than diffusion into the aqueous phase surrounding the monolayer, concluding that protons were being transferred via hydrogen bonds between the lipid headgroups. However, on the addition of a buffer containing a high concentration of K^+ ions, this preferentially lateral proton diffusion was completely lost. Lange *et al.* (1975), had also noted a rapid motion of protons along lipid headgroups using proton NMR. At a pH below 5, reconstituted phospholipid vesicles containing phosphatidyl ethanolamine exhibited a resonance which was assigned as the amino proton resonance from the ethanolamine. Analysis of data led the authors to surmise that protons were moving rapidly along the bilayer via a hydrogen-bonded network between the amino-groups on the phospholipids, and interposed water molecules. The perturbation of this lateral localised proton motion has been suggested as a explanation for the effects of anaesthetics such as halothane or chloroform which have been shown to inhibit oxidative phosphorylation without affecting the proton electrochemical gradient (Rottenberg, 1983). The anaesthetics are postulated to bind to the membrane preventing protons from interacting with the F_oF_1-ATPase.

The 'semi-localised hypothesis' (Kell, 1979) postulates conduction of protons generated by electron transport, via localised channels both across the membrane and laterally, by charge-relay systems, which may be through chains of water molecules absorbed to the membrane (Grotthus transfer), or by proton tunnelling. Most of the functional proton current does not diffuse to the bulk phase. This theory has recently been updated (Westerhoff *et al.*, 1984) and proposes that the primary proton pumps (electron transfer components) and secondary pumps (F_oF_1-ATPases) are localised and form coupling units relying

on 'cross talk', proton motion, directly between the two pumps, constituting a 'mosaic protonic coupling'.

Slater *et al.*, (1985), have proposed a 'collision hypothesis' whereby protons are transferred directly between redox carriers and the F_oF_1-ATPase. This theory is based mainly on the observations of Hackenbrock *et al.* (see Gupte *et al.*, 1984 and Schneider *et al.*, 1980) and their illustration of lateral mobility of redox complexes in membranes of megamitochondria and rates of electron transfer in mitochondrial-liposome fusions. The large redox proteins were shown to be randomly dispersed, free to diffuse laterally and were not confined as large macromolecular structures. Slater *et al.* (1985) suggest the redox complexes transfer electrons and retain protons, becoming 'energised'. Protons can then be directly transferred from the energised complexes either to the F_oF_1-ATPase or occasionally to the bulk phase. However, topographical arrangements of membranes from the photosynthetic bacteria *Halobacterium halobium* include a separation of bacteriorhodopsin and F_oF_1-ATPase (Helgerson *et al.*, 1983) and a similar separation of photosystem II and CF_o-F_1 ATPase exists in chloroplasts (Anderson, J.M., 1981), observations which have not been fully reconciled by Slater *et al.*

The 'localised' hypothesis of Robertson and Boardman (1975) proposed that anhydrous protons may be stabilised within the lipid phase of the membrane as HCl, which is freely soluble in a lipophilic environment. The Cl^- ions are bound in a dehydrated form at the oxidised iron centres of cytochromes or non-haem iron. During respiration the quinol transfers electrons and the resultant protons are trapped by the chloride ions. HCl is then free to diffuse through the membrane until colliding with the F_oF_1-ATPase and transferring the proton intramembranously to the complex. Nichols and Deamer (1980), reported no increase in proton/hydroxide permeability across phospholipid membranes in the presence of varying concentrations of HCl, which suggests the protons would not be readily lost on contact of the HCl with either bulk phase. However, the abstraction of the proton from HCl to the F_oF_1-ATPase would be energetically demanding..

1.4 (ii) Conversion of proton electrochemical energy

All the theories mentioned above recognise the fundamental proton electrochemical nature of the coupling between oxidation and photosynthetic phosphorylation. However, they are concerned more with the transmission of the energy from redox components to ATP synthetic complexes, and do not address the problem of how the energy derived from the proton electrochemical

gradient is directed through the intramembranous F_o-moiety, to the active site of the F_1-sector in the aqueous phase. This can be effected by one of two ways;

a) The protons are delivered directly to the active site possibly via an aqueous pore or channeled as a dehydrated form, through a long charge relay system or 'proton wire' (Nagle and Morowitz, 1978; Nagle and Tristram-Nagle, 1983) or a combination of these. These protons may then be involved directly in the chemistry of ADP and Pi condensation (Mitchell, 1974, 1976; see below).

b) The protons do not reach the active site but are involved in fuelling a form of conformational change which can be coupled over a long distance.

Mitchells' original chemiosmotic molecular mechanism proposed that F_1 catalysis involves dehydrated protons sequestered from the aqueous well within the F_o. A more recent hypothesis (Mitchell, 1985), termed the 'rolling well and turnstile mechanism', envisages a hexameric F_1 structure of alternating α and β subunits rotating in opposite directions with a single central γ-subunit also rotating. A hydrolytic site is formed when specific domains of the α- and β-subunits are juxtaposed and protons are released from the F_o when this site is in close proximity to the 'open' domain of the γ-subunit which acts as a proton gate. The rotation between essentially repelling subunits is believed to produce a torque which can be used to pull substrates in to the active site and release products.

This model attempts to reconcile some observations. Firstly, several groups have suggested the possibility of γ functioning as a proton gate (Williams, N. *et al.*, 1984; Ketcham *et al.*, 1984; Futai and Kanazawa, 1983), and secondly, the catalytic site may be formed at the interface between α- and β-subunits.

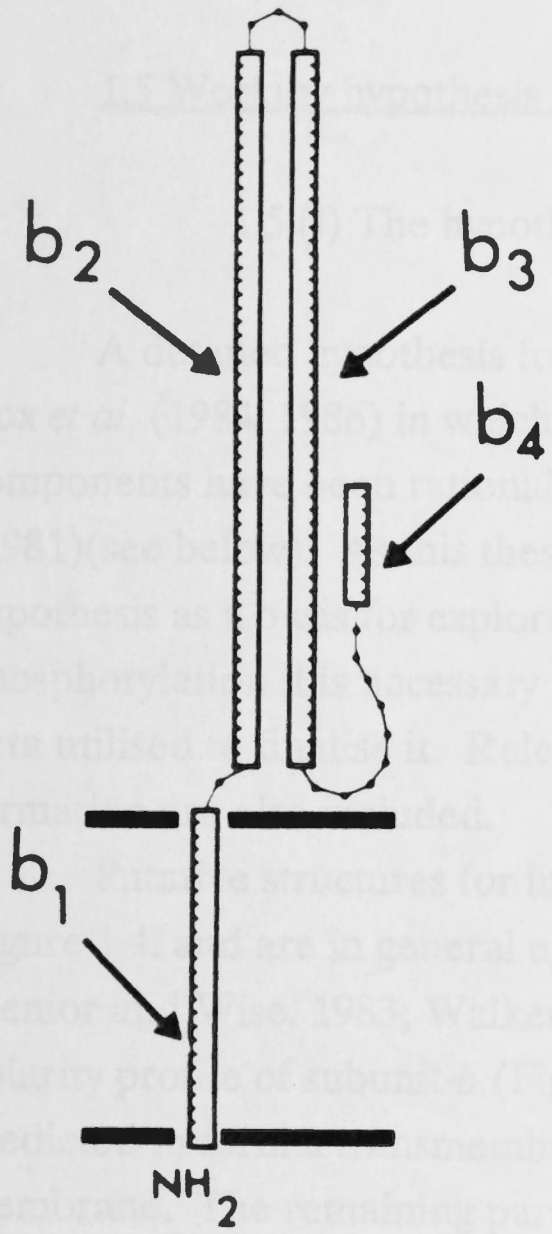
A recent model by Scarborough (1986) attempts to marry certain aspects of models from Mitchell, Boyer (see below), and Williams (see below). The chemistry of ATP production at the active site is similar to that suggested by Mitchell (1974), involving a pentavalent transition state. The F_o is seen as an aqueous pore delivering protons to the active site after a ligand induced hinge-bending type conformational change concomitantly traps the substrate in the active site.

Williams (1987), states there is no evidence that protons can approach the active site, although his earlier hypotheses had suggested a direct coupling of relatively dehydrated protons in a fixed water pore with the active site (Williams, 1978b, 1975). He suggests the protons which enter the F_o must be dehydrated as

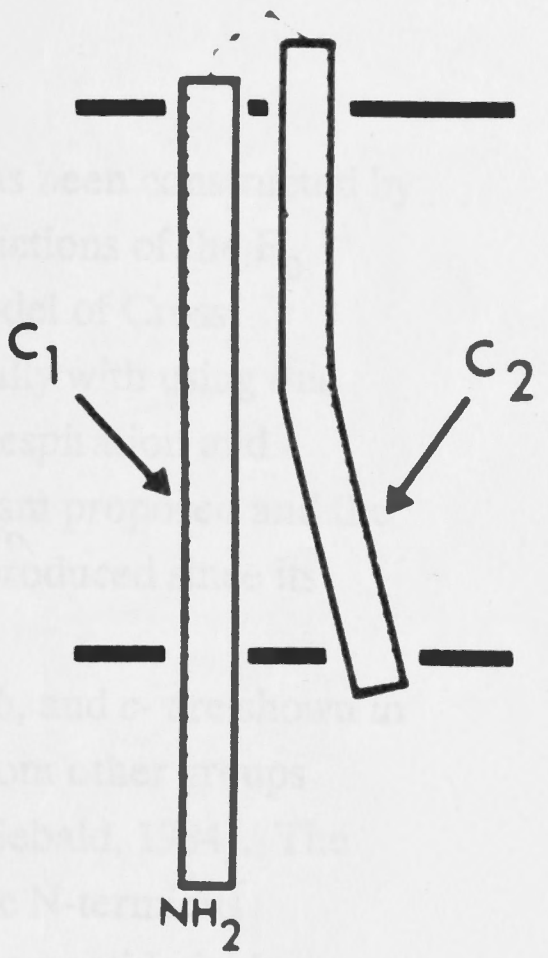
the selectivity of the process could not be guaranteed if larger hydrated forms were being translocated via an aqueous pore. Williams also states that it is unlikely the active site could be kept inaccessible to protons from the aqueous phase or that the flow could be sufficiently 'insulated' to continue through part of the F_1 without being dissipated. In a comparison with various kinases, Williams proposes a conformationally coupled hypothesis suggesting the F_1 acts as a 'hinge-bending enzyme', with the ion gradient energy being converted to mechanical energy via a hinged movement of two transmembranous helices of the F_o (Williams, 1986). This movement drives the conformational change within the catalytic site, simultaneously releasing product and revealing the substrate binding site. The analogy with general kinase enzymes is extended by pointing out that all adenine binding site domains characterised are constructed of β-sheets, proton motion through which would be extremely difficult.

Boyer (1975), maintains the proton gradient can drive phosphorylation by inducing a conformational change at the active site. Originally, this change was seen to be coupled to exposure of charged groups on the protein to different sides of the membrane. However, this theory has been continually updated to accommodate new data. A three site catalytic mechanism was proposed on the basis of catalytic cooperativity (Gresser *et al.*, 1982; Cross, 1981) with the protonmotive force loosely implicated in rotating the β-subunits with respect to the static inner core of the F_1. Kandpal and Boyer (1987), attempted to cross-link subunits -β and -γ with a photocleavable reagent to explore the possibility of proton energised rotation between the minor subunit and the major subunits. Surprisingly, this experiment was attempted in purified F_1-ATPase only, and not the complete complex. The results were inconclusive. Grubmeyer *et al.* (1982), estimated the equilibrium constant for catalysis of the reaction ATP ADP + Pi to be approximately 0.5 in solubilised MF_1 and also reported an MF_1 high-affinity binding-site for ATP of $10^{12}.M^{-1}$. The demonstration that ATP could be formed at the high affinity binding sites with almost no loss of free energy suggested the major energy requirement in oxidative phosphorylation is for the release of the tightly bound ATP. More recently, Penefsky (1985), by measuring the binding and release of [γ-^{32}P] labeled ATP, reported a similar high-affinity binding-site in the intact MF_oF_1-ATPase complex in submitochondrial particles. The binding affinity was markedly decreased when sufficient concentrations of oligomycin or DCCD were added to the particles to produce a concomitant 90% inhibition of ATP-hydrolysis. Both oligomycin and DCCD are believed to effect oxidative phosphorylation or ATP hydrolysis by binding within the F_o-moiety of the complex, prompting Penefsky to postulate that the two inhibitors may function by causing a conformational change on binding within the F_o which

Fig 1.4. Predicted secondary structure for the *E. coli* F_o-F_1-ATPase subunits -*b* and -*c* (Cox *et al.*, 1984). The helical regions of the *b*- and *c*-subunits are designated b_1, b_2, b_3, b_4 and c_1, c_2. The amino terminal of each subunit is shown. (a) Predicted secondary structure of subunit-*b*. Helix b_1, the N-terminal helix is strongly hydrophobic and is depicted in the membrane. Helices b_2, b_3 and b_4 protrude into the cytoplasm and are believed to bind to subunits within the F_1-ATPase. (b) Predicted secondary structure for subunit-*c*. Each subunit is predicted to enter the membrane in a helical configuration, with a slight bend in helix 2 at Pro-64. Supporting data for these structures is detailed in section 1.5.(i).



(a)



(b)

results in impaired binding of the substrate at the active site. The author also suggests that during ATP synthesis, a reversible conformational change occurs, initially within the F_O, possibly through ionisation of one or more amino-acids, which is then transmitted to the F_1, resulting in liberation of the tightly bound ATP.

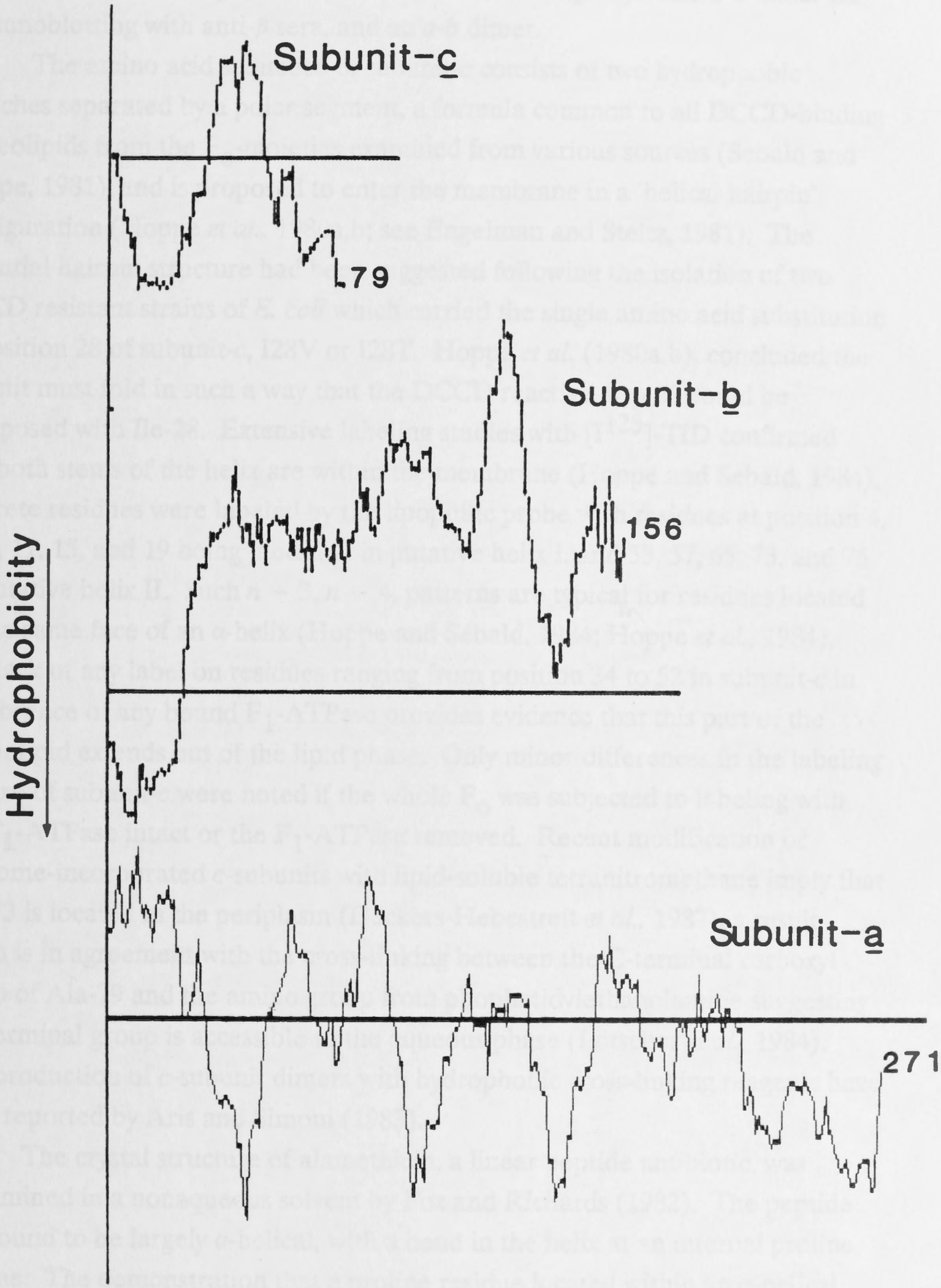
1.5 Working hypothesis and experimental approach

1.5.(i) The hypothesis

A detailed hypothesis for coupled ATP synthesis has been constructed by Cox *et al.* (1984, 1986) in which theoretical structural predictions of the F_O components have been rationalised with respect to the model of Cross (1981)(see below). As this thesis is concerned fundamentally with using this hypothesis as a basis for exploring the coupling nature of respiration and phosphorylation it is necessary to detail the exact mechanism proposed and the data utilised to finalise it. Relevant data which has been produced since its formation are also included.

Putative structures for intramembranous subunits -*b*, and *c*- are shown in Figure 1.4. and are in general agreement with proposals from other groups (Senior and Wise, 1983; Walker *et al.,* 1982b; Hoppe and Sebald, 1984). The polarity profile of subunit-*b* (Fig 1.5) reveals a hydrophobic N-terminus predicted to form a transmembranous α-helix anchoring the peptide in the membrane. The remaining part of the molecule is polar and is believed to constitute a stalk which juts out into the aqueous phase. Proteolytic studies of F_1-depleted complexes revealed subunit-*b* to be highly susceptible to degradation from the C-terminus. This cleavage prevented any binding of F_1, but did not affect proton translocation through the F_O, whilst subunit-*b* was protected from proteolysis by the bound F_1-ATPase (Perlin *et al.,* 1983; Hoppe *et al.,* 1983; Perlin and Senior, 1985; Steffens *et al.,* 1987). Labeling with the photoactivatable lipophilic probe [I^{125}]-TID (3-trifluoromethyl-*m*-(iodophenyl)diazirine) revealed selective modifications from residue 3 to residue 26 of subunit-*b*, with a 'shallow' probe (a phospholipid derivative bearing a phenylazido precursor, which will label residues at the periphery of the membrane, (see Sebald and Hoppe, 1984) labeling residues 2 and 21. As has been previously mentioned (Chapter 1, section 1.2.(ii)) subunit-*b* is present in two copies per complex. The two copies are in close proximity within the complex as they can be cross-linked (Hermolin *et al.,* 1983; Aris and Simoni,

Fig 1.5. Hydropathy profiles of *E. coli* F_oF_1-ATPase subunits *-a, -b* and *-c*. Each polypeptide is viewed as a continuous α-helix and the water-to-oil transfer free energies for successive 19 amino acid segments plotted as a function of the first amino acid in a given segment. The horizontal line represents a free energy of insertion of 0. Areas under this line represent hydrophobic regions and those above the line are calulated as hydrophilic (Engelman *et al.*, 1982). The numbers of residues in each polypeptide are given at the end of each plot.

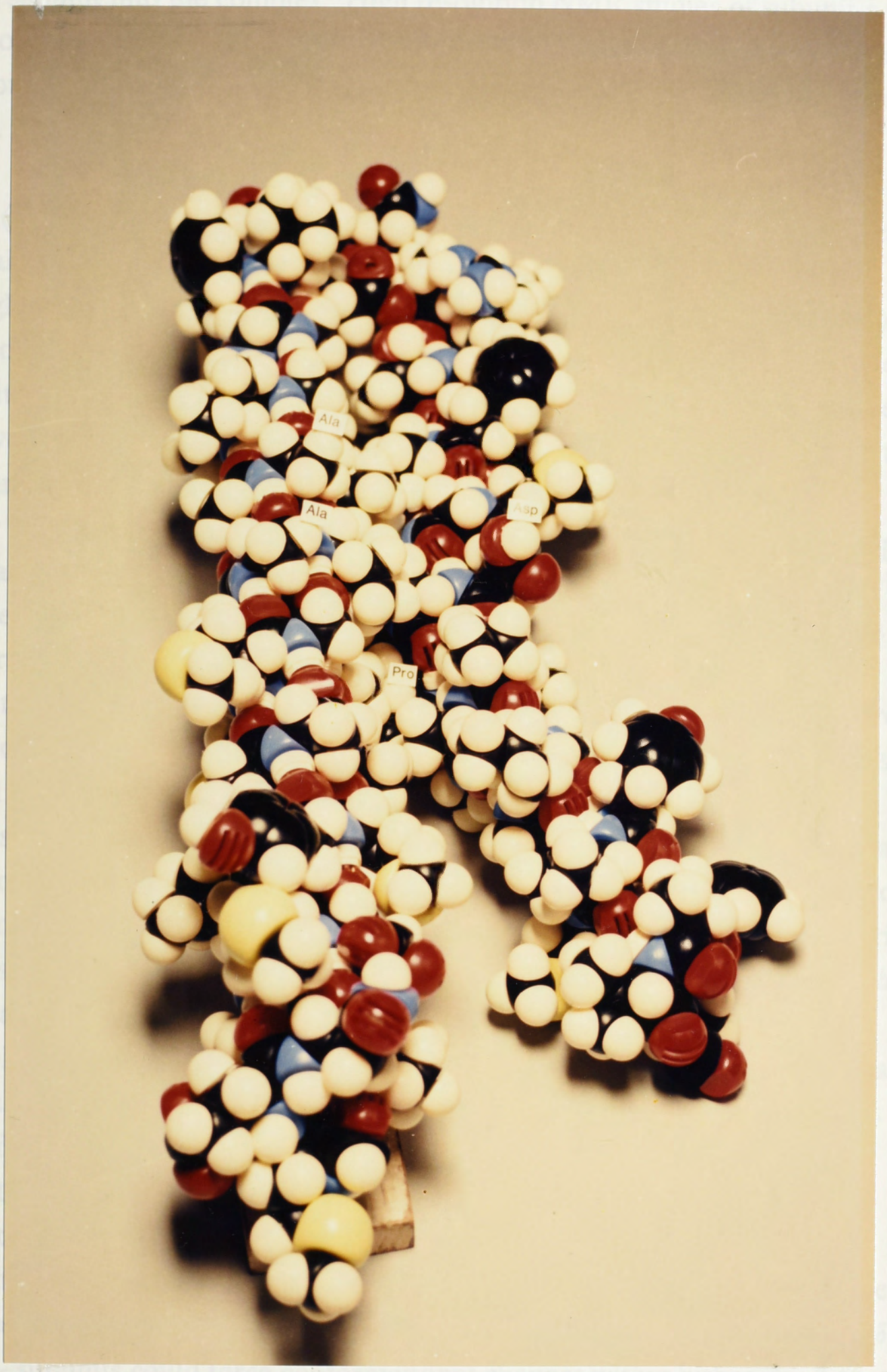

Subunit-c
79
Subunit-b
156
Hydrophobicity
Subunit-a
271

1983). The crosslinking results from Aris *et al.* also highlighted a *β-b* dimer on immunoblotting with anti-*β* sera, and an *a-b* dimer.

The amino acid sequence of subunit-*c* consists of two hydrophobic stretches separated by a polar segment, a formula common to all DCCD-binding proteolipids from the F_o-moieties examined from various sources (Sebald and Hoppe, 1981), and is proposed to enter the membrane in a 'helical hairpin' configuration (Hoppe *et al.,* 1980a,b; see Engelman and Steitz, 1981). The potential hairpin structure had been suggested following the isolation of two DCCD resistant strains of *E. coli* which carried the single amino acid substitution at position 28 of subunit-*c*, I28V or I28T. Hoppe *et al.* (1980a,b), concluded the subunit must fold in such a way that the DCCD reactive Asp-61 could be juxtaposed with Ile-28. Extensive labeling studies with [I^{125}]-TID confirmed that both stems of the helix are within the membrane (Hoppe and Sebald, 1984). Discrete residues were labeled by the lipophilic probe with residues at position 4, 8, 10, 11, 15, and 19 being modified in putative helix I, and 53, 57, 65, 73, and 76 on putative helix II. Such $n + 3, n + 4$, patterns are typical for residues located on the same face of an α-helix (Hoppe and Sebald, 1984; Hoppe *et al.,* 1984). The lack of any label on residues ranging from position 34 to 52 in subunit-*c* in the absence of any bound F_1-ATPase provides evidence that this part of the proteolipid extends out of the lipid phase. Only minor differences in the labeling pattern of subunit-*c* were noted if the whole F_o was subjected to labeling with the F_1-ATPase intact or the F_1-ATPase removed. Recent modification of liposome-incorporated *c*-subunits with lipid-soluble tetranitromethane imply that Tyr-73 is located in the periplasm (Deckers-Hebestreit *et al.,* 1987), a result which is in agreement with the cross-linking between the C-terminal carboxyl group of Ala-79 and the amino group from phophatidylethanolamine suggesting the terminal group is accessible to the aqueous phase (Lötscher *et al.,* 1984). The production of *c*-subunit dimers with hydrophobic cross-linking reagents have been reported by Aris and Simoni (1983).

The crystal structure of alamethicin, a linear peptide antibiotic, was determined in a nonaqueous solvent by Fox and Richards (1982). The peptide was found to be largely α-helical, with a bend in the helix at an internal proline residue. The demonstration that a proline residue located within an α-helical region may cause an internal bend, was cited as evidence for an internal bend in helix II of the *E. coli* F_oF_1-ATPase subunit-*c* at Pro-64, and was illustrated by the molecular model of the proposed subunit-*c*, constructed with Corey-Pauling-Koltun (CPK) units (Fig 1.6). . Evidence for a bend in the C-terminal helix (helix II) of subunit-*c* has also been inferred through the mutational analysis of Fimmel *et al.* (1983) The substitution of Pro-64 by Leu results in an uncoupled

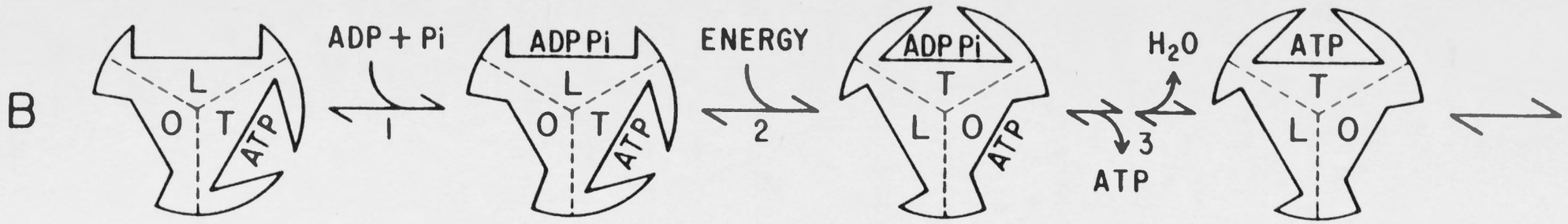
Fig 1.6 Space-filling model of the predicted tertiary structure of the *c*-subunit. The molecule is depicted as a helical hairpin (see text). The bend in helix II caused by proline at position 64 can be clearly seen. The model is arranged such that the residue labeled Asp, the DCCD-binding residue Asp-61, is facing upwards.


Ala
Ala
Asp
Pro

phenotype. Partial revertants of this strain were isolated which harboured a second mutation at position 20, Ala-20 to Pro. The authors conclude that the bend formed by an intramembranous proline is required for normal integration of the subunit into the complex. High resolution NMR studies of subunit-*c* show the molecule has extensive α-helical regions and indicate a region of motional freedom near the C-terminal consistent with the predicted bend (Moody *et al.*, 1987).

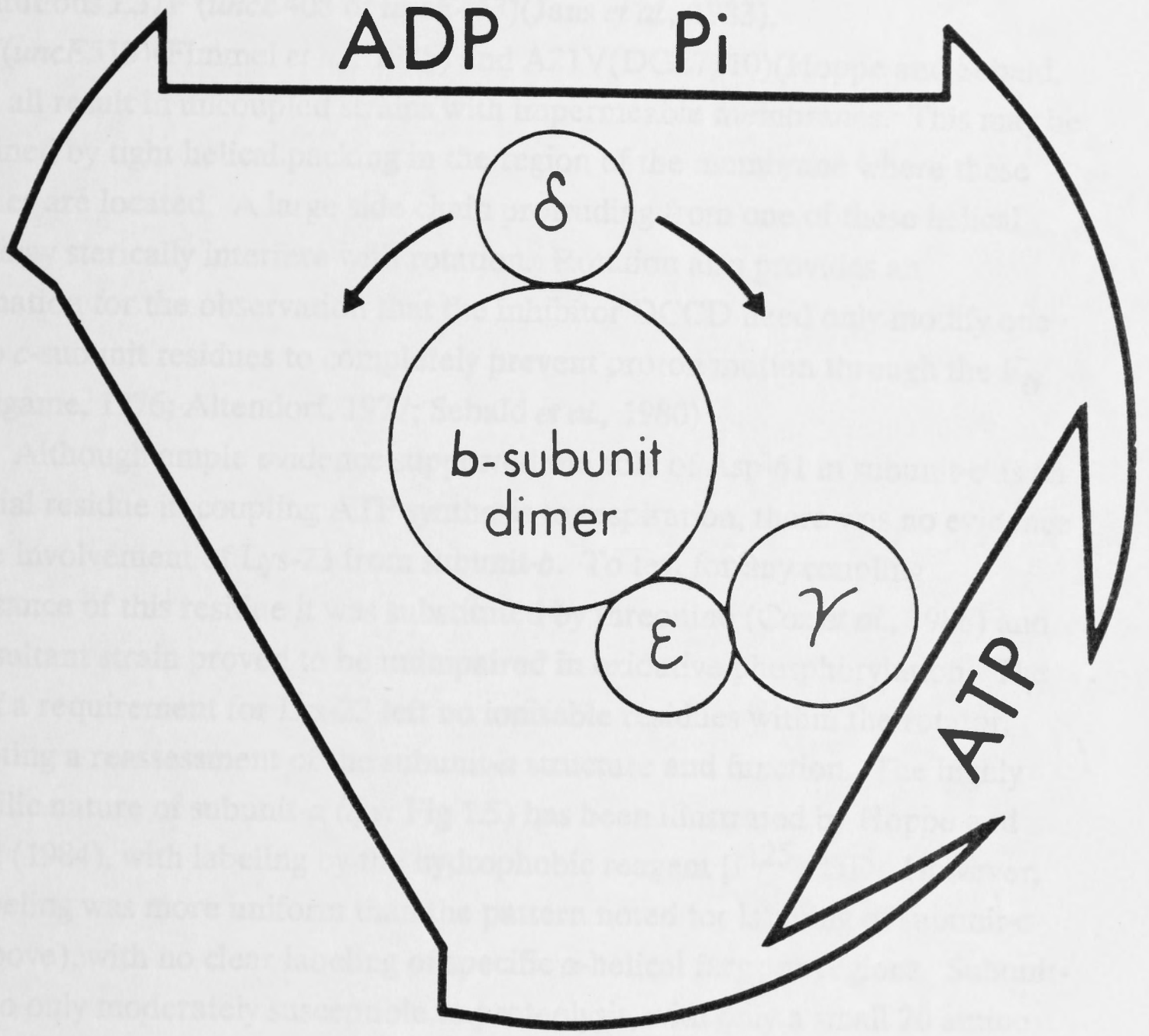
The original model was proposed to account for the kinetic studies of Cross (1981), in which three interacting catalytic sites, on energisation, undergo cyclical interconversions between three forms; a loose-binding non-catalytic site, through a tight-binding catalytic to a low affinity non-catalytic state (Fig 1.8). The conformational change in the F_1-ATPase is produced by rotation of the *b*-subunits and associated minor subunits of the F_1. This rotation was suggested to be driven by sequential protonation and deprotonation of residue Lys-23 in the *b*-subunit dimer and residue Asp-61 in a surrounding ring of *c*-subunits, the energy for the protonation/deprotonation steps being derived from delta μ_H+. An analogous situation occurs in proton-driven bacterial flagellar rotation where the internal 'rotator' moves within an external 'stator' on the generation of a membrane potential. However, the mechanism of flagellar rotation is not understood (McNab, 1986; McNab and Aizawa, 1986). A purely structural role was alloted to subunit-*a*, as all 27 mutations affecting this subunit characterised, proved to be chain-terminating (L. Hatch personal communication). No missense mutations in subunit-*a* had been reported in the literature. Translation of the rotation of subunit-*b* with respect to the external *c*-subunit multimer to conformational changes within F_1 is effected by the rotation of an asymmetric minor subunit arrangement attached to the long aqueous helices of subunit-*b* within a static ring of an alternating α-, β- subunit hexamer (Fig 1.7.) The hydrophilic portions of the subunit-*b* dimer independently constitute a helical hairpin, extending approximately 8 nm in to the cytoplasm. One of the arms of each of the helices participates in intermolecular interactions between *b*-subunits, with another arm involved in direct interaction with the two minor subunits -δ and -ε, possibly by cationic bridging. The γ-subunit is known to interact tightly with the ε-subunit (Dunn, 1982) and is proposed to add to the asymmetry of the rotor by binding with ε. On rotation, the proximity of the δ-, or γ-/ε- subunits could force conformational changes within either -α, -β or -α/-β interface, cycling through three different sites in exactly the cooperative fashion suggested by Cross (1981) or Boyer (see Gresser *et al.*, 1982). Several groups have shown an asymmetry of the β-subunits in the F_1-ATPase after

Fig 1.7 The cyclic energy-dependent binding change mechanism for ATP synthesis as detailed by Cross (1981). The scheme recognises catalytic cooperativity (Kayalar *et al.*, 1977; Gresser *et al.*, 1982) and assumes three interacting sites per F_1-ATPase. Upon energisation in step 2, the catalytic sites of the F_1-ATPase are interconverted between three forms: L, loose binding for ligands and catalytically inactive : O, open site with very low affinity for ligands and catalytically inactive : T, tight binding site for ligands and catalytically active. The scheme shows one third of the enzyme cycle.



The cyclic energy-dependent binding change mechanism. Cross (1981).

Fig 1.8. Diagrammatic representation of the arrangement of the *b*-subunit dimer and associated -δ and -ε/-γ subunits of the F_1-ATPase, in relation to the three alternating catalytic sites of the -α/-β subunit hexamer. The catalytic sites interconvert through the three conformations of substrate binding, catalysis and product release (the energy-dependent step), as proposed by Cross (1981). It is proposed (see text) that the site progression is promoted by rotation of the subunit-*b* dimer, and concomitant juxtaposition of the attached minor F_1-subunits to the respective catalytic sites. No inference however, should be drawn between the position of the minor subunits of the F_1-ATPase and the particular active site conformation.


ADP
Pi
δ
b-subunit
dimer
ε
γ
ATP

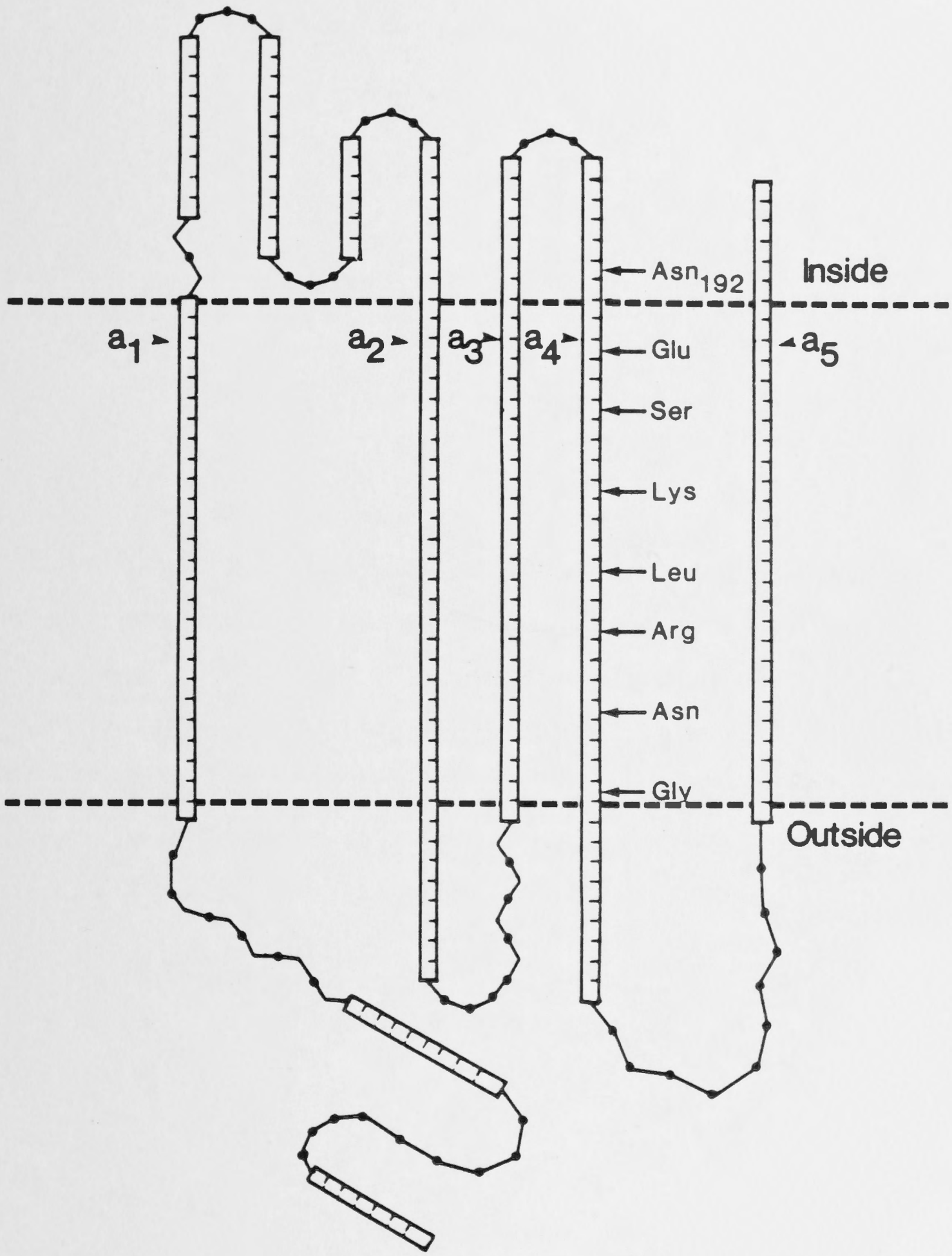
inhibition of catalysis (Lotscher and Capaldi, 1984; Stan-Lotter and Bragg, 1986; Melese and Boyer, 1985)

A rotational hypothesis of the sort outlined above can explain the deleterious affects of amino acid substitutions characterised in subunit-*c*. Several mutants carry amino acids with larger side chains than the normal residue. The substitutions L31F (*uncE*408 or *uncE463*)(Jans *et al.,* 1983), A25T(*uncE*513)(Fimmel *et al.,* 1985) and A21V(DG27/10)(Hoppe and Sebald, 1984) all result in uncoupled strains with impermeable membranes. This may be explained by tight helical packing in the region of the membrane where these residues are located. A large side chain protruding from one of these helical faces may sterically interfere with rotation. Rotation also provides an explanation for the observation that the inhibitor DCCD need only modify one or two *c*-subunit residues to completely prevent proton motion through the F_o (Fillingame, 1976; Altendorf, 1977; Sebald *et al.,* 1980)

Although ample evidence supported the role of Asp-61 in subunit-*c* as an essential residue in coupling ATP synthesis to respiration, there was no evidence for the involvement of Lys-23 from subunit-*b*. To test for any coupling importance of this residue it was substituted by threonine (Cox *et al.*, 1986) and the resultant strain proved to be unimpaired in oxidative phosphorylation. The lack of a requirement for Lys-23 left no ionisable residues within the rotator, prompting a reassessment of the subunit-*a* structure and function. The highly lipophilic nature of subunit-*a* (*q.v.* Fig 1.5) has been illustrated by Hoppe and Sebald (1984), with labeling by the hydrophobic reagent [I^{125}] TID. However, the labeling was more uniform than the pattern noted for labeling of subunit-*c* (see above), with no clear labeling of specific α-helical faces or regions. Subunit-*a* is also only moderately susceptible to proteolysis, with only a small 20 amino acid fragment being removed from the cytoplasmic side by subtilisin (Hoppe *et al.,* 1983). Secondary structures proposed for subunit-*a* have included six or seven transmembrane helices (Walker *et al.*, 1984; Hermolin *et al.*, 1983; Hoppe and Sebald, 1984; Senior, 1983). The *a*-subunit was predicted by Cox *et al.* (1986), to fold with five intramembranous helices (Fig 1.9.). Analogous subunits from several other species can be predicted to share a similar structure, with a remarkably conserved region proposed to comprise helix IV. Primary sequences of subunit-*a* analogues from many diverse species have been published and their homology around proposed helix IV is shown in Fig 1.10.

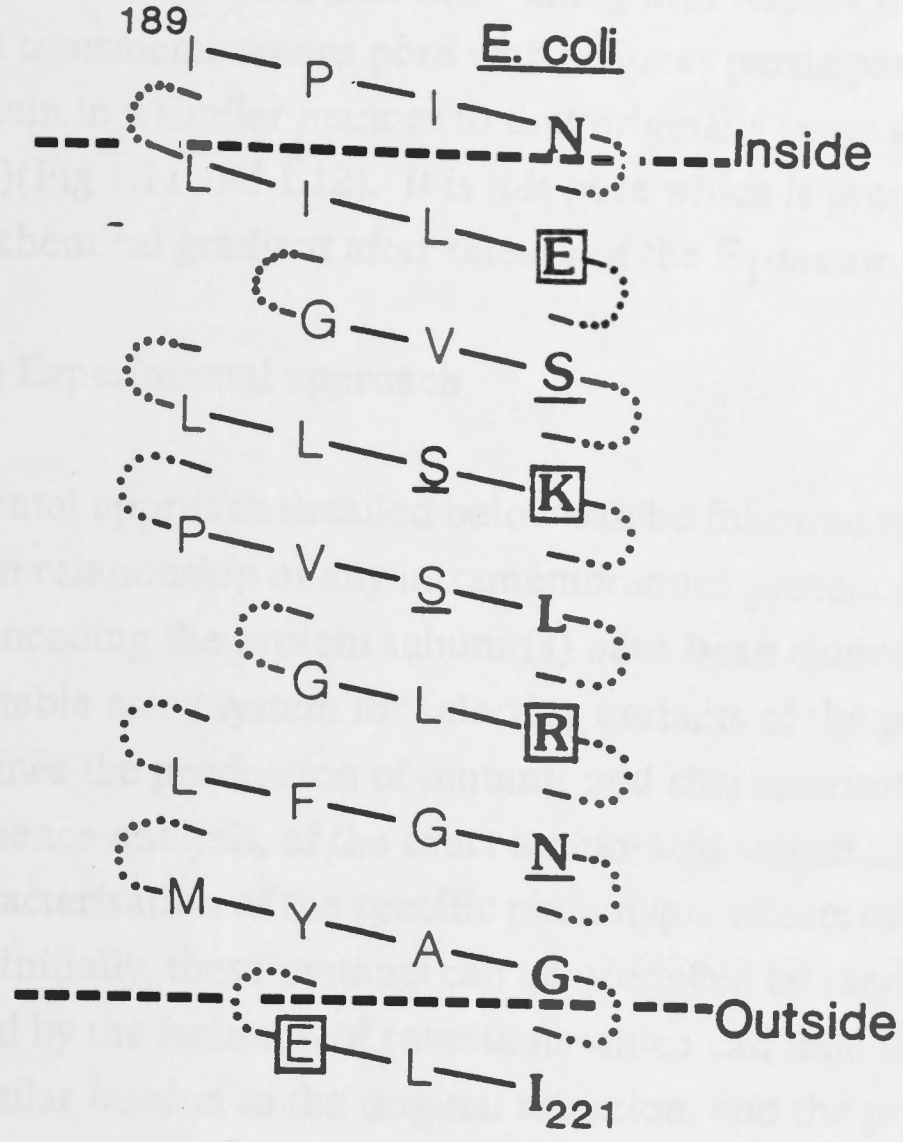
The 'working model' at the time of commencement of the work described in this thesis is depicted in figures 1.9, 1.11 and 1.12. The rotator consists of subunits -*a,* and -*b* within the membrane, moving against a static ring of *c*-subunits, such that residues on subunit-*a* helix IV form a transmembranous pore

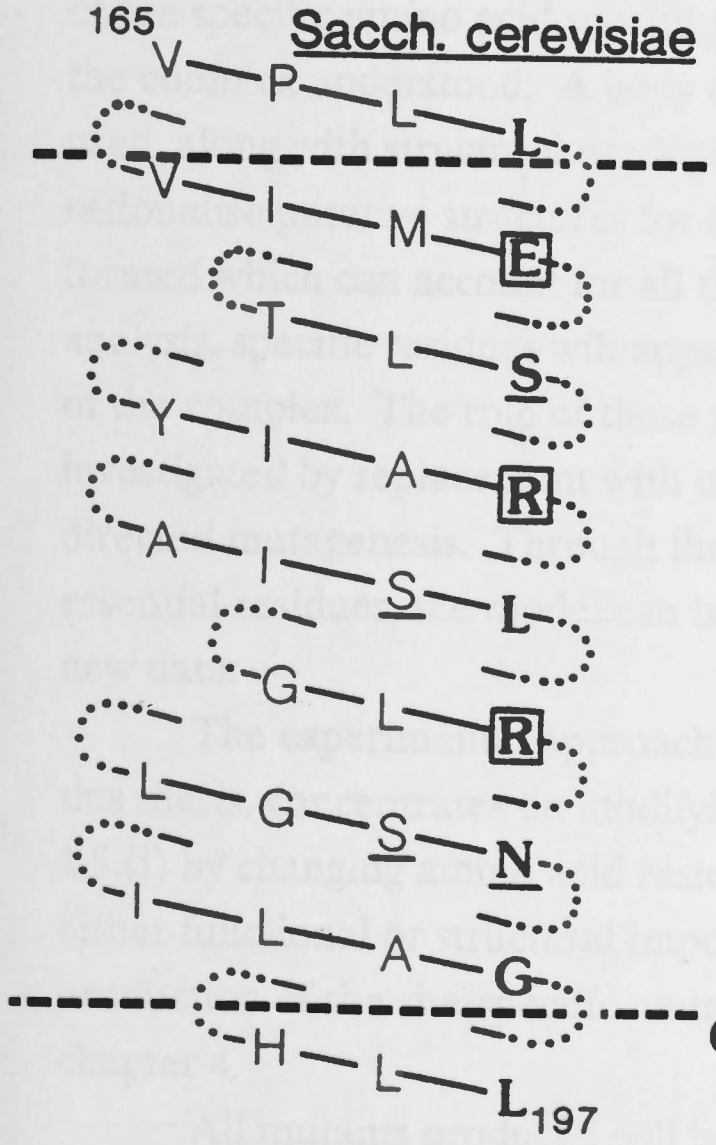
Fig 1.9 Proposed secondary structure of the *E. coli* F_oF_1-ATPase subunit-*a* (Cox *et al.*, 1986). The structure was deduced from the amino acid sequence by consideration of the polarity profile, the application of Chou-Fasman rules for secondary structure prediction (Chou and Fasman, 1978)(with the modification that those portions of proteins located within the membrane are α-helical), and with regard for the effect of the membrane potential on the charge distribution of the assembled protein (a preponderance of basic amino acids on the cytoplasmic side of the membrane). Comparisons were also made with subunit-*a* analogues from mitochondria and chloroplasts from diverse sources.

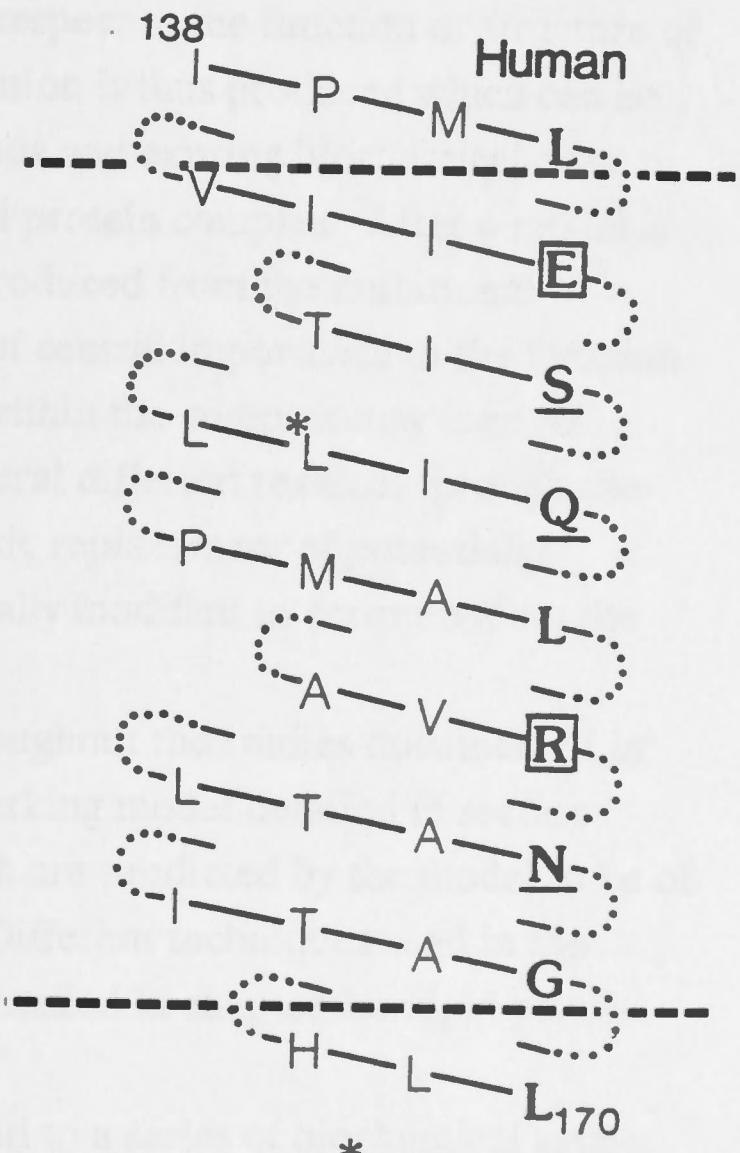


α-SUBUNIT : *E. coli*

Fig 1.10. Conserved polar residues of the amphipathic helical face of helix IV of the *E. coli* F_o subunit-*a* and the *Saccharomyces cerevisiae* and human analogue, ATPase-6 (Cox *et al.*, 1986). Residues are numbered at the N- and C- terminal of each putative helix, with ionisable residues boxed and polar residues underlined.


189
E. coli
I P I N
Inside
L I L E
G V S
L L S K
P V S L
G L R
L F G N
M Y A G
E L I221
Outside


165
Sacch. cerevisiae
V P L L
Inside
V I M E
T L S
Y I A R
A I S L
G L R
L G S N
I L A G
H L L197
Outside


138
Human
I P M L
V I I E
T I S
L *L I Q
P M A L
A V R
L T A N
I T A G
H L L170
*F in bovine

each time they are adjacent to any of the Asp-61 residues on the multiple *c*-subunits. One face of the amphipathic helix IV along with Asp-61 from the *c*-subunits constitute a transmembranous pore with residues participating in a hydrogen-bonded chain in a similar manner to that originally suggested by Nagle and Morowitz (1978)(Fig 1.11 and 1.12). It is this pore which is proposed to dissipate the electrochemical gradient after release of the F_1-sector.

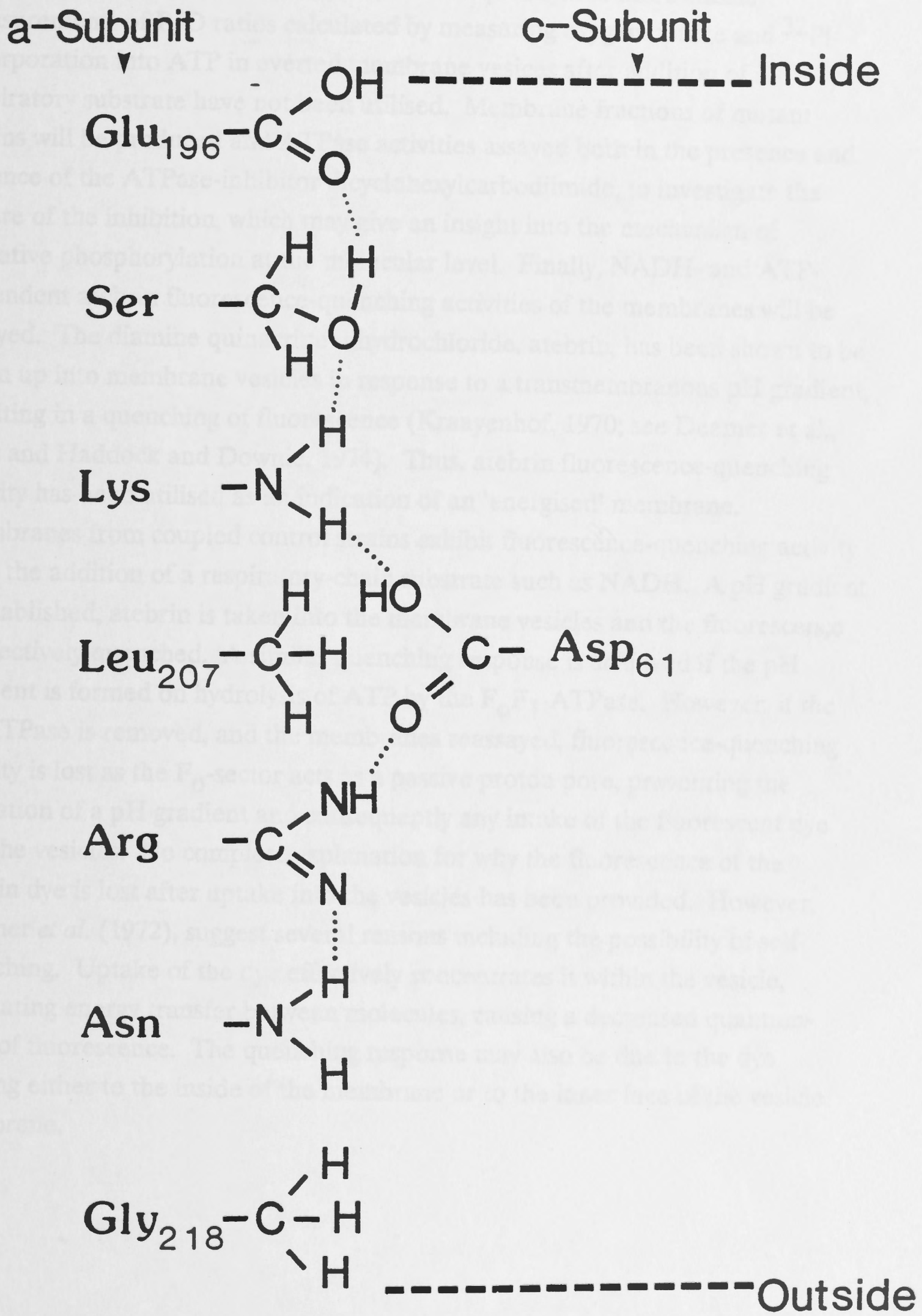
1.5.(ii) Experimental approach

The experimental approach detailed below can be followed in studying the structure-function relationship of any intramembranous protein complex, provided the genes encoding the protein subunit(s) have been cloned and sequenced, and a suitable assay system for selecting mutants of the protein exists. This approach combines the production of mutants and characterisation, through DNA or protein sequence-analysis, of the exact amino-acid substitution(s), with the biochemical characterisation of the specific phenotypic effects of the amino-acid substitution(s). Initially, these mutants can be produced by random mutagenesis, followed by the isolation of revertants which can then be characterised in a similar fashion to the original mutation, and the precise nature of the specific amino acid substitution with respect to the function or structure of the complex understood. A body of information is thus produced which can be used, along with structural prediction methods and existing biochemical data, to rationalise putative structures for the overall protein complex. After a model is formed which can account for all the data produced from the mutational analysis, specific residues will appear to be of central importance to the function of the complex. The role of these residues within the complex may then be investigated by replacement with one or several different residues through site-directed mutagenesis. Through the systematic replacement of potentially essential residues, the model can be continually modified to accommodate the new data.

The experimental approach used throughout the studies documented in this thesis, concentrates on modifying the working model detailed in section 1.5.(i) by changing amino acid residues which are predicted by the model to be of either functional or structural importance. Different techniques used in the production of the site-specific mutants are detailed in chapter 3 and, in part, in chapter 4.

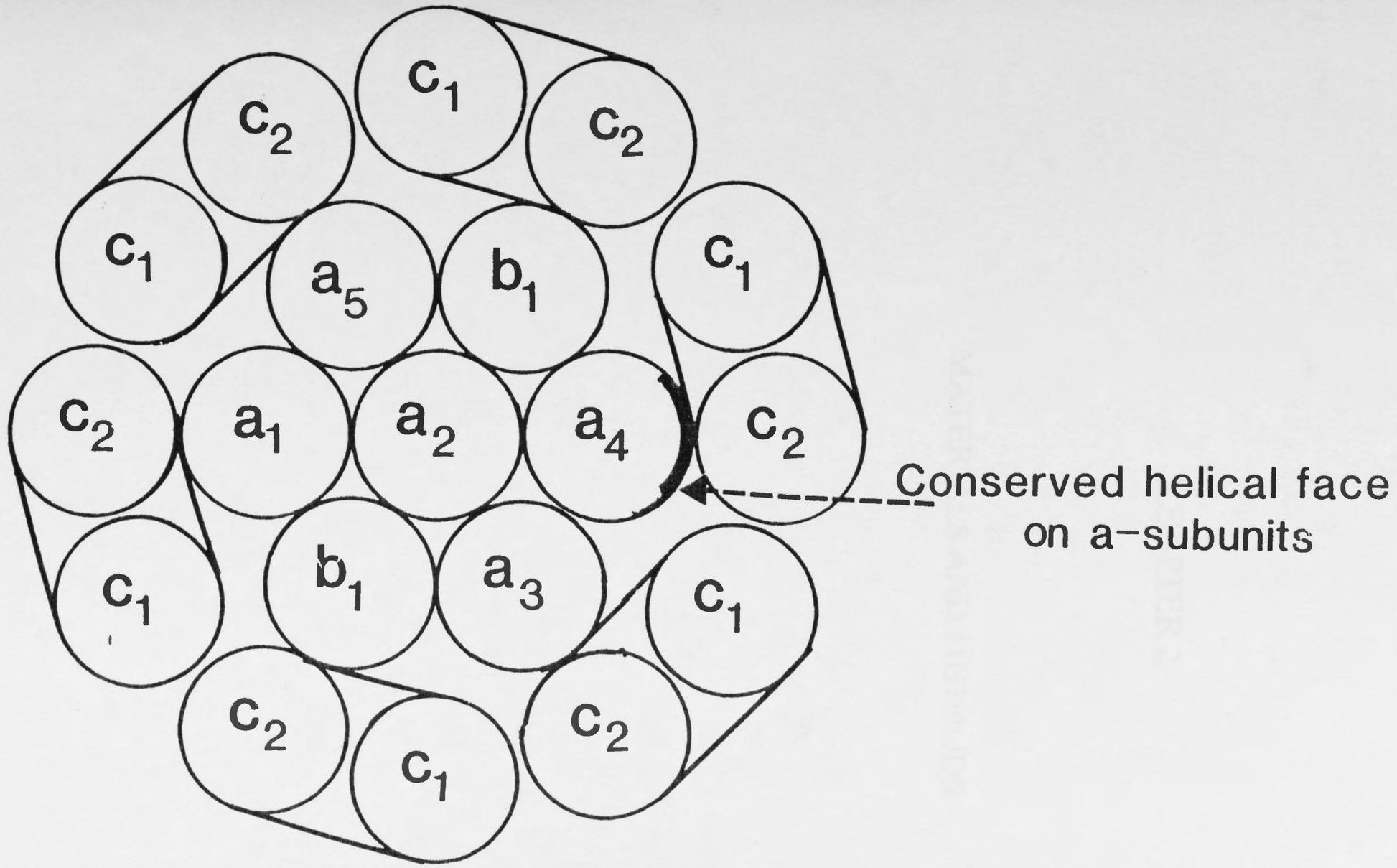
All mutants produced will be subjected to a series of biochemical assays to characterise the mutant at the phenotypic level. Growth yields will be assayed on the mutant strains. The growth yields of cells per mole of glucose is a

Fig 1.11. Proposed formation of a proton pore following interaction of the polar groups on subunit-*a* of *E. coli* F_oF_1-ATPase and the Asp-61 of subunit-*c* (Cox *et al.,* 1986)(see text). Residues on the conserved helical face of the subunit-*a* helix IV are shown interacting with the Asp-61 residue in subunit-*c* to constitute a hydrogen-bonded network which is predicted to facilitate movement of protons across the membrane.


a-Subunit
c-Subunit
Inside
OH
Glu196
O
H
H
Ser
O
H
H
Lys
N
H
H
HO
Leu207
H
C
H
C
Asp61
H
O
NH
Arg
C
N
H
Asn
N
H
H
Gly218
H
H
Outside

measure of the efficiency of the conversion of growth substrate in to cellular mass and appears to be closely related to the efficiency of oxidative phosphorylation (Stouthammer, 1977). Consequently, the more classic measurements of P/O ratios calculated by measuring oxygen uptake and ^{32}Pi incorporation into ATP in everted membrane vesices after addition of a respiratory substrate have not been utilised. Membrane fractions of mutant strains will be produced and ATPase activities assayed both in the presence and absence of the ATPase-inhibitor dicyclohexylcarbodiimide, to investigate the nature of the inhibition, which may give an insight into the mechanism of oxidative phosphorylation at the molecular level. Finally, NADH- and ATP-dependent atebrin fluorescence-quenching activities of the membranes will be assayed. The diamine quinacrine dihydrochloride, atebrin, has been shown to be taken up into membrane vesicles in response to a transmembranous pH gradient, resulting in a quenching of fluorescence (Kraayenhof, 1970; see Deamer et al., 1972 and Haddock and Downie, 1974). Thus, atebrin fluorescence-quenching activity has been utilised as an indication of an 'energised' membrane. Membranes from coupled control strains exhibit fluorescence-quenching activity after the addition of a respiratory-chain substrate such as NADH. A pH gradient is established, atebrin is taken into the membrane vesicles and the fluorescence is effectively quenched. A similar quenching response is achieved if the pH gradient is formed on hydrolysis of ATP by the F_oF_1-ATPase. However, if the F_1-ATPase is removed, and the membranes reassayed, fluorescence-quenching activity is lost as the F_o-sector acts as a passive proton pore, preventing the formation of a pH gradient and consequently any intake of the fluorescent dye into the vesicles. No complete explanation for why the fluorescence of the atebrin dye is lost after uptake into the vesicles has been provided. However, Deamer *et al.* (1972), suggest several reasons including the possibility of self-quenching. Uptake of the dye effectively concentrates it within the vesicle, facilitating energy transfer between molecules, causing a decreased quantum-yield of fluorescence. The quenching response may also be due to the dye binding either to the inside of the membrane or to the inner face of the vesicle membrane.

Fig 1.12. Possible packing of the transmembranous helices of the *a*- and *b*- subunits of the *E. coli* F_O-moiety within a surrounding ring of *c*-subunits , allowing interaction between the *a*- and *c*- subunits. The number of *c*-subunits shown is six, but estimates vary from between 6-12 (*q.v.* Chapter 1, section 1.2.(ii)). An increased number of *c*-subunits could be accommodated in this model.


c1
c2
c1
c2
c1
a5
b1
c2
a1
a2
a4
c2
Conserved helical face
on a-subunits
c1
b1
a3
c1
c2
c1
c2

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and Enzymes

All chemicals and enzymes were of the highest purity commercially available. $\alpha[^{32}P]$ deoxyadenosine triphosphate and $\gamma[^{32}P]$ adenosine triphosphate were obtained from Amersham (Australia) Pty. Ltd. Dideoxynucleotides and deoxynucleotides were from either Boehringer Mannheim (Australia) Pty Ltd, or Biotechnology Research Enterprises South Australia Pty. Ltd. Oligodeoxyribonucleotides were either purchased from the Centre for Science and Industrial Research Organisation (CSIRO) Division of Plant Industry, Canberra, Australia. or donated as a kind gift by Dr.A.E. Senior, Rochester Medical School, Rochester, New Jersey, U.S.A. *E. coli* DNA modifying enzymes DNA ligase, pol III holoenzyme and single-stranded binding protein were kindly donated by Dr.N.E.Dixon, Research School of Chemistry, Australian National University, Canberra. Restriction enzymes and other DNA modifying enzymes were purchased from Amersham (Australia) Pty Ltd., Boehringer Mannheim, Australia, Bethesda Research Laboratories (U.S.A.) or Biotechnology Research Enterprises South Australia Pty. Ltd (BRESA).

2.2 Media used for growth of bacteria

2.2.(i) Media

All media were sterilised by autoclaving at 121^{o}C for 20 min.

2.2.(ii) Growth supplements

All growth supplements, prepared as concentrated solutions, and sterilised separately, were added to sterile medium as required. Glucose or succinate, unless otherwise specified, were maintained at 33 mM final concentration. Other supplements used are indicated (Final concentration):

1 μM Thiamine (Vitamin B1)
0.7 mM L-Arginine
0.32 mM L-Leucine
0.70 mM L-Threonine
30 μM 2,3 Dihydroxybenzoate
0.2 mM Uracil
0.05% w/v Casein hydrolysate

The following antibiotics were used:

10 μg.ml^{-1} Tetracycline
90 μg.ml^{-1} Chloramphenicol

2.2.(iii) Luria Broth

Luria broth, described by Luria and Burrous (1957), consisted of (per litre):

10 g Bacto-Tryptone
5 g Yeast extract
10 g Sodium chloride

with the pH of the solution being adjusted to 7.0 with sodium hydroxide prior to autoclaving.

2.2.(iv) Luria-glycerol

Luria-glycerol, used for the storage of bacterial strains at -20^{0}C, consisted of Luria broth containing 20% v/v glycerol.

2.2.(v) Minimal media

The minimal medium used for growth of strains was similar to that described by Monod et al. (1951) as medium 56, and contained (per litre):

10.6 g Potassium hydrogen orthophosphate
6.1 g Sodium dihydrogen orthophosphate
0.2 g Magnesium sulphate
2.0 g Ammonium sulphate

The medium was prepared as a stock solution at five times the above concentration, with chloroform added to .05% v/v. A metal ion supplement described by Gibson et al (1977a) was also added to give a final concentrations of:

1.78 μM Ferric chloride
2.45 μM Calcium chloride
1.0 μM Manganous sulphate
13.9 μM Zinc sulphate
0.68 μM Cobaltous sulphate
4.69 μM Boric acid

This was kept as a 1000 fold concentration and added to the salts medium prior to sterilisation.

2.2.(vi) Nutrient media

The media utilised throughout this study, referred to as 'nutrient' media, contained (per litre):

10 g Beef extract
10 g Peptone
3g Yeast extract
5 g Sodium chloride

with the solution being adjusted to pH 7.5 with sodium hydroxide before autoclaving.

2.2.(vii) Solid media

Solid media was prepared by adding 2% w/v Bacto-agar to the liquid media. For minimal media the agar was autoclaved separately from the salts solution, in 1/2 final volume distilled water.

2.3 Bacterial strains and plasmids

All bacterial strains were derived from *E. coli K12*. All bacterial strains and plasmids used in this study are listed in Table 2.1 and Table 2.2 respectively.

2.3 (i) Growth measurements in liquid media

Turbidity of cell cultures was used as a measure of cell density. Cultures were measured against a blank of the same medium using a Klett-Summerson

TABLE 2.1 Strains of *E.coli* K12 used in this study

STRAIN	RELEVANT GENOTYPE	NOTES OR REFERENCES
JM101	*lac - pro supE traD$_{36}$ F' proAB lacI^{Q} M15*	
K37	*Hfr supD*	
AN346	*argH ilvC pyrE entA*	(Butlin *et al* ., 1973).
AN727	*uncB402 argH pyrE entA recA*	*uncB* reference strain used in complementation analysis and as recipient strain for plasmids carrying *uncB* substitutions in chapters 6,7, and 8. (Gibson *et al*., 1977a).
AN802	*uncC424 argH pyrE entA recA*	*uncC* reference strain used in complementation analysis. (Gibson *et al*., 1977a.)
AN818	*uncD409 argH pyrE entA recA*	*uncD* reference strain used in complementation analysis.
AN887	*uncB^{-}Mu::413 argH pyrE entA recA*	Polar mutant isolated as described by Gibson *et al* (1978). Used as recipient strain for plasmids carrying *uncB* substitutions in chapters 3, 4, and 5.
AN888	*uncB^{+}Mu::416 argH pyrE entA recA*	Polar mutant isolated as described by Gibson *et al* (1978). Produces normal levels of subunit-*a*.
AN943	*uncE429 argH pyrE entA recA*	*uncE* reference strain used in complementation analysis. (Downie *et al*., 1979).
AN1273	*uncG428 argH pyrE entA recA::Tn10*	*uncG* reference strain used in complementation analysis, and as the parent strain in selection of plasmids carrying the complete *unc* operon . (Downie *et al*., 1980).

TABLE 2.1 (Cont/d) Strains of *E.coli* K12 used in this study

STRAIN	RELEVANT GENOTYPE	NOTES OR REFERENCES
AN1440	*uncF469 argH pyrE entA recA::Tn10*	uncF reference strain used in complementation analysis. (Downie *et al.*, 1981.)
AN1453	*uncB402 thr leu hsdR hsdM recA::Tn10*	C600 derivative (Chapter 3)
AN1460	pAN45/*uncB⁻Mu::413 argH pyrE entA recA*	Coupled control strain used in chapter 3. AN887 transformed by pAN45. (Downie *et al.*, 1980)
AN1873	pAN51/*uncE429 argH pyrE entA recA*	Coupled control strain used in chapter 8. AN943 transformed by pAN51.
AN1952	pAN174/ *thr leu ilvC recA*	C600 derivative carrying the plasmid pAN174 (Chapter 4).
AN2015	*uncH241 argH pyrE entA recA::Tn10*	Obtained by transduction of the *uncH241* allele from strain RH141 (obtained from R.D.Simoni) into AN346. *uncH* reference strain used in complementation analysis.
AN2402	pAN51/*uncB402 argH pyrE entA recA*	Coupled control strain used in chapter 7 and 8. AN727 transformed by pAN51. (Lightowlers *et al.*, 1988b)
AN2584	pAN174/*uncB⁻Mu::413 argH pyrE entA recA*	Uncoupled control strain used in chapters 4 and 5.AN887 transformed by pAN174.
AN2585	pACYC184/*uncB⁻Mu::413 argH pyrE entA recA*	Uncoupled control strain used in chapter 3. AN887 transformed by pACYC184.

TABLE 2.1 (Cont/d) Strains of *E.coli* K12 used in this study

STRAIN	RELEVANT GENOTYPE	NOTES OR REFERENCES
AN2608	pAN378/*uncB⁻Mu::413 argH pyrE entA recA*	Obtained by transfomation of strain AN887 by pAN378 (Chapter 4).
AN2629	pAN385/*uncB⁻Mu::413 argH pyrE entA recA*	Obtained by transformation of strain AN887 by pAN385. (Chapter 3).
AN2680	pAN436/*uncB⁻Mu::413 argH pyrE entA recA*	Coupled control strain used in chapters 4 and 5. AN887 transformed by pAN436.
AN2690	pAN430/*uncB⁻Mu::413 argH pyrE entA recA*	Obtained by transformation of strain AN887 by pAN430 (Chapter 5).
AN2706	pAN433/*uncB⁻Mu::413 argH pyrE entA recA*	Full revertant of the *uncB540* allele (Chapter 4).
AN2707	pAN434/*uncB⁻Mu::413 argH pyrE entA recA*	Partial revertant of the *uncB452* allele (Chapter 5).
AN2708	pAN435/*uncB⁻Mu::413 argH pyrE entA recA*	Partial revertant of the *uncB452* allele (Chapter 5).
AN2709	pAN436/*uncB402 argH pyrE entA recA*	Coupled control strain used in chapters 5-8. AN727 transformed by pAN436. (Lightowlers *et al.*, 1988a).
AN2712	pAN437/*uncB402 argH pyrE entA recA*	Obtained by transformation of strain AN727 by pAN437 (Chapter 6). (Lightowlers *et al.*, 1988b)
AN2735	pAN378/*uncB402 argH pyrE entA recA*	Obtained by transformation of strain AN727 by pAN378 (Chapters 3 and 5). (Lightowlers *et al.*, 1988a).

TABLE 2.1 (Cont/d) Strains of *E.coli* K12 used in this study

STRAIN	RELEVANT GENOTYPE	NOTES OR REFERENCES
AN2736	pAN174/*uncB402 argH pyrE entA recA*	Uncoupled control strain used in chapters 5-8. AN727 transformed by pAN174. (Lightowlers *et al.*, 1988a).
AN2757	pAN451/*uncB402 argH pyrE entA recA*	Obtained by transformation of strain AN727 by pAN451 (Chapter 7). (Lightowlers *et al.*, 1988a).
AN2760	pAN430/*uncB402 argH pyrE entA recA*	Obtained by transformation of strain AN727 by pAN430 (Chapter 5).
AN2765	pAN455/*uncB402 argH pyrE entA recA*	Obtained by transformation of strain AN727 by pAN455 (Chapter 8).
AN2766	pAN456/*uncB402 argH pyrE entA recA*	Obtained by transformation of strain AN727 by pAN456 (Chapter 7).
AN2768	pAN413/*uncB402 argH pyrE entA recA*	Obtained by transformation of strain AN727 by pAN413 (Chapter 6). (Lightowlers *et al.*, 1988b)
AN2769	pAN416/*uncB402 argH pyrE entA recA*	Obtained by transformation of strain AN727 by pAN416 (Chapter 7).
AN2770	pAN385/*uncB402 argH pyrE entA recA*	Obtained by tranformation of strain AN727 by pAN385 (Chapters 3 and 5). (Lightowlers *et al.*, 1988a).
AN2773	pAN415/*uncB402 argH pyrE entA recA*	Obtained by transformation of strain AN727 by pAN415 (Chapter 6).

TABLE 2.1 (Cont/d) Strains of *E.coli* K12 used in this study

STRAIN	RELEVANT GENOTYPE	NOTES OR REFERENCES
AN2774	pAN45/*uncB402 argH pyrE entA recA*	Coupled control strain used in chapters 6 and 7. AN727 transformed by pAN45. (Lightowlers *et al.*, 1988a).
AN2792	pAN467/*uncB402 argH pyrE entA recA*	Obtained by transformation of strain AN727 by pAN467 (Chapter 7). (Lightowlers *et al.*, 1988b).
AN2814	pAN482/*uncB402 argH pyrE entA recA*	Obtained by transformation of strain AN727 by pAN482 (Chapter 8).

TABLE 2.2 (Cont/d) Plasmids used in this study

PLASMID	RELEVANT GENETIC LOCI	NOTES OR REFERENCES
pAN455	*cat uncB569,E581F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$	Plasmid carries base substitutions in both *uncB* and *uncE* resulting in the two mutations R210K in subunit-*a*, D61E in subunit-*c*. The plasmid carries pAN174 as vector (Chapter 8).
pAN456	*cat uncB582E*$^+$*F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$	Plasmid carries an *uncB* base substitution resulting in a L207A mutation . The plasmid carries pAN174 as vector (Chapter 7).
pAN461	*uncB*$^+$*E*$^+$*F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$	M13 mp9 phage replicative form carrying a 4.4 kb *Hin*d III-generated insert used to produce all of the site-directed mutants in this study (Chapter 4).
pAN467	*cat uncB587E*$^+$*F*$^+$*H*$^+$*A*$^+$	Plasmid similar to pAN51, but carries an *uncB* base substitution resulting in an E219Q mutation (Chapter 7).
pAN482	*cat uncB*$^+$*E581F*$^+$*H*$^+$*A*$^+$	Plasmid similar to pAN51, but carries an *uncE* base substitution resulting in a D61E mutation (Chapter 8).

TABLE 2.2 (Cont/d) Plasmids used in this study

PLASMID	RELEVANT GENETIC LOCI	NOTES OR REFERENCES
pAN416	*cat uncB568E⁺F⁺H⁺A⁺G⁺D⁺C⁺*	Plasmid carries an *uncB* base substitution resulting in an N214G mutation. The plasmid carries pACYC184 as vector (Chapter 7).
pAN430	*cat uncB568E⁺F⁺H⁺A⁺G⁺D⁺C⁺*	Plasmid carries *uncB* base substitutions resulting in an R210K mutation . The plasmid carries pAN174 as vector (Chapter 5).
pAN434	*cat uncB571E⁺F⁺H⁺A⁺G⁺D⁺C⁺*	Plasmid carries a five base deletion in the *uncB* gene. Otherwise identical to plasmid pAN430 (Chapter 5).
pAN435	*cat uncB572E⁺F⁺H⁺A⁺G⁺D⁺C⁺*	Plasmid carries an additional substitution in *uncB* to plasmid pAN430 which codes for the L259M mutation. Otherwise identical to plasmid pAN430 (Chapter 5).
pAN436	*cat uncB⁺E⁺F⁺H⁺A⁺G⁺D⁺C⁺*	Plasmid carries pAN174 instead of pACYC184. Otherwise identical to plasmid pAN45 (Chapter 4).
pAN437	*cat uncB573E⁺F⁺H⁺A⁺G⁺D⁺C⁺*	Plasmid carries an *uncB* base substitution resulting in an K203I mutation . The plasmid carries pAN174 as vector (Chapter 6).
pAN451	*cat uncB577E⁺F⁺H⁺A⁺G⁺D⁺C⁺*	Plasmid carries an *uncB* base substitution resulting in a H245L mutation . The plasmid carries pAN174 as vector (Chapter 7).
pAN452	*cat uncB578E⁺F⁺H⁺A⁺G⁺D⁺C⁺*	Plasmid derivative of pAN430 detailed in Chapter 4.

TABLE 2.2 Plasmids used in this study

PLASMID	RELEVANT GENETIC LOCI	NOTES OR REFERENCES
pACYC184	*cat tet*	Chang and Cohen, (1978).
pAN36	*cat uncD*$^+$*C*$^+$	Downie *et al.*, 1980.
pAN51	*cat uncB*$^+$*E*$^+$*F*$^+$*H*$^+$*A*$^+$	Downie *et al.*, 1980.
pAN45	*cat uncB*$^+$*E*$^+$*F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$	Downie *et al.*, 1980.
pAN174	*cat tet*	Plasmid vector from plasmid pAN51 - equivalent to plasmid pACYC184 except that it contains a 100 base pair deletion (Jans, 1984)(Chapter 4).
pAN378	*cat uncB540E*$^+$*F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$	Plasmid carries an *uncB* base substitution resulting in an R210Q mutation . The plasmid carries pAN174 as vector (Chapter 4)·
pAN385	*cat uncB546E*$^+$*F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$	Plasmid carries two *uncB* base substitutions resulting in the double mutation, K167Q, K169Q. The plasmid carries pACYC184 as vector (Chapter 3)·
pAN413	*cat uncB566E*$^+$*F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$	Plasmid carries an *uncB* base substitution resulting in an E196A mutation. The plasmid carries pACYC184 as vector (Chapter 6).
pAN415	*cat uncB567E*$^+$*F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$	Plasmid carries an *uncB* base substitution resulting in a K203Q mutation . The plasmid carries pAN174 as vector (Chapter 6)·

colourimeter fitted with a blue filter. Cell density was expressed in arbitrary Klett units where 10^8 cell.ml^{-1} was approximated to a reading of 40 Klett.

2.3.(ii) Growth and maintenance of bacteria

For large scale culture, cells were grown in minimal medium supplemented with a carbon source, essential requirements, antibiotic (where suitable), and casamino acids (0.5% w/v). Plates of nutrient agar, supplemented with the appropriate antibiotic if required, were inoculated from the storage culture and incubated at 37^{o}C overnight. The cells from one plate were used to inoculate a litre of liquid media. Large scale culture production was in ten litre batches containing the appropriate supplements. One litre of an overnight culture was used as inoculum for growth in a 14 litre New Brunswick fermenter. Small scale, typically 10 ml, cultures were grown in 125 ml side-arm flasks.

2.3.(iii) Large-scale isolation of plasmid DNA

Large-scale isolation of amplifiable plasmid DNA was attempted using the method of Selker *et al.* (1977). One litre lots of minimal media supplemented with 0.5% (w/v) casamino acids, 30 mM glucose, auxotrophic requirements, and the appropriate antibiotic to select for plasmid retention, were inoculated with the plasmid-bearing strains. Growth was monitored by measuring the turbidity of the cultures, and 300 mg of spectinomycin was added per litre of media when the density of the culture produced a Klett reading of 100 units. Cultures were grown overnight for optimal plasmid amplification.

The cells were harvested by centrifugation at 5000 rpm for 10 min (using a Sorvall RC-2B or RC-5C centrifuge and GS3 rotor), and resuspended in (per litre of culture) 20 ml of 25% sucrose (w/v), 50 mM Tris-Cl pH 8.0 and 62.5 mM EDTA. Lysozyme (40 mg) was added, and after 5 min incubation on ice, 4 ml of 0.25 M EDTA was slowly added. After a further 5 min, 32 ml of Triton buffer (3% w/v Triton X-100 , 50 mM Tris-Cl pH 8.0, 62.5 mM EDTA) was added to solubilise the membranes. After 15 min, the lysate was centrifuged at 15000 rpm for 20 min (Sorvall RC-2B or RC-5C, SS34 rotor). The supernatant was decanted and NaCl and PEG-6000 were added to final concentrations of 1 M and 10% (w/v), respectively. Precipitation was allowed to occur for at least 2 hrs, and the precipitate was harvested by centrifugation at 4000 rpm for 10 min.

The pellet was resuspended in TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA) and treated with 200 μl of protease (Type VII from *Bacillus amyloliquefaciens*, 20 mg.ml^{-1} in TE, previously autodigested at 80^{o}C for 10 min) for 1 hr at 37^{o}C.

The suspension was made up to 7 ml by the addition of TE, and 7.2 gms of caesium chloride was added. The solution was centrifuged at 15000 rpm for 20 min to remove residual protein debris, and 300 μl of an ethidium bromide solution (10 mg.ml^{-1}) was added. The gradient was transferred to a polyallomer 'quickseal' ultracentrifuge tube (Beckman) which was filled to its capacity with liquid paraffin and sealed using the Beckman 'quickseal' system. The gradient was then placed, with a balance tube, in a Spinco fixed angle 50Ti, or 75Ti rotor and centrifuged at 35000 rpm for at least 48 hrs at 15^{o}C in a Beckman L8-70 or L8-65 Ultracentrifuge.

After centrifugation, the satellite plasmid DNA band was visualised using ultra-voilet light, and extracted from the polyallomer tube with a sterile 1 ml disposable syringe connected to an 18 G x 1.5" needle. Ethidium bromide was removed from the plasmid DNA by three extractions with isoamyl alcohol. The aqueous layer was retained and dialysed overnight at 4^{o}C against 1.5 litres of one tenth concentration TE to remove the caesium chloride. Plasmid DNA concentration was estimated by absorbance of the dialysed preparation at 260 nm, with an A_{260} reading of 1 approximating to a DNA concentration of 40 μg.ml^{-1}. Plasmid DNA was routinely stored at -20^{o}C at concentrations between 0.1-0.5 μg.ul^{-1}.

2.3.(iv) Small-scale isolation of plasmid DNA

A rapid, small-scale plasmid preparation by alkaline extraction was routinely used for analytical purposes. The method was essentially that of Silhavy *et al.* (1984). A single colony of a strain carrying the appropriate plasmid was used to inoculate 10 ml of luria media, supplemented with the appropriate antibiotic, and was grown overnight at 37^{o}C. Cells were harvested by centrifugation at 5000 rpm for 10 min (SE12 rotor), and were resuspended in 0.4 ml of lysozyme solution (50 mM glucose, 10 mM EDTA, 20 mM Tris-Cl, pH 8.0. Immediately prior to use, lysozyme was added to 5 mg.ml^{-1} concentration). Half of the resuspended, lysozyme-treated suspension was then transferred to two separate 1.5 ml Eppendorf tubes, and incubated on ice for 5 min.

To each of the Eppendorf tubes, 0.4 ml of NaOH-SDS stock solution was added (0.2 M NaOH, 1% SDS (w/v)), the mixture vortexed gently and again incubated on ice for 5 min. Potassium acetate stock solution (3 M potassium acetate, 28.5% (v/v) glacial acetic acid, pH 4.8), 0.3 ml, was further added to each tube, the mixture again vortexed gently, and then frozen in dry-ice for 5 min. The mix was allowed to thaw to room temperature before removing the cell debris by centrifugation in a microfuge (Eppendorf, or Hettich microliter) at

15000 rpm for 15 min. From each of the tubes, 0.75 ml of supernatant was carefully removed and transferred to clean Eppendorf tubes. The DNA was precipitated by the addtion of 0.45 ml of isopropanol to each tube and standing in dry-ice for 5 min. The DNA was allowed to thaw to room temperature and was harvested by centrifugation in the microfuge for 5 min. The supernatant was removed, and the pellet rinsed with 2 ml of cold, 70% ethanol. Finally, the pellet was dried under vacuum, and each pellet was resuspended in 50 μl of TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). Any contaminating RNA was digested by the addition of RNase A to a final concentration of 10 $\mu g.ml^{-1}$ (Pancreatic RNase A was kept as a $1mg.ml^{-1}$ stock solution in 1 ml aliquots at -20^{o}C in 10 mM Tris-Cl, pH 7.5, 15 mM NaCl, and had been boiled at 100^{o}C for 15 min to remove any DNase contamination).

2.4.Techniques used in the propagation and isolation of bacteriophage M13 replicative form and single-stranded form

2.4.(i) Large-scale preparation of single-stranded M13 bacteriophage

One litre of minimal salts medium containing 30 mM glucose supplemented with 0.5% (w/v) casamino acids was inoculated with 10 ml of an overnight culture of strain K37. After the culture had grown sufficiently to give a Klett reading of 40-50 Klett units, the culture was infected with approximately 10^9 plaque forming units of bacteriophage per ml of infected culture, and was grown in the infected state for 16 hrs with aeration. The infected cells were harvested by centrifugation for 10 min at 5000 rpm (GSA rotor) at 4^{o}C. The supernatant was chilled to 4^{o}C and the bacteriophage precipitated by the addition of one tenth of a volume of 5 M NaCl and one tenth of a volume of 50% (w/v) PEG 6000. After mixing, and sitting at 4^{o}C for at least 15 min, the phage were harvested by centrifugation for 15 min at 12000 rpm in an SS-34 rotor.

The precipitated phage were resuspended in 5% of the original culture volume in TE buffer, pH 8, containing 0.5% sarkosyl, precipitated a second time, and finally resuspended in 1% of the original culture volume in TE buffer. An estimate of the final yield of infective particles was obtained by titring the resuspended phage preparation and was routinely 10^{14}-10^{15} plaque forming $units.ml^{-1}$. Phage retained as stock were kept at 4^{o}C.

2.4.(ii) Phage titre and soft agar overlay technique

Estimations of bacteriophage M13 titres were made by direct plating of serial dilutions of the phage stock. Phage was serially diluted into TE (usually $1:10^6$, $1:10^9$,$1:10^{11}$ and $1:10^{13}$ dilutions), and 100 μl from each dilution were mixed with 200 μl of a dense culture of indicator cells (strains K37 or JM101). To each of the mixtures of diluted phage and indicator cells, 3 ml of liquified soft agar was added, and the mix was kept at 45^{o}C. The soft agar contained, (per litre):

10 g Bacto-Tryptone
5 g Yeast extract
10 g Sodium chloride
7.5 g Bacto-agar
(pH 7.5)

and was retained in solid, 50 ml aliquots which were melted by microwave shortly before use, and allowed to equilibrate to 45^{o}C in a water bath. After addition of the phage and indicator cells to the soft agar, each mixture was poured onto a plate made from Luria broth (LB plate, solidified by the addition of 15 gms of Bacto-agar per litre of Luria). Once the mix had been poured onto the plates, the agar was left to solidify for 5 min and the plates were then incubated, inverted, at 37^{o}C. Plaques were visible after 6 hrs, but were routinely left overnight before the plaques were counted, and an estimate of the phage titre made.

2.4.(iii) Small-scale isolation of bacteriophage M13

For small-scale isolation of bacteriophage, 2 ml of Luria broth was inoculated with 20 μl of an overnight culture of strain K37 or strain JM101. A single phage plaque was picked off a plate with a sterile toothpick, and the toothpick placed into the Luria medium. After overnight shaking at 37^{o}C, the infected cells were removed by centrifugation of approximately 1.5 ml of the culture at 15000 rpm in a microfuge, and the supernatant retained. The phage titre of the supernatant was estimated, and varied between 10^9-10^{11} plaque forming units.ml^{-1}.

2.4.(iv) Preparation of bacteriophage M13 replicative form DNA

The large scale isolation of the duplex replicative form DNA was essentially the same method used for the large scale isolation of plasmid DNA detailed in section 2.3.(iii). One litre of minimal salts medium containing 30 mM glucose supplemented with 0.5% (w/v) casamino acids was inoculated with 10 ml of an overnight culture of strain K37. The growth of the culture was monitored until a Klett reading of approximately 150-160 units was attained. Bacteriophage was then added to a final concentration of about 10^{10} plaque forming units.ml^{-1}. To maximise the yield of phage replicative form, the infected cells were harvested 90 min after infection. Once the cells had been collected the procedure for phage replicative form isolation was identical to the procedure detailed in section 2.3.(iii).

A small-scale isolation of bacteriophage M13 was used for analytical purposes and was similar to the procedure for small-scale isolation of plasmid DNA detailed in section 2.3.(iv). Minimal salts medium containing 30 mM glucose supplemented with 0.5% (w/v) casamino acids (10 ml) was inoculated with strain K37 or strain JM101 and when the turbidity of the culture reached a Klett reading of 150 units the cells were infected with phage to a final concentration of about 10^{10} plaque forming units.ml^{-1}. After 90 min the cells were harvested, and double-stranded DNA isolated in an identical manner to that detailed in section 2.3.(iv).

2.5 DNA manipulations

2.5.(i) Restriction, dephosphorylation and ligation of DNA

DNA was cleaved by a suitable restriction endonuclease in one of the following buffer systems:

Low salt buffer: 10 mM Tris-Cl, pH 7.5 10 mM $MgCl_2$
1 mM dithiothreitol

Medium salt buffer: 50 mM NaCl, 10 mM Tris-Cl, pH 7.5
10 mM $MgCl_2$, 1 mM dithiothreitol

High salt buffer: 100 mM NaCl, 50 mM Tris-Cl, pH 7.5
10 mM $MgCl_2$, 1 mM dithiothreitol

Ten fold concentrations of each buffer were prepared by autoclaving the solutions. Digests using endonuclease *Cla* 1 were attempted in the low salt buffer, digests using endonucleases *Hin*d III, *Bam* H1, *Pst* 1 and *Mlu* 1 in the medium salt buffer, and *Eco* R1 in the high salt buffer. All digests were carried out at 37^{o}C (except *Pst* 1, which was at 30^{o}C), with sufficient enzyme to totally cleave the DNA in a period of one hour. In cloning experiments which required partial cleavage of DNA, several different amounts of the required endonuclease were added to a standard, normally 200 ng, amount of DNA, and were incubated for 30 sec, 1 min, 2 min and 5 min. For analytical purposes, between 200 ng and 1 μg of DNA were cleaved in a total volume of either 10 or 20 μl.. Cloning experiments routinely required cleavage of sufficient vector DNA to give a concentration of DNA of 15 ng.μl^{-1} in the ligation mixture, and sufficient donor DNA to give a concentration of 10-20 ng.μl^{-1} in the ligation mixture. Before ligation, the endonuclease used to cleave the DNA was inactivated either by heating at 65^{o}C for 10 min, or by a series of equal volumes of phenol:phenol/chloroform:chloroform extractions. The phenol used in all DNA manipulations was redistilled, melted at 65^{o}C, and 8-hydroxyquinoline added to a final concentration of 0.1%. The phenol was then extracted several times with an equal volume of 1 M Tris-Cl, pH 8.0, and then extracted with an equal volume of 0.1 M Tris-Cl, pH 8.0, 0.2% (v/v) β-mercaptoethanol until the pH of the aqueous phase was higher than 7.6. The chloroform solution was kept as a mixture of chloroform/isoamyl alcohol (24:1 v/v). Digested DNA, after treatment with phenol, was precipitated with an equal volume of ethanol and one tenth of a volume of 3 M sodium acetate, and incubated in dry ice for a minimum of 5 min. Precipitated DNA was rescued by centrifugation in a microfuge at 15000 rpm for 5 min, and the pellet washed with 1 ml of cold 70% ethanol, 0.1 mM EDTA. The pellet was finally dried under vacuum by a bench dessicator for 2 min.

In several cloning experiments, alkaline phosphatase was used to aid in the construction of a desired plasmid, by dephosphorylation of vector DNA. The method of dephosphorylation used was similar to that of Maniatis *et al.* (1982), and was normally used to produce a stock of dephosphorylated vector which could be retained and used in several experiments. After digestion and precipitation of the DNA (normally 10 μg), 1 unit of calf intestinal alkaline phosphatase (0.01 unit of alkaline phosphatase is needed to remove the terminal phosphates from 1 pmole of 5' ends of DNA) was added to 100 μl of reaction buffer containing 50 mM Tris-Cl pH 8.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine and the DNA. The reaction mix was incubated at 37^{o}C for 30 min,

and another unit of phosphatase was added. After a further 30 min, the reaction was terminated by the addition of an equal volume of phenol prepared as above. Three further phenol extractions were followed by a phenol/chloroform (1:1) and chloroform extraction, and then by a similar method of ethanol precipitation to that detailed above.

Ligation of suitably cleaved, and occasionally dephosphorylated, DNA was attempted in 100 μl volume, containing:

10 mM $MgCl_2$
10 mM dithiothreitol
50 mM Tris-Cl, pH 8.0
2 mM ATP

The phage T4 DNA ligase enzyme was added, and the ligation mix incubated at 20^{o}C for between 2-5 hrs, or at 12^{o}C overnight.

2.5.(ii) Transformation of bacterial strains by plasmid DNA

Two methods of transformation were used. The more routine $CaCl_2$ method was used when the production of an extremely high number of transformant colonies were not necessary. To maximise transformation efficiency with ligation mixes, the high efficiency transformation procedure of Hanahan was used.

The former procedure was based on that described by Lederberg and Cohen (1974). After inoculation of 20 ml of Luria broth containing 30 mM glucose, the culture was shaken at 37^{o}C until a Klett reading of 100 units was attained (mid-log phase growth). Cells were harvested by centrifugation at 5000 rpm in an SS-34 rotor for 10 min at 4^{o}C, the supernatant discarded, and the cells resuspended in one half volume of ice cold $CaCl_2$ (10 ml). Following a period of 30 min on ice, the cells were again centrifuged at 5000 rpm for 10 min (SS-34 rotor), the supernatant discarded, and the cell pellet resuspended in one twentieth of the original volume in $CaCl_2$ (1 ml). After standing for one hour on ice, the competent cells were aliquoted (200 μl aliquots) in to sterile, polystyrene round-bottomed tubes. Plasmid DNA, 500 ng, was added to each aliquot of competent cells, gently mixed, and left on ice for 20 min. The cells were then heat-shocked at 42^{o}C for 2 min and 1 ml of sterile Luria-glucose added. After a one hour incubation at 37^{o}C to allow full recovery of the cells and to permit expression of plasmid-encoded genes, the cells were recovered by centrifugation in a bench centrifuge at 3000 rpm for 5 min, resuspended in 1 ml of mineral-salts medium, recentrifuged, and resuspened in 200 μl of the minimal salts medium.

Aliquots were then spread on selective medium and incubated for 1-3 days, inverted, at 37^{o}C.

For the high transformation efficiency procedure, 20 ml of Luria broth containing 20 mM $MgCl_2$ and no added glucose, was inoculated. Cells were harvested after reaching a Klett of 100 units, by centrifugation at 5000 rpm (SS-34 rotor) for 10 min at 4^{o}C, the supernatant discarded, and the cell pellet resuspended in one third volume (6.7 ml) ice cold TFB buffer. The TFB buffer contained (final concentration)

10 mM MES, pH 6.3, 100 mM RbCl
45 mM $MnCl_2.4H_20$, 10 mM $CaCl_2.2H_20$
3 mM hexamine cobalt chloride

The buffer was stored at 4^{o}C, and sterilised by passing through a 0.45 μm pore-size filter. MES buffer was adjusted to pH 6.3 by the addition of 10 M KOH, and was stored as a 1 M stock solution at -20^{o}C. After resuspension in TFB buffer, the cells were maintained on ice for 15 min and were again harvested by repeating the previous centrifugation procedure. The pellet was then resuspended in one twelfth of the volume of the original suspension (1.6 ml) of ice cold TFB buffer. After resuspension, 56 μl of dimethyl sulphoxide was added and the mixture incubated for 5 min on ice. 56 μl of 2.25 M dithiothreitol was added, and the cells incubated for 10 min on ice. A further 56 μl of dimethyl sulphoxide was added and again the cells were incubated for 5 min on ice. 210 μl aliquots of the competent cels were then placed into polystyrene round-bottomed tubes and DNA was added routinely in four different amounts, 25 ng, 50 ng, 100 ng and 250 ng, in 10 μl volumes. The mixes were incubated for a further 30 min on ice, and then heat-shocked at 42^{o}C for 2 min. 800 μl of Luria broth containing 30 mM Glucose was then added to each tube, and the cells incubated at 37^{o}C for one hour to allow expression of the plasmid-borne genes. Cells from the tranformation mix were then harvested and treated in a manner identical to the procedure detailed for the $CaCl_2$ method above.

2.5.(iii) Tranformation of bacterial strains by bacteriophage replicative form: production of phage plaques and selection for recombinant phage.

For the transformation of cells of a male strain of bacteria by phage replicative form DNA, cells of either strain K37 or strain JM101 were made competent by the modified method of Hanahan (1983) detailed above. 210 μl aliquots of the competent cels were placed into polystyrene round-bottomed tubes and DNA was added routinely in four different amounts, 25 ng, 50 ng, 100 ng and 250 ng, in 10 μl volumes. The mixes were incubated at 45^{o}C for 2 min, after which 3 ml of soft agar and 150 μl of a dense culture of indicator cells were added to each tube. The soft agar was then poured onto LB plates in the manner detailed in section 2.4.(ii).

In experiments where attempts were made to insert restriction endonuclease-generated fragments into bacteriophage M13, selection for recombinant bacteriophage was made by adding colour indicators to the soft agar along with the ligated phage replicative form and the indicator cells. This method was based on the procedure of Messing *et al.* (1981), whereby any fragment of DNA inserted into the polylinker region of the mp series of M13 bacteriophage would inactivate the β-galactosidase enzyme. BCIG, 5-bromo-4-chloro-3-indolyl-βD galactopyranoside, is recognised as a substrate by the enzyme, and one of the cleavage products is blue. In the presence of a *lac* operon inducer such as IPTG, β-galactosidase is produced, the BCIG is cleaved, and a blue colouration results. However, if a fragment of DNA is inserted within the gene coding for the β-galactosidase enzyme no blue colouration results. Thus, when selecting for recombinant bacteriophage, 25 μl of a 20 mg.ml^{-1} stock solution of IPTG and 25 μl of a 25 mg.ml^{-1} stock solution of BCIG were added to the soft agar containing the phage replicative form DNA, competent cells of strain JM101, and 150 μl of JM101 cells from a dense overnight culture, before overlaying LB plates with the soft agar mix. Recombinant phage were picked from clear plaques after at least a 12 hr incubation period.

2.5.(iv) Isolation and electrophoresis of DNA in agarose gels

Agarose gel electrophoresis was routinely used to analyse the construction of plamids, both after restriction endonuclease digest, and uncleaved plasmids, and occasionally was used to extract specific endonuclease-generated fragments to facilitate cloning. After large-scale plasmid preparations (*q.v.*section 2.3.(iii)),

the preparations were checked for any contamination of chromosomal DNA by agarose gel electrophoresis.

Agarose gels of between 0.4-1 % agarose were used to separate DNA, with the majority of gels being 0.7%. 300 mls of buffer containing 1 x TBE (89 mM Tris-borate, 89 mM boric acid, 20 mM EDTA), the appropriate amount of agarose, and 500 μg.litre^{-1} ethidium bromide were heated for 5 min in a microwave oven, and cooled to approximately 65^{o}C before pouring into a flat bed electrophoresis tank of dimensions 16 cm x 17 cm. Wells of 9 mm x 6 mm x 1 mm were formed in the gel. After approximately one hour, samples were added to the wells. The samples contained (per well): enough DNA to be visualised by UV light after gel electrophoresis (normally 200 ng for each fragment, such that 200 ng of an uncut plasmid would be added, whilst 600 ng of a digest comprising three similarly sized fragments would be added. If small fragments produced from a restriction digest were to be analysed, enough DNA would be cleaved to produce 100-200 ng of the smallest fragment, and all the digested DNA would be electrophoresed), 0.04% (w/v) bromophenol blue, 6.5% (w/v) sucrose in a total volume of 30 μl. The samples were subjected to gel electrophoresis in a gel running buffer containing 89 mM Tris-borate, 89 mM boric acid, 20 mM EDTA(1 x TBE) at room temperature at 30 mA (approx. 60V) for 6 hrs, or at 20 mA overnight. The DNA was visualised using a transilluminator (Ultra-Violet Products, San Gabriel, Ca, U.S.A.) and the gel photographed through a red filter.

For the isolation of restriction endonuclease-generated DNA fragments, two methods were used. One utilised DEAE paper, and one, low melting point agarose. For the former method, after electrophoresis as above, the DNA fragment to be isolated was visualised by a hand-held UV lamp, and a small piece of agarose in extremely close proximity to the fragment was excised. A strip of DE-81 paper was then cut which was a similar size to the piece of agarose, and the paper was washed for 30 min in 2.5 M NaCl. After a five min rinse in 1 x gel running buffer, the DE-81 paper was stuck onto the piece of agarose, and the agarose replaced into the gel such that the DNA fragment could be further electrophoresed onto the DE-81 paper. Once the fragment had been electrophoresed onto the paper, the paper was removed and rinsed in distilled water for 5 min before being transferred to a 1.5 ml eppendorf tube. The paper was then crushed with a gilson C-20 pipette tip after the addition of 600 μl of 1.5 M NaCl in TE. The crushed paper was incubated at 37^{o}C for 2-3 hrs and the paper pelleted by a brief centrifugation. A small hole was made in the bottom of the eppendorf tube by a 27.5 gauge needle and the eppendorf tube placed in the top of a 5 ml disposable polystyrene round-bottomed tube which also had a small

hole punctured in its side by the needle. The salt solution was gently centrifuged through the hole in the eppendorf tube and was retained by the polystyrene tube by a brief spin in a bench centrifuge. The sample was added back to the paper, and the centrifugation repeated into a clean polystyrene tube. Precipitation of the rescued DNA fraction was effected by adjusting the salt concentration of the sample to 1 M NaCl, and by the addition of 2 volumes of ice cold ethanol. The DNA was harvested after incubation in dry ice for at least two hours, by centrifugation in a microfuge at 15000 rpm for 5 min. The pellet was washed with 1 ml of 70% ethanol, 0.1 mM EDTA, and dried using a bench dessicator.

Fragment isolation utilising low melting-point agarose was effected by electrophoresing the endonuclease-generated fragments through 300 ml of low melting-point agarose which was set at 4^{o}C. Essentially, the gel production and gel running conditions were identical to conditions detailed above, except the gel was run at 4^{o}C. After visualisation with a hand-held UV lamp, the piece of agarose containing the DNA fragment was excised from the gel and melted at 65^{o}C for 15 min. 150 μl aliquots of the melted agarose were placed in 1.5 ml eppendorf tubes and 300 μl of a 50 mM Tris-Cl pH 8, 0.5 mM EDTA solution were added, along with 300 μl of phenol prepared as in section 2.5.(i). After vortexing, the mixtures were centrifuged in a microfuge (15000 rpm) for 5 min. The top, aqueous phase was removed from each tube, transferred to a fresh tube, and again phenol extracted. The centrifugation and phenol extraction were repeated until the aqueous phase was clear. This phase was then extracted with an equal volume of a phenol/chloroform mix (1:1), centrifuged, and the aqueous phase extracted with an equal volume of chloroform. The purified DNA fragment was then precipitated by the addition of two volumes of cold ethanol, and one tenth volume of 3 M sodium acetate. DNA was harvested after incubation of the mixture in dry-ice for 2 hrs, by centrifugation in a microfuge (15000 rpm) for 5 min, the pellet washed with 70% ethanol, 0.1 mM EDTA, and dried in a bench dessicator.

2.6 DNA sequencing

Nucleotide sequences were determined by a method based on the dideoxy chain-terminating method of Sanger *et al.* (1977), using [α-^{32}P]dATP, and is detailed below.

2.6.(i) Isolation and preparation of template DNA

The method of template isolation was essentially that of Schreier and Cortese (1979). Single-stranded bacteriophage M13 mp9 carrying the relevant DNA fragment, were isolated from 2 ml of culture as detailed in section 2.4.(iii). To 0.8 ml of supernatant carrying the bacteriophage in a 1.5 ml eppendorf tube, 0.2 ml of 20% PEG 6000 in 2.5 M NaCl was added and mixed, and the mixture incubated at room temperature for 15 min. Agglutinated phage were collected by centrifugation in a microfuge (15000 rpm) for 5 min. The supernatant was carefully decanted, and all traces of PEG removed from the eppendorf tube with cotton-tipped tooth picks, and with a drawn out capillary tube. The pellet was resuspended in 100 μl of buffer containing 20 mM Tris-Cl pH 7.5, 10 mM NaCl, 0.1 mM EDTA, and treated with 50 μl of Tris-saturated phenol(*q.v.*section 2.5.(i)) and 50 μl of chloroform. After gentle vortexing, the mixture was centrifuged for 2 min in a microfuge, and 80 μl of the aqueous phase removed. The single-stranded DNA was precipitated with 2 volumes (160 μl) of cold ethanol, followed by storage at -20^{o}C for at least 30 min. After harvesting the DNA by centrifugation in a microfuge for 5 min, the pellet was rinsed twice with 1 ml of cold 70% ethanol, 0.1mM EDTA, and dried *in vacuo* for 1 min. The single-stranded DNA was finally dissolved in 40 μl of distilled water, and stored at -20^{o}C until ready for use.

2.6.(ii) Primer annealing and sequencing reactions

In a total volume of 10 μl, approximately 600 ng of purified single-stranded template DNA (normally between 3-8 μl of the 40 μl from 2.5.(i)), were mixed with 4 ng of the relevant oligonucleotide used as a sequencing primer, in a buffer containing 66 mM Tris-Cl pH 8.0, 10 mM $MgCl_2$. The mixture was incubated at 65^{o}C for 5 min, and the oligonucleotide annealed to the template by incubating the mix at 37^{o}C for 30 min. Whilst the annealing was occurring, the sequencing reaction mixes were made. Eight mixes were stored as stock solutions at -20^{o}C, and were made from 10 mM stock solutions of the four DNTPs and four didNTPs.

The mixes were:

G^o:	5 μM dGTP	A^o:	25 μM dGTP
	25 μM dTTP		25 μM dTTP
	25 μM dCTP		25 μM dCTP
	1 μM dATP		1 μM dATP
T^o:	25 μM dGTP	C^o:	25 μM dGTP
	2.5 μM dTTP		25 μM dTTP
	25 μM dCTP		10 μM dCTP
	1 μM dATP		1 μM dATP

and the dideoxynucleotide mixes were:

ddGTP: 50 μM	ddATP: 50 μM
ddTTP: 100 μM	ddCTP: 100 μM

The G, A, T, and C sequencing reaction mixes contained 2 μl of the N^o mix, and 2 μl of the corresponding ddNTP. After annealing, to each of the four reaction mixes were added 1 μl of 0.1 M dithiothreitol, 1 μl of [α-^{32}P]dATP (approx. 111 Tbq.mmol^{-1}; 370 Mbq.ml^{-1};2000-3000 Ci.mmol^{-1}), 1 μl of DNA pol 1 large fragment, and finally, 3 μl of the annealing mix to each of the four sequencing reaction mixes. The mixes were incubated at 37^{o}C for 10 min, and then 1 μl of a chase solution (1 mM of each of dGTP, dATP, dTTP and dCTP) was added to each of the reaction mixes. After a further 20 min at 37^{o}C, the reactions were terminated by the addition of 4 μl of loading dye (80% (v/v) recrystallised formamide, 50 mM Tris-borate pH 8.3, 1 mM EDTA, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue) and heating at 85^{o}C for 10 min.

2.6.(iii) Gel electrophoresis of sequencing reaction mixes

Immediately after heating at 85^{o}C, the reaction mixes were placed on ice. Gel electrophoresis of the reaction mixes was performed through gels containing 6%, 8% or 20% (w/v) acrylamide gels, and were run on vertical slab gel apparatus. The gels were 175 mm wide, 365 mm long, and 0.2 mm thick, with sample wells of approx. 6 mm width produced by the inclusion of a 'sharks-tooth' comb after polymerisation of the gel, and contained 1 x TBE buffer (89 mM Tris-borate pH 8.3, 89 mM boric acid, 20 mM EDTA), 50% (w/v) urea, and the appropriate acrylamide concentration (from a 40% w/v stock solution containing 38% acrylamide and 2% bisacrylamide) in 60 ml volume. 20% gels were

polymerised by the addition of 100 µl ammonium persulphate (10% w/v) and 75 µl TEMED. The more routinely used 6% gels were polymerised by the addition of 150 µl ammonium persulphate (10% w/v), and 75 µl TEMED. 2 µl of each of the four sequencing reaction mixes were applied to separate wells at the gel interface by drawn out capillary tubes. Gels were run at 30 Watts (approx. 1500 Volts) for 2 hr periods before the next sample loading. By staggering the loadings, different regions of DNA sequence could be obtained if required. After sufficient electrophoresis, the gel was removed from the glass plate retainers by sticking to a sheet of Whatman 3MM paper, and were then dried by heating at 80^{o}C under vacuum on a BIO-RAD model 483 slab-dryer. Gels were exposed at room temperature in a Kodak X-Omatic C-2 cassette without an intensifying screen and were developed after 12-24 hrs using the Kodak M20 X-Omat processor. DNA sequences were then read.

2.7 Techniques used in site-directed mutagenesis

2.7.(i) Preparation of synthetic oligonucleotides

Synthetic oligodeoxyribonucleotides were prepared for use as deoxynucleotide primers by the method of Sanchez-Pescador and Urdea (1984). The oligonucleotides were synthesised using phosphoramidite derivatives and were either purchased from the Centre for Science and Industrial Research Organisation (CSIRO) Division of Plant Industry, Canberra, Australia. or donated as a kind gift by Dr.A.E.Senior Rochester Medical School, Rochester, New Jersey, U.S.A. Removal of the oligonucleotide from the solid support was achieved by treatment with 2 ml of ammonium hydroxide for 6 hr at room temperature, followed by heating at 65^{o}C overnight. One drop (approx. 20 µl) of the solution was evaporated to dryness by placing on a watchglass, treated with 80% acetic acid for 20 min, then precipitated by the addition of 1 ml of diethyl ether. The white precipitate was pelleted by centrifugation, dried for 1 min under vacuum, and dissolved in 1 ml of water. The absorbance at 260 nm was recorded, and the solution diluted to give a stock solution of 40 ng.µl^{-1}.

2.7.(ii) Phosphorylation of the synthetic oligonucleotide and preparation of a radiolabelled probe.

Synthetic oligonucleotides used for the formation of site-specific base-substitutions were phosphorylated with phage T4 polynucleotide kinase. 40 ng of the prepared oligonucleotide was phosphorylated in a 10 μl volume containing 66 mM Tris-Cl pH 8.0, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM ATP and 1 μl of polynucleotide kinase (Amersham, Australia Pty. Ltd). The reaction was allowed to prodeed at 37^{o}C for 1 hr, and where required, the DNA was precipitated by the addition of 2 vol of ethanol and one tenth vol sodium acetate, incubated at -70^{o}C for at least 5 min, harvested by 5 min centrifugation in a microfuge (15000 rpm) and dried under vacuum.

For the production of a radiolabelled oligonucleotide probe used in the selection of phage DNA carrying specific base-substitutions, 400 ng of the relevant oligonucleotide was phosphorylated by the addition of 4 μl of [γ-^{32}P] labelled ATP (approx. 110 Tbq.mmol^{-1}; 370 Mbq.ml^{-1}; 2000 Ci.mmol^{-1}) in a total volume of 30 μl containing the identical constituents to the phosophorylation buffer detailed above except for the omission of the unlabelled ATP. The reaction was allowed to proceed for 1 hr at 37^{o}C before termination by the addition of one third of a volume of formamide loading dye (*q.v.*section 2.6.(ii)). To remove unincorporated radiolabel and smaller oligonucleotides, the radiolabelled oligonucleotide mix was subjected to polyacrylamide gel electrophoresis through a 20% vertical acrylamide gel. The gel constituents were similar to those for sequencing gels detailed in section 2.6.(iii). However, gels of 0.8 mm thickness were used, and wells of approximately 1.5 cm x 1.5 cm were formed in the gel. The labelled oligonucleotide mix was electrophoresed through the gel at 30 Watts for 3-4 hrs, using 1 x TBE as running buffer. One glass retaining plate was then removed, and the position of the oligonucleotide within the gel visualised by exposing the gel to Kodak XRP-5 film for 2 min, and subsequent development after marking the film. After development, the oligonucleotide was located by orienting the film with repect to the marks, and the gel piece containing the [γ-^{32}P]-labelled oligonucleotide carefully excised with a scalpel. The oligonucleotide was then removed from the gel piece by leaching into 600 μl of TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA) during incubation for at least 2 hr (preferably overnight) at 65^{o}C.

2.7.(iii) Preparation of filters and hybridisation with the radiolabelled oligonucleotide

DNA was immobilised onto nitrocellulose filters tially by the method of Maniatis *et al.* (1982). Nitrocellulose filters (Bartelt Instruments Pty. Ltd. West Heidelberg, Victoria. Australia, BA85 filters, 82 mm diameter 0.45 μm pore size), were placed onto agar plates and the transformation mix plated directly onto the filters. After 24 hrs the colonies were clearly visible, and the filter was removed after marking by stabbing through the filter and into the agar to enable easy location of specific colonies at a later time. The filter was then placed, colony-side up, on a piece of Whatman 3MM paper. A second filter was laid over the first, and gently pressed to enable transfer of some of the cells from each colony. This was repeated with a third filter. The second and third filters were placed onto fresh agar plates, colony-side up, and the colonies allowed to grow for a further 12 hrs. The filters were then removed and placed, colony-side up, onto a sheet of 3MM paper soaked in 10% (w/v) SDS solution for 3 min. The filters were then transferred to a second sheet of 3MM paper, soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl). After 5 min, the filters were transferred to a third sheet of 3MM paper that was saturated in neutralising solution (1.5 M NaCl, 0.5 M Tris-Cl pH 8.0) and left for a further 5 min. Finally the filters were rinsed in 50 ml of 6 x SSC (0.9 M NaCl, 90 mM sodium citrate pH 7.0), and left to air dry for 1 hr before baking the DNA onto the filters over 2 hr at 80^{o}C in a vacuum oven. After immobilising the DNA, the replica filters were immersed in prehybridisation buffer for 2 hr. The buffer contained 5 x Denhardts solution (1% w/v ficoll, 1% w/v polyvinylpyrrolidone, 1% bovine serum albumin, stored as a 50 x stock solution at -20^{o}C), 6 x SSC (0.9 M NaCl, 90 mM sodium citrate), 0.5% (w/v) SDS and 100 μg.ml^{-1} sonicated salmon sperm DNA, denatured by heating at 90^{o}C for 10 min prior to its addition to saturate any nonspecific DNA-binding sites on the nitrocellulose filters. 5 ml of buffer were used for each filter, and the prehybridisation reaction was left for 2 hrs at 65^{o}C.

After prehybridisation, the buffer was removed, the filters washed briefly in 0.9 M NaCl, 90 mM sodium citrate, and then placed into the hybridisation buffer (5 x Denhardts solution, 6 x SSC, 5 ml per filter). The radiolabelled oligonucleotide probe was added after heating for 5 min at 80^{o}C to prevent any potential secondary structure formation. Hybridisation was carried out overnight for 14 hrs at a precalculated temperature (*q.v.* chapter 3.2.(iv)). Filters were removed from the hybridisation chamber and washed in three changes of 50-100

ml of 0.9 M NaCl, 90 mM sodium citrate buffer at room temperature. Filters were then air-dried and visualised by autoradiography after a 2 hr exposure at -70^{o}C in a Kodak X-Omatic cassette fitted with an intensifying screen. After development of the film, the filters were further washed in 0.9 M NaCl, 90 mM Na citrate at 10^{o}C hotter increments and re-exposed until possible mutant colonies could be discerned.

Plaque hybridisations were performed using essentially the same technique as for colony hybridisation detailed above. Nitrocellulose filters were laid onto overlays which contained phage plaques, and left for 1 min. A second, replica, filter was then laid onto the plate and left for 150 secs. Both filters were marked by a needle to aid in identification of specific plaques. The nitrocellulose filters were air-dried, DNA-side up, and then baked immediately at 80^{o}C for 2 hrs. Prehybridisation and hybridisation were essentially identical to the procedure detailed above for colony hybridisation.

Dot-blot hybridisation involved spotting 2 μl aliquots of bacteriophage DNA isolated from 20-200 individual phage preparations by the method detailed in section 2.6.(i) onto two separate nitrocellulose filters, and the DNA immobilised by baking at 80^{o}C for 2 hrs in s vacuum oven. Prehybridisation and hybridisation procedures were identical to those detailed above.

2.8 Preparation of cell membranes and biochemical assays

2.8.(i) Preparation of cell membranes

Membranes were prepared as described by Cox *et al* (1973), and Senior *et al* (1979). After harvesting of cells at a Klett of 200 units from twenty litres of culture using a continuous flow, Sharples harvester, the cells were washed in STEM buffer (250 mM sucrose, 100 mM TES buffer, pH 7.0, 0.25 mM EGTA, 20 mM magnesium acetate). After centrifugation at 5000 rpm for 10 min, the cells were resuspended in STEM buffer containing 40 mM ε-amino caproic acid (EACA) and 1 mg.ml^{-1} *p*-aminobenzamidine(PAB), 2ml.gm^{-1} wet weight of cells. The cells were then passed through a RIBI cell fractionater, operated by myself, Doug Abigail, or Russell Taylor (Department of Biochemistry, John Curtin School of Medical Research, Australian National University), at 137 MPa. Cell debris was removed by centrifugation at 20000 rpm in an SS-34 rotor for 1 hr, the supernatant carefully decanted and then centrifuged at 60000 rpm (254,000xg) in a 60 or 70Ti fixed-angle rotor for 2 hrs. The pellet contained two layers, and the upper, red-brown layer was resuspended in a 50 mM TES buffer

pH 7.0 containing 0.5% (w/v) EACA and 1 $mg.ml^{-1}$ PAB. The membranes were harvested by centrifugation at 60000 rpm (254,000xg) for 1 hr and the pellet resuspended in a 5 mM TES buffer pH 7.0 containing:

15% glycerol
0.5 mM EDTA
0.5 mM dithiothreitol
0.5% (w/v) EACA
1 $mg.ml^{-1}$ PAB

The membranes were washed again in this buffer, and finally resuspended in 2.5 ml of the same 5 mM TES buffer for each 10 litres of original culture.

To remove the F_1-ATPase from the membrane, 1 ml of the membrane preparation was dialysed against 2 litres of the 5 mM TES buffer with the omission of the *p*-aminobenzamidine. The dialysis was maintained for 24 hrs at 4^oC, with one buffer change after 8 hrs. Membranes stripped of the F_1-ATPase were then harvested by centrifugation for 1 hr at 60000 rpm (254,000xg), and were resuspended in 1 ml of the 5 mM TES buffer without the addtion of the *p*-aminobenzamidine. For each mutant produced, at least three independent preparations of membranes were undertaken, with at least one set being prepared with a concomitant set of membranes from a suitable coupled and uncoupled control strain.

2.8.(ii) Atebrin fluorescence-quenching assay

Atebrin fluorescence-quenching was measured as described by Gibson *et al* (1977a). Quenching of atebrin (quinacrine dihydrochloride) was measured at 30^oC in an Aminco-Bowman fluorimeter using the wavelength pair 450 nm/520 nm for excitation and emission. The fluorimeter was connected to a time base recorder, with chart speed set at 1 $cm.min^{-1}$. Approximately 1 mg of membrane protein was incubated in:

10 mM Hepes (pH 7.5 with HCl)
5 mM $MgCl_2$
300 mM KCl

in a final volume of 2.5 ml. The fluorescence of the buffer plus membranes was designated as the zero fluorescence. After addition of atebrin to a final concentration of 4 μM the intensity of fluorescence was then arbitrarily

designated as 100 units. NADH was added to 2 mM (Final concentration), and the decrease in fluorescence recorded. The time taken for membranes to use the oxygen in solution was taken as a standard measure for each set of membranes, with the assumption that NADH oxidase levels would be similar. Thus, membranes were added until a level was reached whereby the oxygen in solution was exhausted after 1 min. After the solution had gone anaerobic, sodium cyanide (2.5 mM final concentration) was added to the buffer to poison the respiratory chain, restoring the fluorescence. 1 mM ATP (Final concentration) was added and the fluorescence recorded. Finally, the uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (4 μM final concentration) was added to abolish any ATP-induced fluorescence-quenching activity.

2.8 (iii) Assay of ATPase activity and DCCD inhibition

Mg-ATPase activity was assayed essentially as described by Gibson *et al* (1977a). The reaction mixture contained:

10 mM ATP
50 mM Tris-Cl, pH 8.0
5 mM $MgCl_2$

in a final volume of 1 ml. After preincubation at 30^{o}C, the reaction was started by the addition of 50-300 μg of membrane protein. The reaction was terminated at specific times by adding 0.5 ml of the reaction mix to 9.5 ml of King's reagent (King, 1930). After 15 min the absorbance at 660 nm was measured and the amount of inorganic phosphate trapped as phophomolybdate determined by comparison with a linear standard curve derived from a known amount of inorganic phosphate.

For the determination of the DCCD-sensitivity of ATPase activity, membranes (300 μg membrane protein) were incubated in 5 ml of the ATPase assay mixture detailed above, together with various concentrations of DCCD, at 30^{o}C. Samples of 0.9 ml were taken at 0, 3, 6, 9, and 12 mins from the mixes incubated in each DCCD concentration and added to 0.1 ml 50% trichloroacetic acid. The amount of inorganic phosphate present at each time point was calculated by the method detailed above (King, 1930), and the rate of ATPase activity determined in the presence of each of the concentrations of DCCD from the linear portion of each curve. Linearity was normally achieved after approximately 3 min. Inhibition for each DCCD concentration was calculated as a percentage of the rate of ATPase activity in the absence of DCCD.

2.8.(iv) Protein determination

Protein concentrations were determined using Folin's phenol reagent (Lowry *et al.*, 1951) with bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo) as standard.

2.9. Protein manipulations

2.9.(i) Two-dimensional gel electrophoresis of membrane preparations

Membranes were prepared for electrophoretic analysis by dripping 5 ml of acetone slowly into 1 ml of the membrane preparation, while vortexing. The mixture was vortexed for a further 2 min and then centrifuged in a bench centrifuge for 5 min at room temperature. The pellet was again extracted carefully with 5 ml of acetone after the supernatant had been discarded and the pellet briefly dried under vacuum. After centrifugation, the pellet was again dried and extracted for a third time with 5 ml of acetone to remove as much phospholipid as possible. The remaining protein was harvested by centrifugation and suspended in approximately 500 μl of Lysis buffer (9.5 M urea, 2% w/v Nonidet NP-40, 2% w/v ampholines (1.2% pH range 5-7 and 0.8% pH range 3.5-10) and 5% v/v β-mercaptoethanol).

The method of two-dimensional electrophoresis employed was essentially that of O'Farrell (O'Farrell, 1975; O'Farrell *et al.*, 1977).

The first dimension, isoelectric focussing, separated polypeptides on the basis of their isoelectric points. The tube gels (5 mm internal diameter by 130 mm) consisted of 9.2 M urea, 2% (w/v) Nonidet NP-40, 4% (w/v) acrylamide/bisacrylamide (from a 30% w/v stock comprising 28.4% acrylamide, 1.6% bisacrylamide) and ampholines with a pH range 5-7 (1.2% w/v) and a pH range 3.5-10 (0.8% w/v). The mixture was polymerised by the addition of 20 μl ammonium persulphate (10%) and 14 μl TEMED per 10 ml. The gel was then overlayed with 8 M urea. Samples of acetone extracted membranes (50-100 μl) were loaded and subjected to electrophoresis for 4 hr starting at 180 Volts and maintaining constant current until the voltage reached 800 Volts (normally about 3 hr), then maintaining constant voltage for the remainder of the run. The cathode (20 mM NaOH) was in the bottom reservoir and the anode (10 mM phosphoric acid) was in the top reservoir. After electrophoresis, the gels were

frozen in SDS sample buffer (2% SDS, 125 mM Tris-Cl pH 6.8) and stored overnight at -20^{o}C.

The second dimension was SDS polyacrylamide gel electrophoresis using a linear polyacrylamide gradient. The gel separated polypeptides on the basis of their molecular weight. The slab gel (90 mM high x 150 mM long x 2 mM thick) comprised a stacking gel and a separating gel. The separating gel consisted of acrylamide and glycerol and was prepared by mixing the following two solutions. The front chamber of the gradient mixer contained 350 mM Tris-Cl p 8.8, 0.01% (w/v) SDS, 11.7% glycerol (v/v), and 22.5% acrylamide (from a 30% w/v stock solution containing 29.2% (w/v) acrylamide and 0.8% (w/v) bisacrylamide) in a volume of 11.75 ml. Polymerisation was initiated by the addition of 15 μl 10% (w/v) ammonium persulphate and 3 μl TEMED. The back chamber contained 350 mM Tris-Cl pH 8.8, 0.01% (w/v) SDS, and 7.5% (w/v) acrylamide (from the above 30% acrylamide stock solution), in a volume of 11.75 ml and was polymerised by the addition of 25 μl 10% (w/v) ammonium persulphate and 5 μl TEMED. The stacking gel (14 ml) was 4.5% (w/v) acrylamide (from the above 30% stock solution), 0.1% (w/v) SDS and 125 mM Tris-Cl pH 6.8. Polymerisation was initiated by the addition of 42 μl 10% (w/v) ammonium persulphate and 14 μl of TEMED. The slab gel was then assembled into the running apparatus and the cylindrical first dimension gel was placed across the top of the stacking gel and secured in place with 1% w/v agarose. Running buffer (25 mM Tris base, 192 mM glycine and 0.1% w/v SDS) was added to each reservoir and the polypeptides were subjected to electrophoresis for 17 hr at 18 mA constant current.

After electrophoresis, the gel slab was immersed in 5.5% trichloroacetic acid, 5.5% sulphosalicylic acid and 11% methanol and incubated at 60^{o}C for 1 hr to fix the proteins within the gel. Proteins were then visualised by staining with 0.11% Coomassie Blue in 25% v/v ethanol, 8% acetic acid for 2 hr and destained for several hours in 25% v/v ethanol, 8% acetic acid. The staining and destaining procedures were repeated and the gels photographed.

CHAPTER 3

IN VITRO SITE-SPECIFIC MUTAGENESIS AND ITS APPLICATION TO THE PRODUCTION OF SUBSTITUTIONS IN THE *unc* GENES *uncB* AND *uncE* CODING FOR SUBUNITS *-a* AND *-c* OF THE *E. coli* F_oF_1-ATPase: FORMATION AND CHARACTERISATION OF THE DOUBLE AMINO ACID SUBSTITUTION IN SUBUNIT-*a*, K167Q,K169Q(*uncB546*)

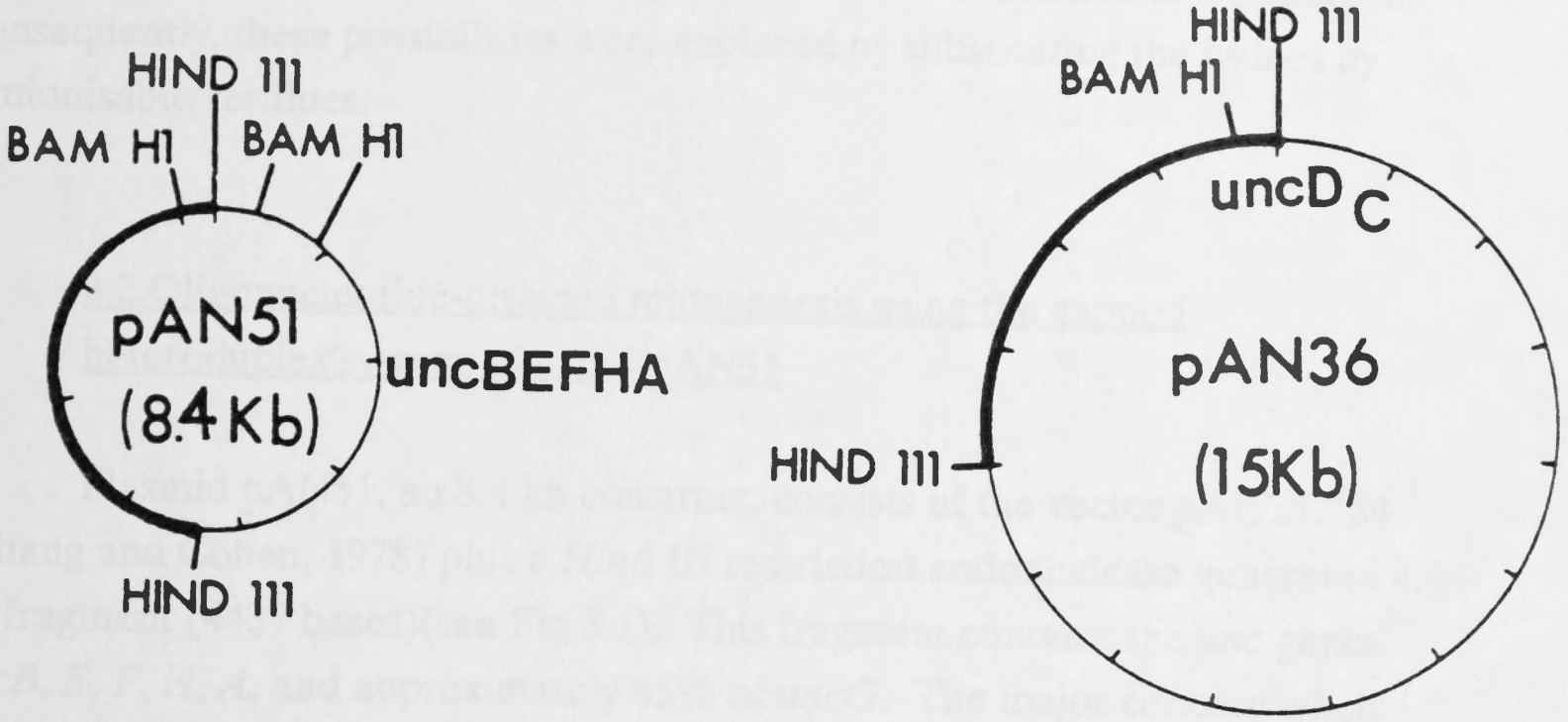
3.1. Introduction

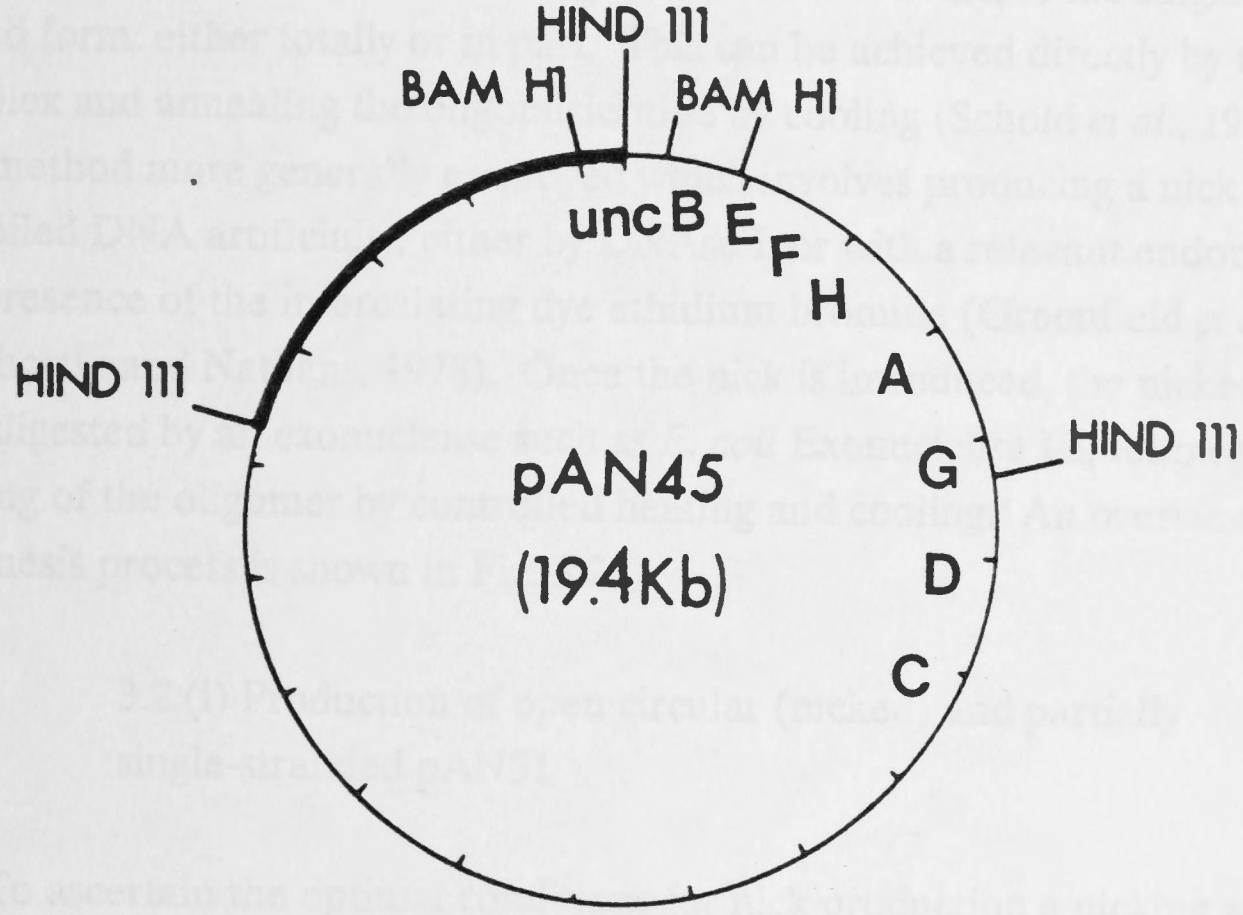
Many mutations have been produced in the *unc* operon in the past sixteen years, and a general indication of the information elicited has been given (*q.v.* chapter 1). Until recently, the mutants were all isolated following alterations of the DNA through random mutagenesis involving either a chemical mutagen such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Butlin *et al.* ,1971,1973;Hoppe *et al.*, 1980), ethyl methanesulphonate (Ito and Ohnishi, 1981; Porter *et al.*, 1985), hydroxylamine (Cain and Simoni, 1986), random viral or transposon insertion (Kanazawa *et al* 1984), or by deleting a specific endonuclease-generated fragment within the *unc* operon (Gay, 1984). *In vitro* site-specific mutagenesis results in defined base transitions, transversions, deletions or insertions in a specific region of DNA, a selectivity which demands a prior knowledge of the sequence of the regions of DNA to be mutated. Thus, by synthesising and artificially introducing small oligonucleotides which carry single or multiple base changes from the original DNA sequence, the *unc* operon can be manipulated such that the potential functional importance of an individual amino acid, or group of amino acids, central to any hypothesis concerning the structure or function of the F_0F_1-ATPase complex can be challenged.

Many different methods of oligonucleotide-directed mutagenesis have been developed over the past five or six years (see Smith, M., 1985). Therefore, when attempting to set up a system of site-directed mutagenesis it is important to use a method or methods which is/are as simple and efficient as possible, and applicable to mutant production throughout the *unc* operon. The procedures most commonly used for mutant production involve an initial formation of a heteroduplex DNA molecule, by annealing a synthetic oligonucleotide to a larger, covalently-closed single-stranded template. The annealed oligomer is then secured by using both a polymerase and a ligase to produce a covalently-closed duplex.

Systems which utilise the formation of a heteroduplex to produce mutations, require an original single-stranded substrate complementary in part to a synthetic oligodeoxyribonucleotide carrying the desired base substitution(s). This single-stranded substrate can be formed by either of two ways. It may originate as a double-stranded plasmid, which has been suitably manipulated to exhibit a relevant 'gap' (Wallace *et al.*, 1980), or by using a filamentous phage (ϕX174 (Hutchison *et al.*, 1978; Razin *et al.*, 1978), M13 (see Zoller and Smith, 1983), or fd (Wasylyk *et al.*, 1980)), which is readily prepared, and in fact propogates, in the single-stranded form. Both forms can easily be made to accommodate the relevant *unc* genes, and both systems were used to attempt to

Fig 3.1. Schematic diagrams of plasmids pAN51, pAN36 and pAN45 showing cleavage sites for endonucleases *Hin*d III and *Bam* H1, and approximate positions of the *unc* genes. The heavy lines indicate the vector portions of the plasmids (pAN174 in plasmid pAN51, and pACYC184 in plasmids pAN36 and pAN45). The calibrations represent intervals of 1 kb.


HIND 111
BAM H1
BAM H1
pAN51
(8.4Kb)
uncBEFHA
HIND 111
HIND 111
BAM H1
uncD C
pAN36
(15Kb)
HIND 111


HIND 111
BAM H1
BAM H1
uncB E F H A G D C
pAN45
(19.4Kb)
HIND 111
HIND 111

produce the double substitution K167Q, K169Q in subunit-*a*. Lysine residues at position 167 and 169 of subunit-*a* in the working model were positioned inside the membrane. If these residues are intramembranous it may be assumed that they fulfil either a functional role, or structural role, being possibly involved in intra or intermolecular charge-pairing to stabilise intramembranous structures. Consequently, these possibilities were explored by substituting the lysines by nonionisable residues.

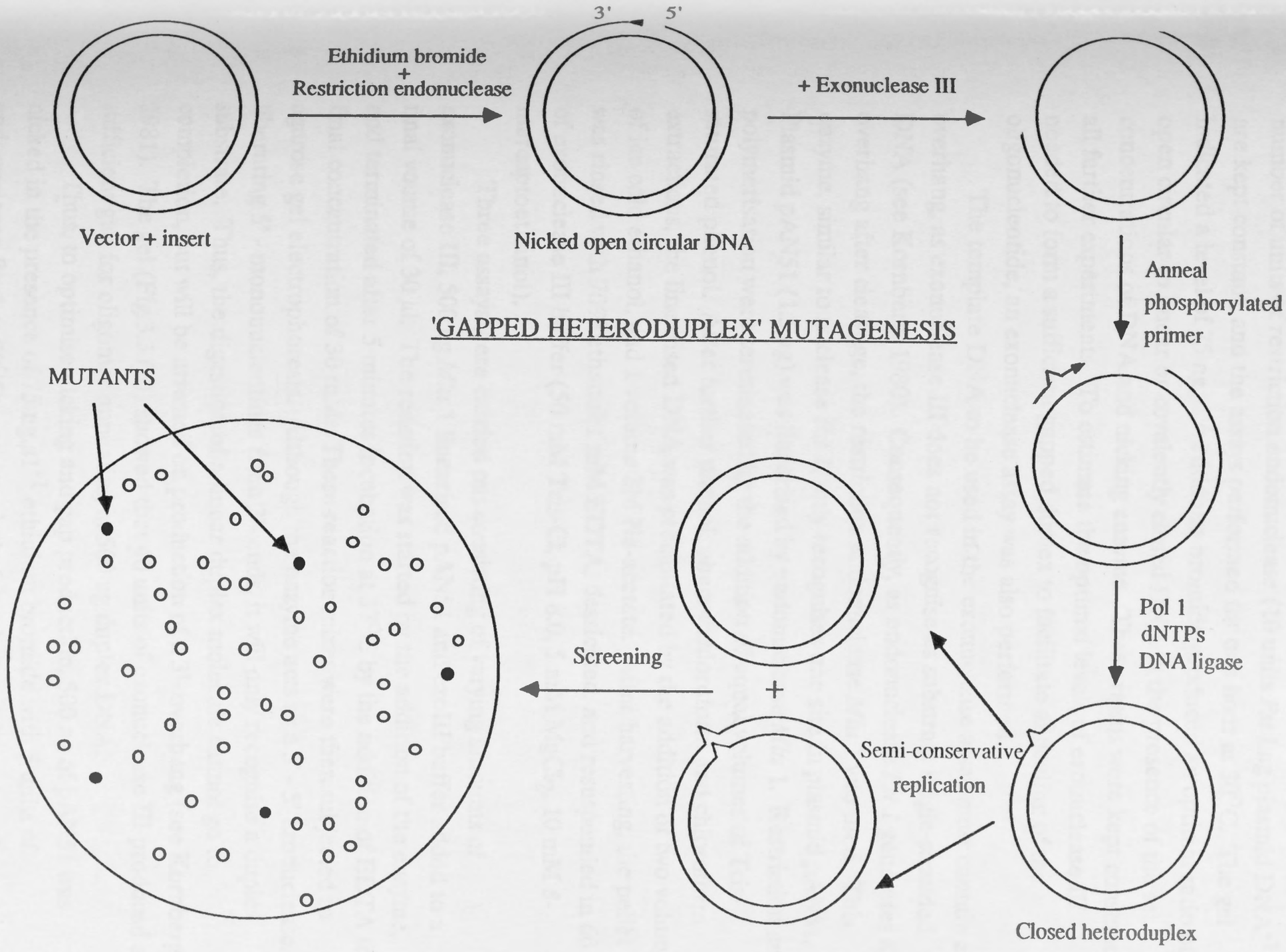
3.2.Oligonucleotide-directed mutagenesis using the'gapped heteroduplex'system: plasmid pAN51

Plasmid pAN51, an 8.4 kb construct, consists of the vector pACYC184 (Chang and Cohen, 1978) plus a *Hin*d III restriction endonuclease generated 4.4 kb fragment (4437 bases)(see Fig 3.1). This fragment contains the *unc* genes *uncB, E, F, H, A*, and approximately 65% of *uncG*. The major concern when using a double-stranded potential template, is its conversion to the single-stranded form, either totally or in part. This can be achieved directly by melting the duplex and annealing the oligonucleotide by cooling (Schold *et al.*, 1984), or by the method more generally employed which involves producing a nick in the supercoiled DNA artificially, either by DNAse I, or with a relevant endonuclease in the presence of the intercalating dye ethidium bromide (Greenfield *et al.*, 1975; Shortle and Nathans, 1978). Once the nick is introduced, the nicked strand can be digested by an exonuclease such as *E. coli* Exonuclease III, followed by annealing of the oligomer by controlled heating and cooling. An overview of the mutagenesis process is shown in Fig. 3.2.

3.2.(i) Production of open circular (nicked) and partially single-stranded pAN51

To ascertain the optimal conditions for nick production a nicking assay was conducted. Plasmid pAN51 was isolated from strain AN2402(*uncB402*/pAN51) and the final DNA concentration estimated. Endonuclease *Pst* I was used as the restriction enzyme to produce the required nick. Endonucleas *Pst* I recognises only one site in pAN51, cutting 40 base pairs 3' to the Lys-169 codon. The close proximity of the nick to the region of interest ensures that the activity of the exonuclease will reveal the annealing site for the oligomer. The mutagenesis was attempted using the oligonucleotide listed in Table 3.1 to produce the double amino-acid substitution K167Q,K169Q in

Fig 3.2 Schematic representation of the "gapped heteroduplex" method of mutagenesis (Vlasuk and Inouye, 1983). The double-stranded vector carrying the DNA fragment of interest is nicked by a suitable restriction endonuclease in the presence of ethidium bromide. The nick is extended in the 3'-5' direction by exonuclease III, revealing a complementary region for a small oligonucleotide carrying the desired base substitution. After annealing, the gap is sealed by DNA polymerase 1 (large fragment) and ligated by T4 DNA ligase. After transformation of a suitable recipient strain by the heteroduplex, the duplex is replicated, producing two forms of homoduplex plasmid, mutant and normal. Colonies carrying the mutant plasmid are then selected by colony hybridisation (q.v.Chapter 2, section 2.7.(iii)).


3'
5'
Ethidium bromide
+
Restriction endonuclease
+ Exonuclease III
Vector + insert
Nicked open circular DNA
Anneal
phosphorylated
primer
'GAPPED HETERODUPLEX' MUTAGENESIS
MUTANTS
Pol 1
dNTPs
DNA ligase
Screening
+
Semi-conservative
replication
Closed heteroduplex

subunit-*a*, corresponding to base substitutions at positions 499 and 505. A typical nicking assay is shown in Fig. 3.3(i). Amounts of plasmid DNA (500 ng) and number of units of restriction endonuclease (10 units *Pst* I.μg plasmid DNA^{-1}) are kept constant, and the assays performed for one hour at 30^{o}C. The gel indicated a level of 75 ng.μl^{-1} ethidium bromide produced an optimal ratio of open circular to linear or covalently closed DNA in the presence of the set concentrations of DNA and nicking enzyme. These ratios were kept constant for all further experiments. To estimate the optimal levels of exonuclease III needed to form a sufficient gapped duplex to facilitate annealing of the oligonucleotide, an exonuclease assay was also performed.

The template DNA to be used in the exonuclease assay must contain a 5' overhang, as exonuclease III does not recognise as substrate single-stranded DNA (see Kornberg, 1980). Consequently, as endonuclease *Pst 1* generates a 3' overhang after cleavage, the restriction endonuclease *Mlu* 1 was used. This enzyme, similar to nuclease *Pst 1*, only recognises one site in plasmid pAN51. Plasmid pAN51 (1.5 μg) was linearised by endonuclease *Mlu* 1. Restriction and polymerisation were terminated by the addition of equal volumes of Tris-saturated phenol. After further phenol, phenol:chloroform and chloroform extractions, the linearised DNA was precipitated by the addition of two volumes of ice cold ethanol, and 1 volume 3M Na-acetate. After harvesting, the pellet was rinsed with 70% ethanol;1 mM EDTA, dessicated, and resuspended in 60 μl of exonuclease III buffer (50 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol).

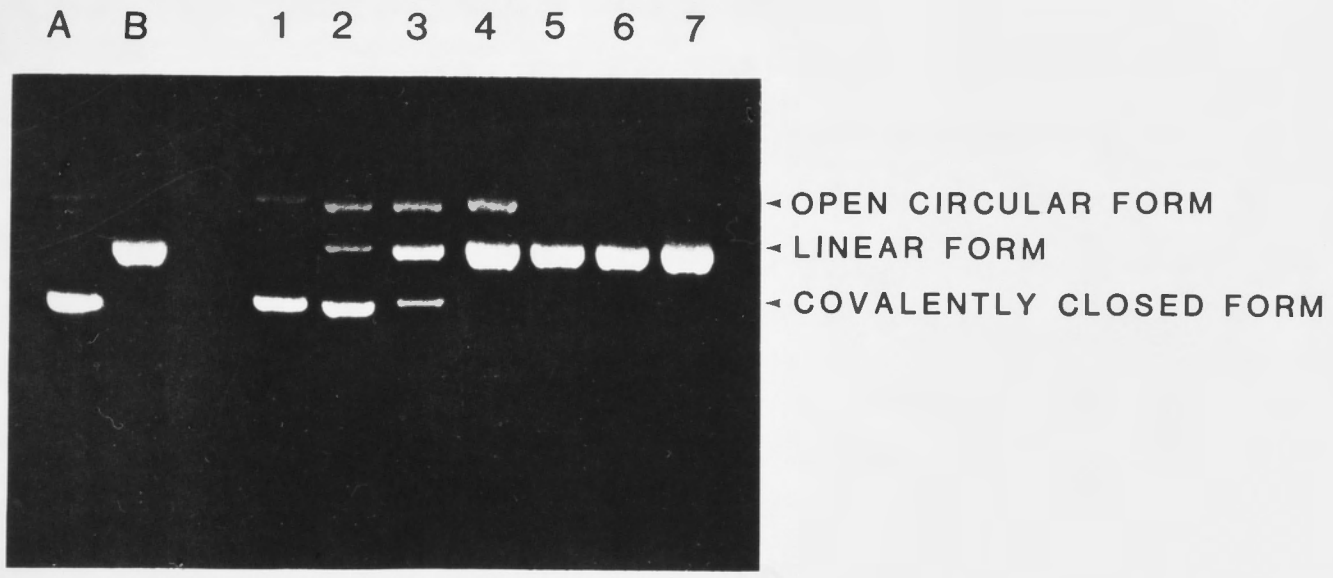
Three assays were carried out consisting of varying amounts of exonuclease III, 500 ng *Mlu* 1 linearised pAN51 and exoIII buffer added to a final voume of 30 μl. The reaction was started by the addition of the enzyme, and terminated after 5 minutes incubation at 37^{o}C by the addition of EDTA to a final concentration of 30 mM. These reaction mixes were then subjected to agarose gel electrophoresis. Although the enzyme acts as a 3' - 5' exonuclease, liberating 5' - mononucleotides from 3'- ends, it will only recognise a duplex substrate. Thus, the digestion of a linear duplex molecule cannot go to completion, but will be arrested on production of a 3'- overhang (see Kornberg, 1981). The gel (Fig.3.3.(ii)) showed that 40 units of exonuclease III produced a sufficient gap for oligomer annealing in 500 ng duplex DNA.

Thus, to optimise nicking and gap production, 500 ng of pAN51 was nicked in the presence of 75 ng.μ1^{-1} ethidium bromide with 5 units of endonuclease *Pst* 1 at 30^{o}C over a period of one hour. After ethanol precipitation, the nicked plasmid DNA was treated with 40 units of Exonuclease III for five minutes at 37^{o}C to produce a partially-gapped plasmid molecule, with

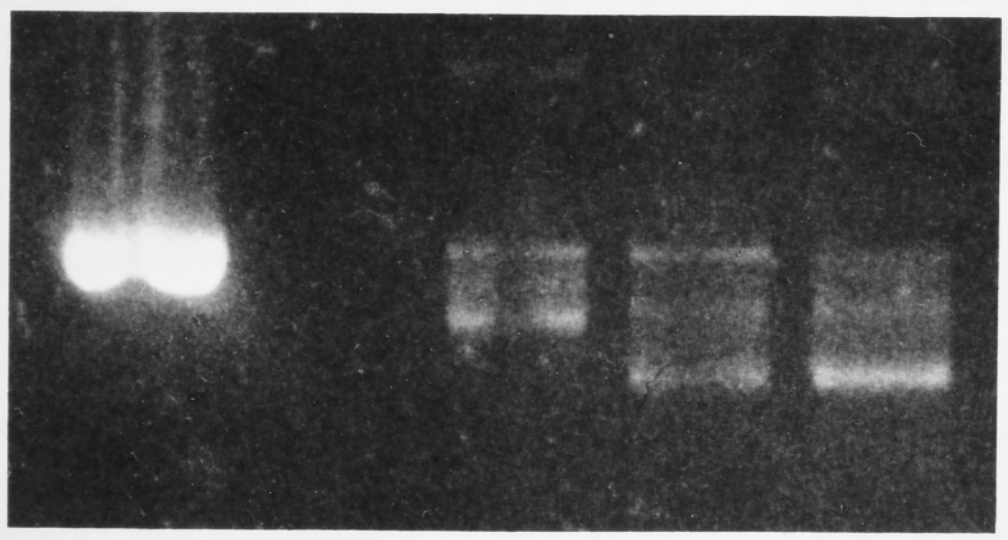
Fig 3.3. Nicking and exonuclease III assays. Fig 3.3.(i) shows a nicking assay performed on plasmid pAN51 as detailed in section 3.2.(i). Samples were electrophoresed through a 0.8% (w/v) agarose gel as described in Chapter 2, section 2.5.(iv). Control lanes; (A) 250 ng of plasmid pAN51 linearised by endonuclease *Pst* 1 in the absence of ethidium bromide; (B) 250 ng of plasmid pAN51 uncut. Samples run in lanes 1 - 7 contained 250 ng of plasmid pAN51 after digestion by nuclease *Pst* 1 (10 units.μg DNA^{-1}) at 37^{o}C for 1 hr in differing concentrations of ethidium bromide. Digestion of the samples were attempted in the following concentrations of ethidium bromide (numbers correspond to the lane numbers in figure 3.3.(i)); (1) 300 ng.μl^{-1}; (2) 150 ng.μl^{-1}; (3) 75 ng.μl^{-1}; (4) 50 ng.μl^{-1}; (5) 25 ng.μl^{-1}; (6) 10 ng.μl^{-1}; (7) 1 ng.μl^{-1}.

Fig 3.3.(ii) shows an exonuclease assay performed on linearised plamid pAN51 as detailed in section 3.2.(i). Samples were electrophoresed through a 0.8% agarose gel as described in Chapter 2, section 2.5.(iv). Control lane, (A) 250 ng of linearised plasmid pAN51. Samples run in lanes 1 - 3 contain 250 ng of linarised pAN51 treated with various amounts of exonuclease III over a 5 min incubation period at 37^{o}C. Digestion of the samples were attempted by the following amounts of exonuclease III (numbers correspond to the lane numbers in Figure 3.3.(ii)); (1) 10 units.μg DNA^{-1}; (2) 40 units.μg DNa $^{-1}$; (3) 80 units.μg DNA^{-1}.

(i)


A
B
1
2
3
4
5
6
7
OPEN CIRCULAR FORM
LINEAR FORM
COVALENTLY CLOSED FORM

(ii)


A
1
2
3

the reaction being terminated by the addition of EDTA to a final concentration of 30 mM.

3.2.(ii) Annealing the oligodeoxyribonucleotide to gapped duplex template, and production of covalently-closed duplex.

After nicking and digestion, the mix was phenol extracted, and the gapped duplex was precipitated and rinsed with 500 μl 70% ethanol;0.1 mM EDTA. 50 ng. of the purified synthetic oligodeoxyribonucleotide (*q.v.*section 2.7.(i), see Table 3.1) carrying an enzymatically transferred [γ-5'] phosphate from ATP (*q.v.*section 2.7.(ii)) was annealed to the gapped duplex in a final volume of 20 μl, containing 60 mM Tris-CL pH 8.0, 10 mM $MgCl_2$, and 10 mM β-mercaptoethanol. The solution was heated to 65^{o}C for 5 mins and the oligomer was annealed at 37^{o}C over a period of 30 mins. Filling, and production of a covalently-closed heteroduplex were undertaken in a final volume of 100 μl, containing:

20 μl heteroduplex mix
1 mM ATP
330 μM of each of the dNTPs
10 mM dithiothreitol
5 units *E. coli* DNA Polymerase (Large fragment)
4 units T4 DNA Ligase

and the mix incubated at 12^{o}C for 12 hours before transformation.

3.2.(iii) Transformation of recipient strain and screening for mutant production.

Strain AN1453*(uncB402)* was chosen as the recipient strain to facilitate immediate complementation analysis of any potential mutants. Aliquots of the incubation mix were used to transform $CaCl_2$ treated cells of strain AN1453(*uncB402*). After plating onto nutrient agar supplemented with chloramphenicol, approximately 1000 resultant transformants were screened for the incorporation of the base substitution by colony hybridisation.

Working with short (20-30 bases) oligodeoxyribonucleotides, Astell *et al.*(1971)(see also Gillam *et al.* 1975), found that when the oligomers carried a single base mismatch from the complementary strand, the melting point (Tm) of the heteroduplex was reduced in comparison to the homoduplex. This instability

TABLE 3.1

SYNTHETIC OLIGONUCLEOTIDES USED IN THE PREPARATION AND SEQUENCE CONFIRMATION OF THE DOUBLE SUBUNIT-*A* SUBSTITUTION K167Q,K169Q.

Preparation of the oligonucleotides is detailed in Chapter 2, section 2.7.(i). Bases which are underlined signify a base substitution from the normal sequence.

Synthetic oligonucleotide	Relevant amino acid substitution	Notes
5'-CAGCATCCAAATGCAAGGCAT-3'	K167Q,K169Q	
5'-GTAAAACGACGGCCAGT-3'	-	Phage M13 universal primer which anneals onto the viral strand approximately 50 bases 5' from the *Hind* III restriction site in the mp9 polylinker.
5'-GGGTCTGTTGTTCCTGGTTT-3'	-	Sequencing primer used for sequencing from nucleotide 300 in the*uncB* gene.

can be utilised, as the oligonucleotide will preferentially remain bound to the plasmid or phage carrying the substitution. This form of genotypic screening also allows the mutants to be identified without the amino acid substitutions necessarily producing a phenotypically distinct mutation. The procedure for colony hybridisation was essentially that of Maniatis *et al*(1983),(*q.v.*section 2.7.(iii)). Replica plates from the transformation experiments were retained, and each plate was used to produce duplicate nitrocellulose filters carrying immobilised DNA from the colonies. Labeling of the relevant oligonucleotide probe with [γ-^{32}P]ATP is set out in Chapter 2.section 2.7.(ii). The optimal temperature for hybridisation was estimated using 4^{o}C for each G or C base within the probe , and 2^{o}C for each A or T.(Suggs *et al.*, 1981), As suggested by Vlasuk and Inouye (1983), a hybridisation temperature of 4^{o}C below this estimate was used, which was 58^{o}C. (see Table 3.1 for base compostition of oligonucleotide). After 14 hours, the filters were removed from the hybridisation chamber and washed in three changes of 0.9 M NaCl, 90 mM Na-citrate over a 10 minute period at room temperature. The filters were air dried, and possible mutants were visualised by autoradiography at -70^{o}C with the aid of an intensifying screen. After an exposure of 2-3 hours, the film was developed. A number of colonies exhibited strong signals, but the results were clarified by washing the filters for one minute in 0.9 M NaCl, 90 mM Na-citrate at 50^{o}C followed by further autoradiography. Four colonies exhibited strong signals. These colonies were identified and rescued from the replica plate, streaked out onto sectors of another nitrocellulose filter, and overlaid onto fresh nutrient agar. Once the sectors had grown, they were again hybridised with the probe. Colonies carrying potentially mutated plasmids were isolated, and their plasmids prepared. The *Hin*d III-generated 4.4 kb fragment carrying *uncB* was subcloned from each of the four into M13 mp9, and confirmation of the mutation attempted by sequencing. However, nucleotide sequence analysis revealed that all four plasmids had retained an unaltered *uncB* gene. A repetition of this experiment highlighted a further seven colonies which, on the basis of a strong hybridisation signal, appeared to contain the mutation. These seven also proved to be normal following plasmid isolation, subcloning, and sequence analysis.

3.3.Oligonucleotide-directed mutagenesis using the single-stranded system: Bacteriophage M13 mp9

After the failure with the 'gapped heteroduplex' method to produce any mutants, an attempt was made to utilise the single-stranded phage template method of mutant production. One advantage of this procedure is the ease of

preparation of template once the gene, or genes of interest have been introduced in to the phage replicative form. Although large inserts, or some specific regions, of DNA may prove unstable when incorporated into the phage (Yannish-Perron *et al.*, 1985), this is usually not a problem, and once introduced, the phage carrying the inserted gene can be stored as the infective, single-stranded form after precipitation and resuspension in TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA), or double-stranded, replicative form DNA can be retained as a stable stock at -20^{o}C. Base substitutions successfully produced, can easily be confirmed by directly sequencing the mutant. Once the substitution has been introduced, the mutated fragment must be subcloned into a suitable vector to facilitate expression of the mutant gene product.

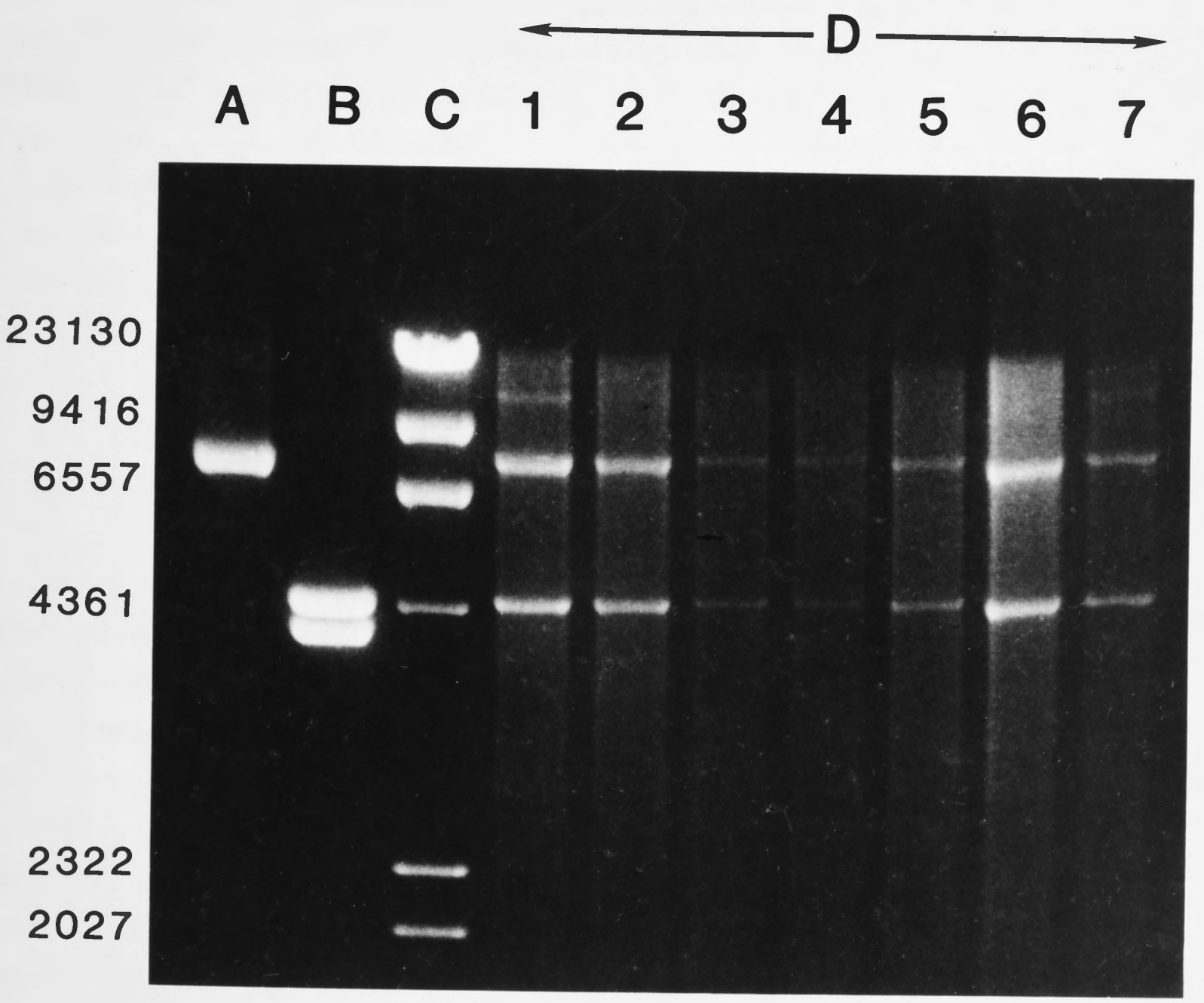
This system of mutagenesis was pioneered by work with the single-stranded phage ϕX174 (Hutchison *et al.*, 1978), but the development of the M13 mp series of phage vectors by Messing and co-workers (see Messing, 1983) has facilitated their easy and efficient manipulation and template production. These vectors have small 'polylinkers' incorporated in their genome which act as unique recognition sites for a large number of commercially available restriction endonucleases. Cloning of fragments into these sites is readily screened by a colouration reaction whereby the blue colour produced on cleavage of 5-bromo-4-chloro-3-indolyl-βD-galactopyranoside(BCIG) by the enzyme β-galactosidase is lost when fragments of DNA are cloned within the reading frame for the galactosidase enzyme (*q.v.*section 2.5.(iii)).

3.3.(i) Production of M13 mp9 carrying a 4.4 kb *Hin*d III-generated fragment coding for the *E.coli* F_0F_1-ATPase subunits -*a*, -*b*, -*c*, δ, and α

The replicative form of phage M13 mp9 was isolated from 1 litre of infected K37(*q.v.*section 2.4.(iv)). The DNA was cleaved by nuclease *Hin*d III, the terminal 5'-phosphates were removed(*q.v.*section 2.5.(i)), and the DNA was retained at a stock concentration of 200 ng.μl^{-1}. Plasmid pAN51 (2 μg) was cut with nuclease *Hin*d III and mixed with 400 ng *of Hin*d III-linearised, phosphatase-treated phage vector in a final volume of 100 μl. One unit of T4 DNA ligase was added and ligation was allowed to proceed at 12^{o}C for 5 hours before transformation of competent JM101 cells (*q.v.*section 2.5.(ii)) to chloramphenicol resistance.

Phage carrying an inserted fragment were selected initially by the colouration reaction outlined above. Single-stranded phage DNA from twelve colourless plaques was isolated (*q.v.*section 2.3.(iv)), and after agarose gel

Fig 3.4. Agarose gel electrophoresis of seven rapid preparations of bacteriophage replicative form containing DNA inserts. Sample DNA of control and phage clone were cleaved with nuclease *Hin*d III and electrophoresed through a 0.7% agarose gel as described in Chapter 2, section 2.5.(iv). Control lanes A, B, and C. (A) 200 ng of phage M13 mp9 replicative form; (B) 500 ng plasmid pAN51; (C) 1 μg of bacteriophage lambda. The values given on the left of the gel correspond to the size (in bases) of the fragments produced by *Hin*d III digest of the lambda DNA. The samples in D (lanes 1 - 7) contain DNA prepared from seven different phage clones by the method detailed in Chapter 2, section 2.3.(iv). All seven preparations contain the 4.4 kb *Hin*d III-generated fragment carrying the *unc* genes *uncB, E, F, H,* and part of *unc G.*


D
A
B
C
1
2
3
4
5
6
7
23130
9416
6557
4361
2322
2027

electrophoresis, seven were shown to carry an insert. Double-stranded replicative form DNA was isolated from the seven phage clones, and the DNA digested with either *Hin*d III (Fig.3.4) or *Cla* I (Fig.3.5). Fig.3.4 shows that the seven phage carried the 4.4 kb fragment and none carried the *Hin*d III-generated vector fragment from pAN51. Analysis of the *Cla* 1-generated fragments in Fig.3.5 enables the orientation of the fragment insert in each of the seven preparations to be mapped. Fig 3.6 shows schematically how the correct orientation of the fragment insert for oligonucleotide annealing was deduced, with the desired orientation facilitating the production of single-stranded phage complementary to the published sequence. Phage carrying the fragment insert in the appropriate orientation (Lanes 1, 2, 4 and 7 of (D) Fig 3.5) was taken and replicative form DNA isolated (from the preparation shown in lane 1). This phage replicative form was designated pAN461.

3.3.(ii) Annealing of oligodeoxyribonucleotides to a single-stranded template, and production of a covalently-closed duplex.

The oligonucleotide carrying the relevant base substitutions (Table 3.1) was assessed for correct single-site annealing by using it as a sequencing primer on the single-stranded template. After confirmation by producing a single sequencing ladder, consistent with the published sequence 3' to the annealing site, production of the duplex was attempted. The method employed was similar to that used in 3.2.(iii), except for the addition of a second oligonucleotide, the M13 universal primer (Table 3.1), which was believed to increase the frequency of mutant production (*q.v.*section 3.4). Purified single-stranded DNA (400 ng) was used as template in the mutagenesis experiment with 40 ng of phosphorylated oligonucleotide and 20 ng of the universal primer.

3.3.(iii) Transformation of recipient strain and screening for mutant production

Efficiency of transformation of strain K37 was optimised by using the method of Hanahan (1983; *q.v.*section 2.5.(ii)). Production of approx. 200-1000 plaques after transformation was achieved with between 25-50 ng DNA after ligation. Plaque hybridisation using the same method of differential-temperature hybridisation with the oligonucleotide probe as used in the colony hybridisation (*q.v.*section 3.2.(iv)) was performed to select for potential mutant phage after immobilisation of the phage DNA onto nitrocellulose filters. However, hybridisation of the probe to the filters was performed by adding the filters to the

Fig 3.5. Agarose gel electrophoresis of seven rapid preparations of bacteriophage replicative form containing DNA inserts. Sample DNA of control lanes A, B, and C and phage clones (D) were cleaved with nuclease *Cla* 1 and electrophoresed through a 0.7% agarose gel as described in Chapter 2, section 2.5.(iv). Control lanes A, B, C and E; (A) 400 ng of M13 mp9 replicative form; (B) 500 ng plasmid pAN51; (C) 200 ng plasmid pACYC184;(E) 1 μg of bacteriophage lambda DNA cleaved with nuclease *Hin*d III. The values given on the right of the gel correspond to the size (in bases) of the fragments produced by *Hin*d III digest of the lambda DNA. The samples in D (lanes 1 - 7) contain DNA prepared from seven different phage clones by the method detailed in Chapter 2, section 2.3.(iv). As the exact recognition sites for nuclease *Cla* 1 in both the 4.4 kb *Hin*d III-generated fragment and M13 mp9 are known, it is possible to define the correct plasmid construction for oligonucleotide hybridisation (*q.v.* section 3.3.(i) and Fig 3.6.).

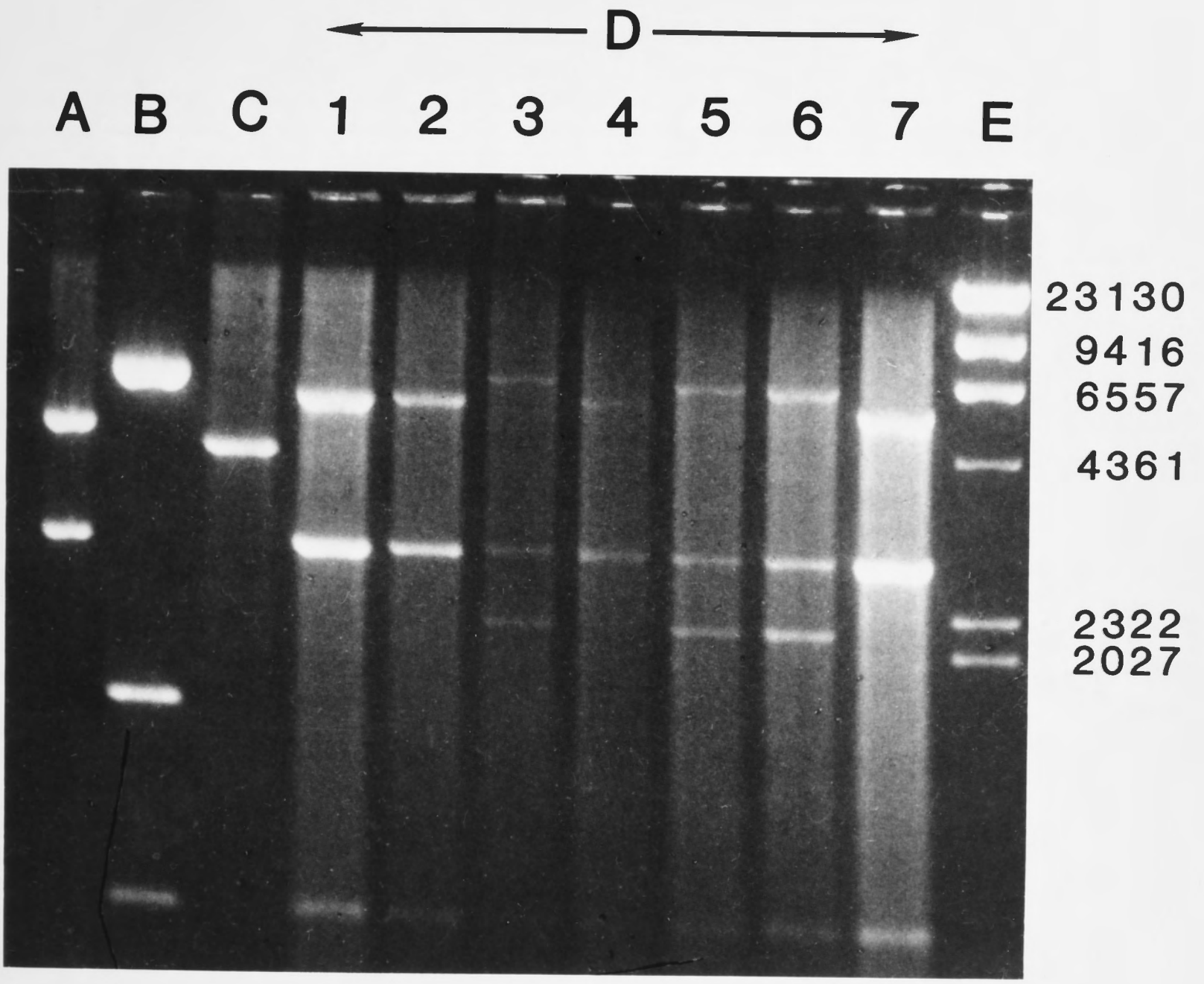
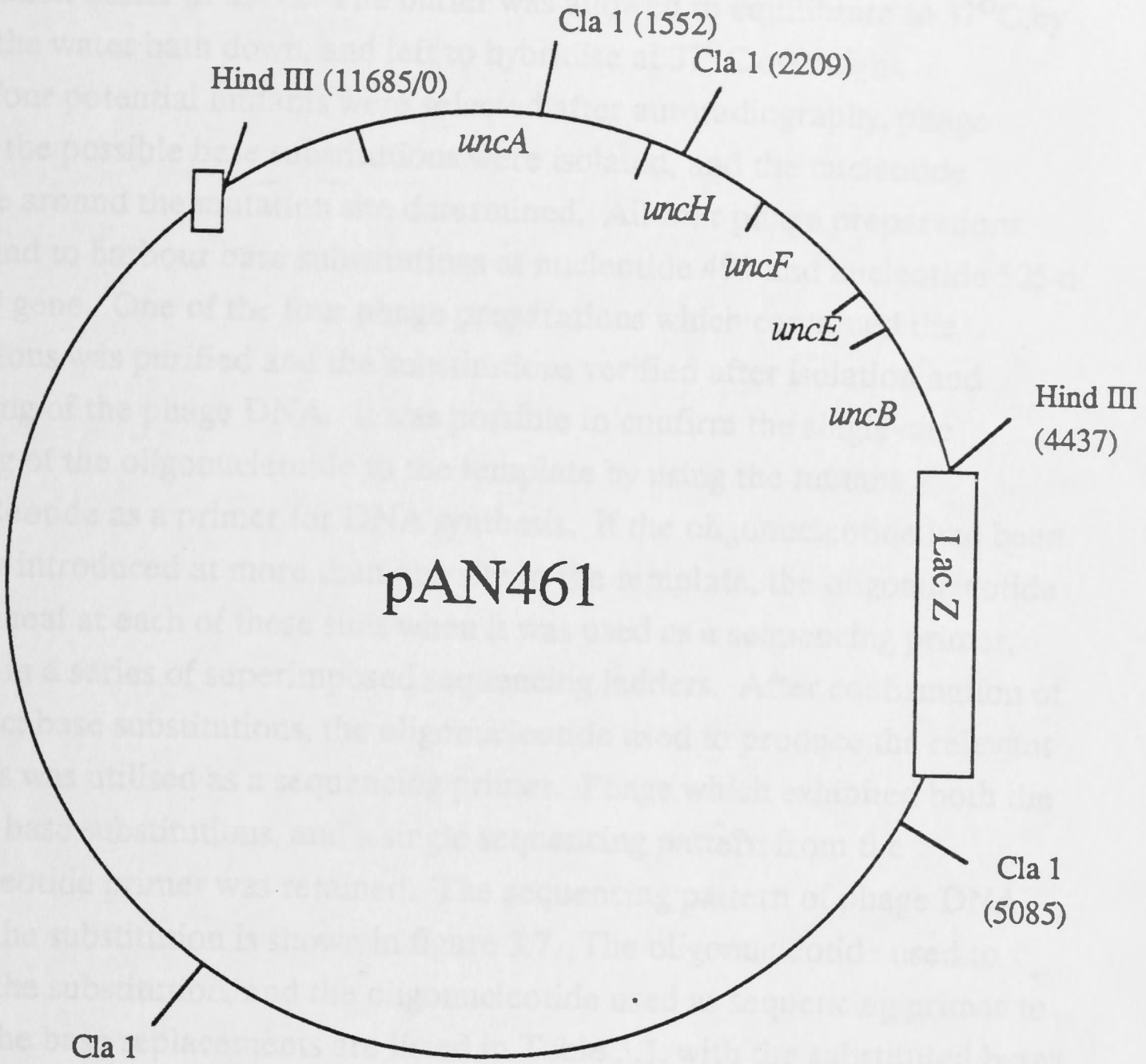
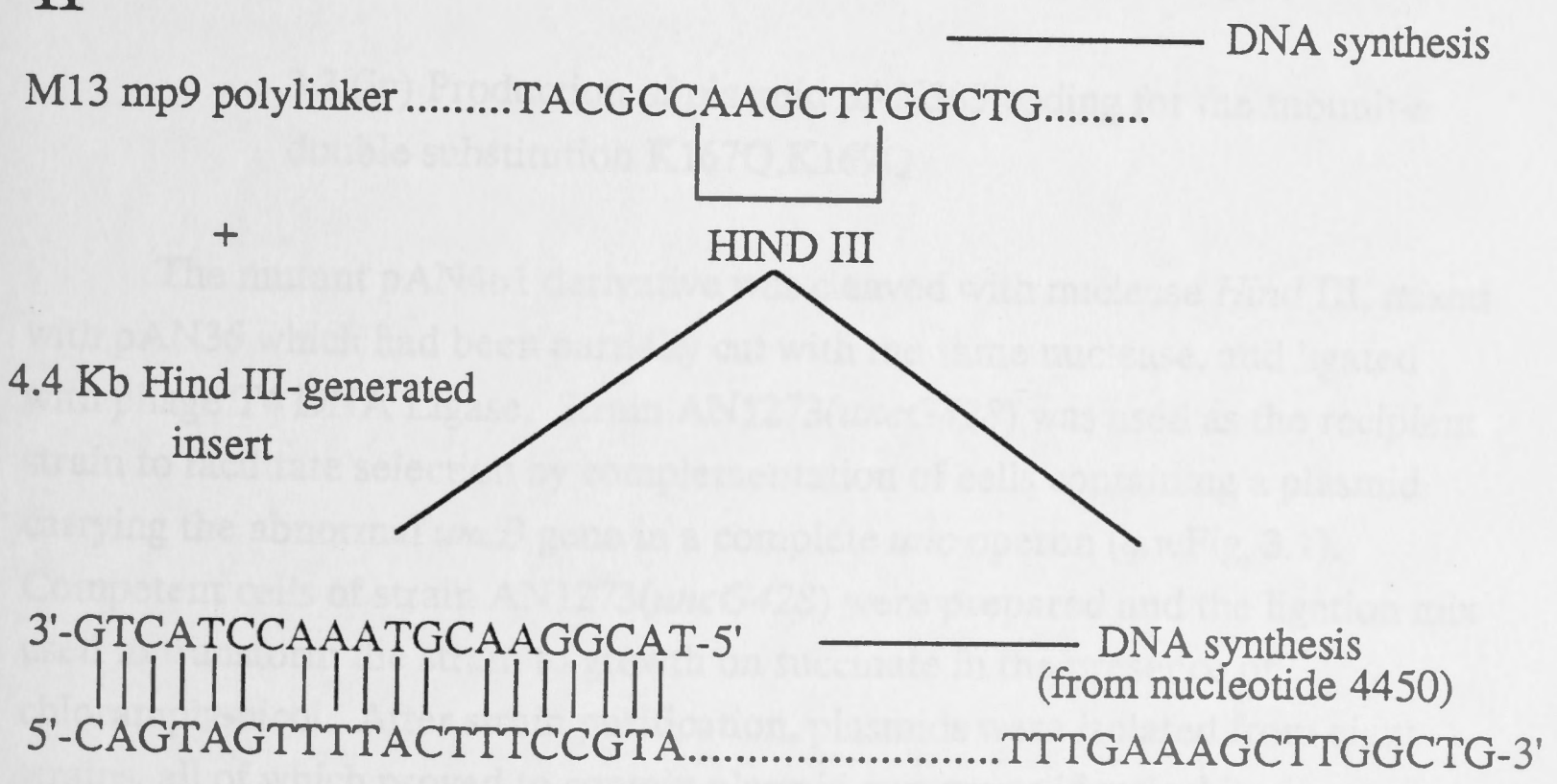

D
A B C 1 2 3 4 5 6 7 E
23130
9416
6557
4361
2322
2027

Fig 3.6 Schematic representation of phage replicative form pAN461 and illustration of the orientation of the 4.4 kb fragment with respect to the phage M13 mp9. Fig 3.6 (i) is a schematic diagram of replicative form pAN461 showing cleavage sites for endonucleases *Hin*d III and *Cla* 1, and approximate positions of the *unc* genes. The two small boxes represent the *lacZ* structural gene which is cleaved on insertion of a DNA fragment into M13 mp9. The numbers flanking the restriction sites correspond to the nucleotide position within the plasmid, with the numbering commencing from the *Hin*d III site at the top left hand position of the diagram. As the mode of replication of the bacteriophage M13 dictates that the same strand always acts as template for synthesis of the nascent strand, it is imperative that the orientation of the fragment insertion will guarantee tha the correct strand will occur in the purified single-stranded template for oligonucleotide annealing. Fig 3.6 (ii) illustrates the correct orientation of the fragment insert within the mp9 polylinker to effect annealing of oligonucleotides which have been the same composition as, and not the complementary sequence to, the published nucleotide sequence. The *Hin*d III-generated 4.4 kb fragment carrying the *unc* genes must be inserted in the orientation illustrated by Fig 3.6 (i). The annealing site for the M13 universal primer is located around position 4437, and sequencing is effected in the direction *uncB* to *uncA*.

I


Cla 1 (1552)
Cla 1 (2209)
Hind III (11685/0)
uncA
uncH
uncF
uncE
uncB
Hind III
(4437)
pAN461
Lac Z
Cla 1
(5085)
Cla 1
(7978)

II


DNA synthesis
M13 mp9 polylinker .....TACGCCAAGCTTGGCTG.....
+
HIND III
4.4 Kb Hind III-generated
insert
3'-GTCATCCAAATGCAAGGCAT-5'
5'-CAGTAGTTTTACTTTCCGTA.....TTTGAAAGCTTGGCTG-3'
DNA synthesis
(from nucleotide 4450)

hybridisation buffer at 65^{o}C. The buffer was allowed to equilibrate to 37^{o}C by turning the water bath down, and left to hybridise at 37^{o}C overnight.

Four potential mutants were selected after autoradiography, phage carrying the possible base substitutions were isolated, and the nucleotide sequence around the mutation site determined. All four phage preparations were found to harbour base substitutions at nucleotide 499 and nucleotide 505 of the *uncB* gene. One of the four phage preparations which contained the substitutions was purified and the substitutions verified after isolation and sequencing of the phage DNA. It was possible to confirm the single-site annealing of the oligonucleotide to the template by using the mutant oligonucleotide as a primer for DNA synthesis. If the oligonucleotide had been originally introduced at more than one site in the template, the oligonucleotide would anneal at each of these sites when it was used as a sequencing primer, resulting in a series of superimposed sequencing ladders. After confirmation of the correct base substitutions, the oligonucleotide used to produce the relevant mutations was utilised as a sequencing primer. Phage which exhibited both the expected base substitutions, and a single sequencing pattern from the oligonucleotide primer was retained. The sequencing pattern of phage DNA carrying the substitution is shown in figure 3.7. The oligonucleotide used to produce the substitution, and the oligonucleotide used as sequencing primer to confirm the base replacements are listed in Table 3.1, with the substituted bases being underlined. The base replacements are highlighted in Fig 3.8, with the altered bases underlined and the substituted amino acids in bold type.

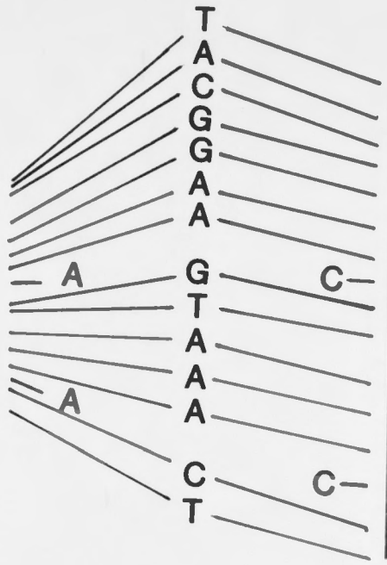
3.3.(iv) Production of plasmid pAN385 coding for the subunit-*a* double substitution K167Q,K169Q

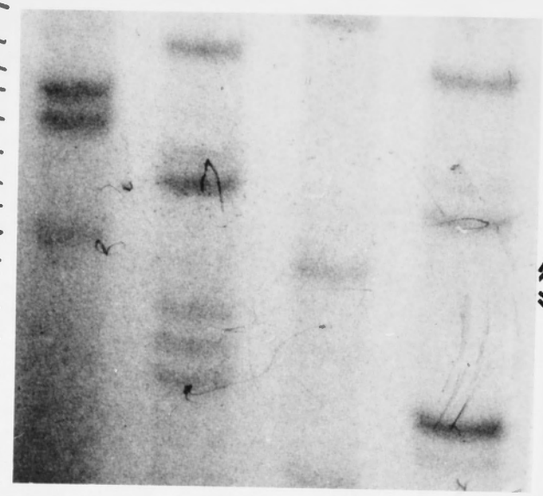
The mutant pAN461 derivative was cleaved with nuclease *Hin*d III, mixed with pAN36 which had been partially cut with the same nuclease, and ligated with phage T4 DNA Ligase. Strain AN1273(*uncG428*) was used as the recipient strain to facilitate selection by complementation of cells containing a plasmid carrying the abnormal *uncB* gene in a complete *unc* operon (*q.v.*Fig. 3.1). Competent cells of strain AN1273(*uncG428*) were prepared and the ligation mix used to transform the strain to growth on succinate in the presence of chloramphenicol. After strain purification, plasmids were isolated from eight strains, all of which proved to contain plasmid constructs identical in size and *Hin*d III restriction pattern analysis to pAN45 (*q.v.*Fig. 3.1). Strain AN887(*uncB*$^{-}$ *Mu::413*) was chosen as the recipient strain as the Mu phage insertion had produced a strain which apparently expressed no *unc* gene products due to its

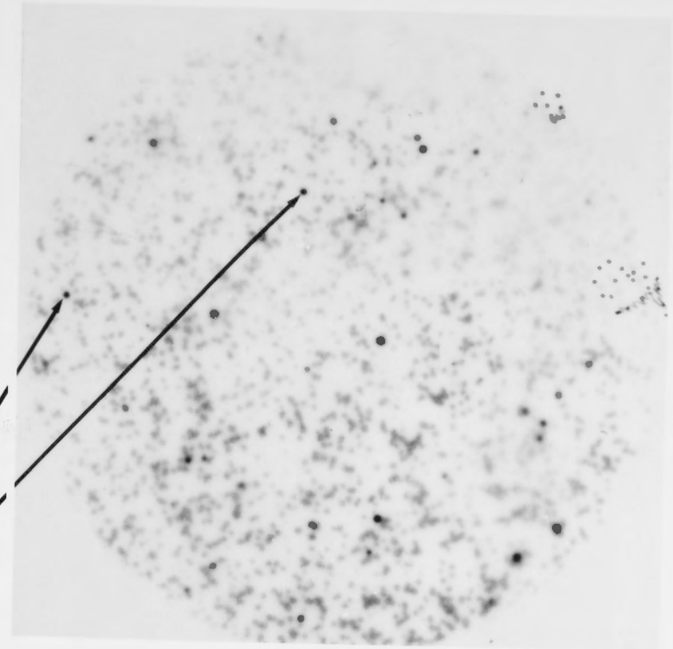
Fig 3.7 Sections of DNA sequencing gels showing the *uncB546* nucleotide substitutions and two nitrocellulose filters after plaque hybridisation and autoradiography. Samples of DNA from both normal and mutant were sequenced and the sequencing reactions electrophoresed through 6% (w/v) polyacrylamide as described in Chapter 2, section 2.6. Sequencing gels may be read in the order G, A, T, C from left to right. The indented nucleotides represent the nucleotides which have been substituted. The filters were obtained by the procedure detailed in Chapter 2, section 2.7. The autoradiographs shown are of filters after plaque hybridisation with the oligonucleotide described in Table 3.1 after end-labelling with [γ-^{32}P]. The filters had been washed at room temperature for 10 min and show clearly the difference in intensity between the signal from mutant and normal plaques.

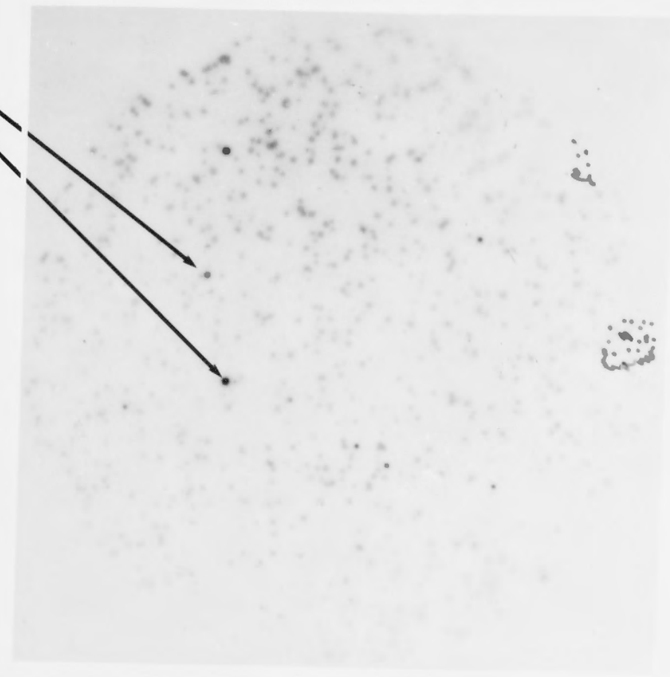
NORMAL

MUTANT
UNCB546









TRIPLET CHANGE AAA → CAA $K_{167} \rightarrow Q$

AAA → CAA $K_{169} \rightarrow Q$

Fig 3.8 Nucleotide sequence of the *uncB* gene coding for the *E. coli* F_0-F_1-ATPase subunit-*a*, together with the amino acid sequence. The sequence shown corresponds to *uncB546* with the replaced nucleotides underlined at positions 499 and 505 and the substituted amino acids printed in bold type.

```
 M  A  S  E  N  M  T  P  Q  D  Y  I  G  H  H  L  N  N  L
ATGGCTTCAGAAAATATGACGCCGCAGGATTACATAGGACACCACCTGAATAACCTT
         10         20         30         40         50
 Q  L  D  L  RL T  F  S  L  V  D  P  Q  N  P  P  A  T  F
CAGCTGGACCTGCGTACATTCTCGCTGGTGGATCCACAAAACCCCCCAGCCACCTTC
 60         70         80         90        100        110
 W  T  I  N  I  D  S  M  F  F  S  N  N  L  G  L  L  F  L
TGGACAATCAATATTGACTCCATGTTCTTCTCGGTGGTGCTGGGTCTGTTGTTCCTG
   120        130        140        150        160        170
 V  L  F  R  S  V  A  K  K  A  T  S  G  V  P  G  K  F  Q
GTTTTATTCCGTAGCGTAGCCAAAAAGGCGACCAGCGGTGTGCCAGGTAAGTTTCAG
      180        190        200        210        220        2
 T  A  I  F  I  V  I  G  F  V  N  G  S  V  K  D  M  Y  H
ACCGCGATTGAGCTGGTGATCGGCTTTGTTAATGGTAGCGTGAAAGACATGTACCAT
30         240        250        260        270        280
 G  K  S  K  L  I  A  P  L  A  L  T  I  F  V  W  V  F  L
GGCAAAAGCAAGCTGATTGCTCCGCTGGCCCTGACGATCTTCGTCTGGGTATTCCTG
  290        300        310        320        330        340
 M  N  L  M  D  I  L  P  I  D  L  L  P  Y  I  A  E  G  V
ATGAACCTGATGGATTTACTGCCTATCGACCTGCTGCCGTACATTGCTGAACATGTA
     350        360        370        380        390        40
 L  G  L  P  A  L  R  N  N  P  S  A  D  V  N  V  T  L  S
CTGGGTCTGCCTGCACTGCGTGTGGTTCCGTCTGCGGACGTGAACGTAACGCTGTCT
0         410        420        430        440        450

 M  A  L  G  V  F  I  L  I  L  F  Y  S  I  Q  M  Q  G  I
ATGGCACTGGGCGTATTTATCCTGATTCTGTTCTACAGCATCCAAATGCAAGGCATC
 460        470        480        490        500        510

 G  G  F  T  K  E  L  T  L  Q  P  F  N  H  W  A  F  I  P
GGCGGCTTCACGAAAGAGTTGACGCTGCAGCCGTTCAATCACTGGGCGTTCATTCCT
    520        530        540        550        560        570
 V  N  L  I  L  E  G  V  S  L  L  S  K  P  V  S  L  G  L
GTCAACTTAATCCTTGAAGGGGTAAGCCTGCTGTCCAAACCAGTTTCACTCGGTTTG
       580        590        600        610        620
 R  L  F  G  N  M  Y  A  G  E  L  I  F  I  L  I  A  G  L
CGACTGTTCGGTAACATGTATGCCGGTGAGCTGATTTTCATTCTGATTGCTGGTCTG
630        640        650        660        670        680
 L  P  W  W  S  Q  W  I  L  N  V  P  W  A  I  F  H  I  L
TTGCCGTGGTGGTCACAGTGGATCCTGAATGTGCCGTGGGCCATTTTCCACATCCTG
   690        700        710        720        730        740
 I  I  T  L  Q  A  F  I  F  M  V  L  T  I  V  Y  L  S  M
ATCATTACGCTGCAAGCCTTCATCTTCATGGTTCTGACGATCGTCTATCTGTCGATG
     750        760        770        780        790        8
 A  S  E  E  H
GCGTCTGAAGAACAT
00         810
```

or within the *uncB* gene (Gibson *et al.*, 1978). One plasmid was retained and used to transform strain AN887(*uncB⁻Mu::413*) to chloramphenicol resistance. This strain was designated AN2629(pAN385, carrying *uncB546*), and will be referred to as AN2629(K167Q,K169Q)

3.4 Characterisation of strain AN2629(K167Q,K169Q)

3.4.(i) Growth characteristics

Strain AN2629(K167Q,K169Q) was assayed along with the coupled control strain AN1460(pAN45/*uncB⁻Mu::413*) and uncoupled control strain AN2585(pACYC184/*uncB⁻Mu::413*) for its ability to utilise succinate minimal medium as sole carbon source and for its growth yield on limiting glucose (Table 3.2). Strain AN2629(K167Q,K169) was capable of growing on succinate and its growth yield was similar to the coupled control strain AN1460(*unc*$^+$).

3.4.(ii) Membrane properties of strain AN2629(K167Q,K169Q)

Membranes were prepared from mutant strain AN2629(K167Q,K169Q), the coupled and the uncoupled control strains. ATPase activities in membranes from the mutant strain AN2629 and the coupled control strain AN1460(*unc*$^+$) were almost identical (Table 3.2). The levels of inhibition of membrane-bound ATPase activities by N-N'-dicyclohexylcarbodiimide were also similar in membranes from strains AN2629 and AN1460. NADH- and ATP- dependent fluorescence-quenching activities were found to be identical in membranes from the mutant strain AN2629(K167Q,K169Q) and coupled control strain AN1460(*unc*$^+$). After removal of the F_1-moiety by washing in low ionic strength buffer in the absence of *p*-aminobenzamidine the NADH- and ATP-dependent fluorescence-quenching activities were lost in membranes from both the mutant strain AN2629(K167Q,K169) and the coupled control strain AN1460(*unc*$^+$)(Table 3.2).

3.5 Discussion

During the past five years, attempts have been made with enzymes that have been structurally well defined such as tyrosyl tRNA synthetase (Fersht *et al.*, 1986a,b), serine proteases (Craik *et al.*, 1984; Wells, J.A. *et al.*, 1987), lysozyme (Alber *et al.* 1985, 1987; Perry and Wetzel, 1986), and dihydrofolate reductase (Howell *et al.*, 1986; Chen, J-T. *et al* 1986) which have all been

TABLE 3.2

GROWTH PROPERTIES AND MEMBRANE-BOUND ATPASE ACTIVITIES OF MUTANT STRAIN AN2629(K167,K169), COUPLED AND UNCOUPLED CONTROL STRAINS.

Growth yields were measured as turbidities (Klett units) after growth on media containing 5mM glucose supplemented with 5% luria and chloramphenicol. The value shown is the the average of at least three attempts. Properties of membrane preparations were determined as described in Chapter 2. For further details of strains see either section 3.4.(i) or tables 2.1 and 2.2

RELEVANT PROPERTY	STRAIN		
	AN2629(K167Q,K169Q)	AN1460(*unc*$^+$)	AN2585(*unc*$^-$)
Growth on succinate	+	+	-
Growth yield(Klett units)	225	230	141
ATPase activity (μmol.min^{-1}.mg protein^{-1})	1.6	1.8	<0.1
DCCD inhibition of ATPase activity(%)	68	69	
NADH-dependent atebrin fluorescence-quenching activity:			
% in intact membranes	84	83	84
% after removal of F_1-ATPase	8	10	84
ATP-dependent atebrin fluorescence-quenching activity:			
% in intact membranes	80	82	0
% after removal of F_1-ATPase	0	0	0

crystallised and their 3-Dimensional structures deduced, to utilise the method of site-directed mutagenesis to investigate the specific role of residues which may be involved in substrate binding, catalytic efficiency or protein stability and to deduce the exact chemical mechanism of catalysis at the active site of these enzymes. Interest in the potential of site-specific mutagenesis has produced highly efficient techniques for which kits are now commercially available. Due to the intramembranous location of enzymes such as *the E. coli* F_0F_1-ATPase, structural information of such clarity is at present not available. However, an attempt can be made to use mutants to probe the structures of membrane-bound proteins.

Of the two systems employed to produce the K167Q,K169Q double replacement, the single-stranded phage template system was found to be the easier to use, and the more successful. The modification whereby a second primer (the universal primer) is added which anneals to a region of template close 5' to the annealing site of the oligonucleotide carrying the base replacements is believed to improve the efficiency of mutant production as the short region of DNA between the two primers is easily filled in by the polymerase, effectively trapping the second oligonucleotide 3' from the universal primer (Zoller and Smith, 1983). Unfortunately, although the double-stranded vector system did produce a low level of potential mutants as judged by the hybridisation results, sequence analysis after subcloning the putative mutant fragments into M13 showed the potential K167Q,K169Q mutants still carried a normal sequence at the mutation sites. The reason for these false positives was unclear, but may have been due to nonspecific binding of the oligomer. Using a double-stranded template immediately lowers the theoretical maximal level of mutant production to 50%, in comparison to the single-stranded system which, due to its method of replication, has a theoretical maximum of 100%. The extremely low percentage of potential mutants, and the level of colonies which gave false positive signals from which the potentially altered fragment had to be subcloned into M13 and sequenced before verification, showed a clear advantage for the single-stranded phage system.

Using the single-stranded template system, at least one percent of phage appeared to contain the K167Q,K169Q mutation. This system facilitates easy discovery of false positives, and any problems with nonspecific annealing of the oligonucleotide can be readily shown by using the oligonucleotide as a sequencing primer.

Analysis of mutant strain AN2629(K167Q,K169Q) revealed the strain was identical both in growth characteristics and membrane enzymic properties to the coupled control strain AN1460(*unc*$^+$). Growth on succinate minimal medium

was unimpaired and growth yields showed the mutant strain was unaffected in its coupling of ATP synthesis to respiration . In the working model detailed in chapter 1, section 1.5.(i), the putative structure of subunit-*a* portrays the two lysine residues -167 and -169 as being intramembranous components of helix III. Engleman and Steitz (1981), estimated the free energy costs of burying individual non-paired ionisable side chains by considering the energy requirements for protonation/deprotonation of the carboxylate/amino group and the transfer of the protonated-carboxyl and deprotonated-amino group from an aqueous to a nonaqueous environment , quoting figures of 8 kcal.mol^{-1} for glutamates or aspartates and 10 kcal.mol^{-1} for lysine residues. The authors claim that the free energy loss of burying a charged residue into the lipid phase of the membrane could be compensated for by the energy gain of transferring an α-helix from an aqueous to a non-aqueous environment. The net energy gain of transferring a helical hairpin of forty residues was calculated by treating the helices as cylinders of 1.5 nm diameter and 3.0 nm length. The removal of a hydrophobic polypeptide from water to a hydrophobic environment has been calculated as an increase of free energy of 25 cal.per Angstrom^2 of surface area available to the solvent. For a helical pair this equates to a hydrophobic free energy favouring partitioning into the lipid phase of 60 kcal.mol^{-1}, more than compensating for the burial of an ionisable group or charge pair.

Analysis of the 3-dimensional structure of 36 globular proteins by Rashin and Honig (1984), revealed 80% of all amino acids with ionisable side chains buried in the hydrophobic interiors of the proteins formed salt-bridges with ionisable side chains from other residues. The remaining 20% were involved in extensive hydrogen-bonding with polar residues. Further to these analyses, Honig and Hubbel (1984), calculated the free energy of transfer of two ionisable residues involved in a charge pair or 'salt-bridge' from water to a hydrophobic medium of dielectric 2-4.. This was approximated at 10-16 kcal.mol^{-1} and was calculated as similar to the free energy of transfer of a pair of ionisable residues involved in hydrogen bonding, suggesting the free energy of transfer of a proton from the charged to the hydrogen bonded pair was negligible. Internally ionisable groups are also stabilised by additional hydrogen-bonds (Nemethy, 1986), contributing approximately 3 kcal.mol^{-1} for each bond. Thus, Honig and Hubbel (1984) propose that most ionisable residues buried in the hydrophobic interior of proteins will exist as charge-pairs.

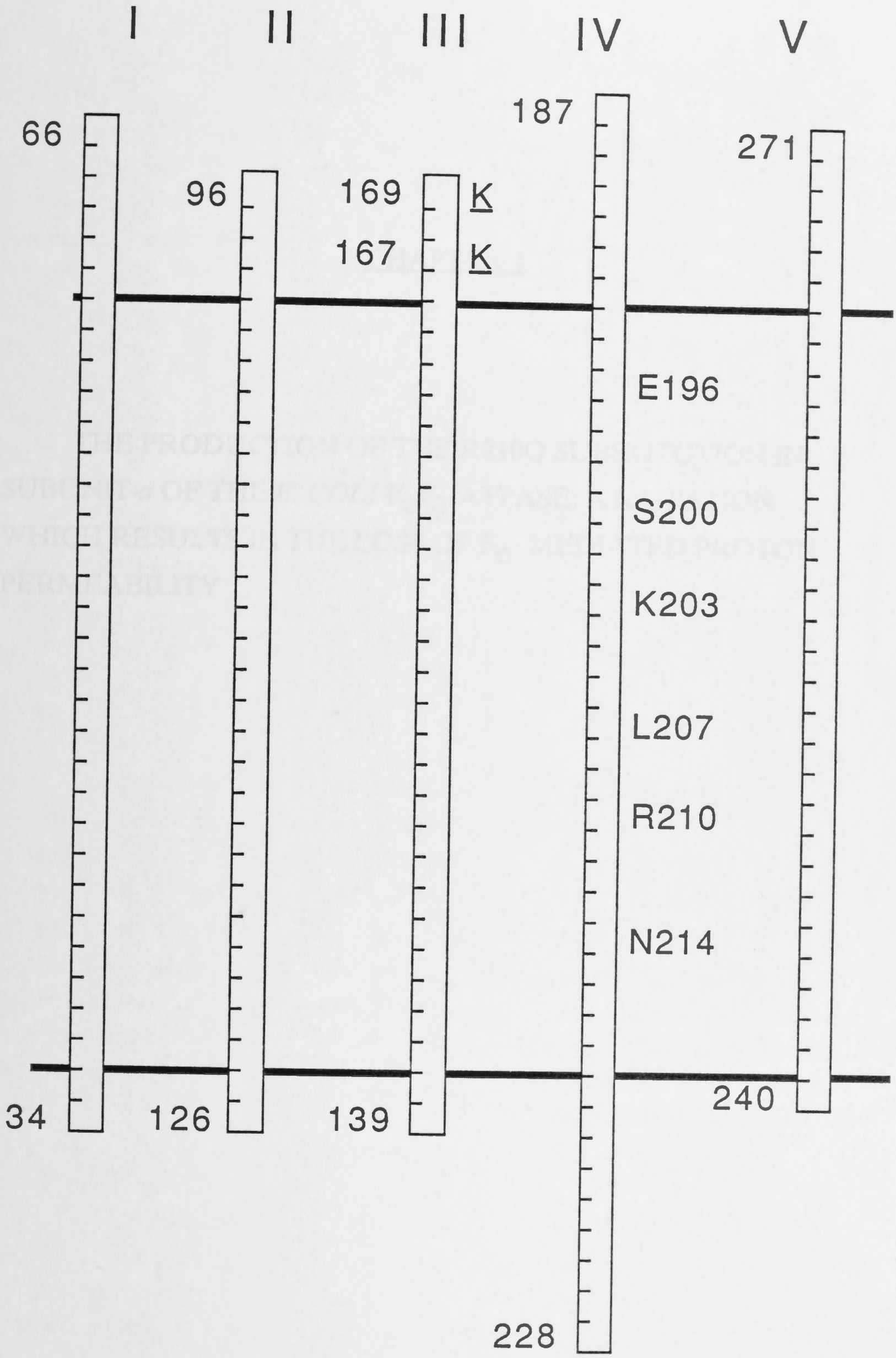
Raman spectroscopic studies of rhodopsin (Oseroff and Callender, 1974) and bacteriorhodopsin (Lewis *et al.*, 1974) highlighted C=N vibrational frequencies consistent with a protonated Schiff base, which is involved in linking the retinal to the ε-amino group of a lysine residue in the opsin moiety. This

frequency alone was perturbed when the measurements were made in deuterated solutions both in the pigments and in isolated protonated Schiff base, a result which could be explained by the increase in mass of the oscillator. These results show conclusively that charged residues can be tolerated within the membrane.

Thus, it appears that it is possible to bury charged residues into a membrane but these residues are extremely likely to be charge-paired, extensively stabilised by hydrogen-bonds (see also Klotz and Franzen, 1962), or, as shown by the example of the Schiff base in bacteriorhodopsin, functionally important. The importance of salt-bridging in stabilising protein tertiary and quaternary structures has been well documented. Thus, if Lys-167, or Lys-169 of subunit-*a* were intramembranous and possibly charge-paired, their substitution with nonionisable amino-acids, such as glutamine, would destabilise the overall structure and possibily perturb assembly or membrane-integration of the complex. Therefore, it is unlikely that the residues are involved in complex stabilisation. Thus, energetic constraints preventing spontaneous insertion of ionisable residues into the lipid phase of membranes suggest that those charged residues which prove to be buried will either be functionally or structurally important. Consequently, it can be concluded that residues Lys-167 and Lys-169 of subunit-*a* are not located within the membrane and the working model must be changed to accommodate this piece of data (see Fig.3.9).

Positioning of Lys-167 and Lys-169 on the membrane cytoplasmic interface may help to stabilise the structure. The transmembrane potential being negative on the cytoplasmic aspect and positive on the periplasmic aspect may assist in defining the transmembrane helices. As illustrated by the model, there is a preponderance of positively charged residues lining the cytoplasmic aspect of the membrane which may function to 'anchor' the helices (Date *et al*, 1980). These positively charged residues may also be involved in ionic interactions with the charged phosphate head group of the phospholipids.

Fig 3.9 Diagrammatic representation of the five predicted intramembranous helices of subunit-*a*. The helices are marked I - V, and the number of the residue at both N- and C- terminals of each putative helix are given. The thick horizontal line towards the top of the page represents the membrane/cytoplasm border, whilst the lower thick line represents the membrane/periplasm border. Helix III have been repositioned with respect to the membrane, with residues Lys-167 and Lys-169 being placed outside the membrane in accord with results documented in Chapter 3.


I
II
III
IV
V
66
96
169 K
167 K
187
271
E196
S200
K203
L207
R210
N214
34
126
139
228
240

CHAPTER 4

THE PRODUCTION OF THE R210Q SUBSTITUTION IN SUBUNIT-*a* OF THE *E. COLI* F_oF_1-ATPASE: A MUTATION WHICH RESULTS IN THE LOSS OF F_o- MEDIATED PROTON PERMEABILITY

Figures 1.11 and 1.12 in the general introduction illustrate the potential positioning of helix IV of subunit-*a* in close proximity to helix II of subunit-*c*, effectively placing Arg-210 and Asp-61 within one turn of an α-helix. The involvement of Asp-61 in proton movement within the F_oF_1-ATPase has been well documented (*q.v.* Chapter 1, section 1.5.(i)). Residue Arg-210, an intrinsic component of the amphipathic face of helix IV in subunit-*a*, is implicated as a member of the putative proton pore, and is completely conserved in subunit-*a* homologues from all diverse species analysed (Table 4.1). Thus, the two residues may be interacting in facilitating the translocation of protons through the F_o-moiety. By substituting a non-ionisable residue for Arg-210, this hypothesis can be challenged. Glutamine was chosen as the residue to replace the arginine, as it has a similar side chain, and can be introduced by a single base substitution in the arginine codon -CGA- to -CAA-(see Table 4.2). This single base change should allow the isolation of full revertants, assuming the substitution produces a phenotypic mutation, confirming that the phenotype is due specifically to the R210Q substitution in subunit-*a*.

4.1. Production of the subunit-*a* amino-acid substitution R210Q

4.1.(i) Introduction

The single-stranded template method of mutant production detailed in chapter 3.3 was employed to produce the R210Q-encoding base substitution using the 20-base synthetic oligomer listed in Table 4.2. The oligomer was annealed to single-stranded template DNA purified from phage isolated after transformation of a culture of K37 with phage replicative form pAN461. Partial heteroduplex molecules were completed by the DNA pol 1 (large fragment) and covalently closed by T4 DNA ligase. After transformation of strain K37 with the potential phage heteroduplex, approximately 5000 plaques were formed. These were subsequently screened, utilising the differential-temperature hydridisation procedure, with the [γ-^{32}P] labeled base-substituted oligonucleotide as probe. No potential mutants were visible, even when the filters were subjected to autoradiography after sequential 2 minute washes at 40^{o}C, 50^{o}C and 60^{o}C. Dot-blot hybridisations (*q.v.* Chapter 2, section 2.7.(iii)) were also attempted on 150 possible mutant phage preparations without any potential mutants being formed. DNA sequence analysis of 40 phage isolates confirmed the observation that no mutants had been produced. Therefore, it was necessary to devise a method of mutagenesis which gave greater efficiency of mutant production.

Table 4.1 Amino acid comparisons of a portion of subunit-*a* (ATPase-*6*, CF_OIV) of the F_O-F_1 ATPase. Amino acid residues 194-221 of the *E. coli* subunit-*a* are compared with residues 142-169 of the human Anderson *et al.,* 1981), bovine (Anderson *et al.*, 1982), mouse (Bibb *et al.,* 1981), rat (Grosskopf and Feldmann, 1981) chinese hamster (Breen *et al.*, 1986) and X. laevis (Roe *et al.*, 1985) ATPase subunit-*6*, with residues 143-170 of the *D. melanogaster* ATPase subunit-*6* (de Bruijn, 1983), with residues 171-198 of the *T. glabrata* ATPase subunit-*6* (Clark-Walker, G.D. personal communication), with residues 170-197 of the *S. cerevisiae* ATPase subunit-*6* (Macino and Tzagoloff, 1980; Novitski *et al.,* 1984), with residues 167-194 of the *A. nidulans* ATPase subunit-*6* (Grisi *et al.*, 1982), with residues 163-190 of the *N. crassa* ATPase subunit-*6* (Morelli and Macino, 1984), with residues 184-211 of the *Synechococcus 6301* subunit-*a* (Cozens and Walker, 1987) and with residues 174-201 of the spinach chloroplast CF_OIV (Hennig and Herrmann, 1986; Hudson *et al.,* 1987). The amino acid in bold type represents the residue substituted in the accompanying chapter.

TABLE 4.1

COMPARISON OF THE AMINO-ACID SEQUENCES OF THE PUTATIVE HELIX IV OF SUBUNIT-*A* (ATPase *6*) FROM SEVERAL SPECIES

Human	I	I	E	T	I	S	L	L	I	Q	P	M	A	L	A	V	**R**	L	T	A	N	I	T	A	G	H	L	L
Bovine	I	I	E	T	I	S	L	F	I	Q	P	M	A	L	A	V	**R**	L	T	A	N	I	T	A	G	H	L	L
Rat[a]	I	I	E	T	I	S	L	F	I	Q	P	M	A	L	A	V	**R**	L	T	A	N	I	T	A	G	H	L	L
X. laevis	I	I	E	T	I	S	L	F	I	R	P	L	A	L	G	V	**R**	L	T	A	N	L	T	A	G	H	L	L
D. melanogaster	C	I	E	T	I	S	N	I	I	R	P	G	T	L	A	V	**R**	L	T	A	N	M	I	A	G	H	L	L
T. glabrata	V	I	E	L	L	S	Y	V	A	R	A	F	S	L	G	L	**R**	L	S	A	N	I	F	S	G	H	L	L
S. cerevisiae	I	I	E	T	L	S	Y	I	A	R	A	I	S	L	G	L	**R**	L	G	S	N	I	L	A	G	H	L	L
A. nidulans	L	I	E	F	I	S	Y	L	S	R	N	V	S	L	G	L	**R**	L	A	A	N	I	L	S	G	H	M	L
N. crassa	L	I	E	F	I	S	Y	L	A	R	N	I	S	L	G	L	**R**	L	A	A	N	I	L	S	G	H	M	L
Synechococcus.6301	P	F	K	I	L	E	D	F	T	K	P	L	S	L	S	F	**R**	L	F	G	N	I	L	A	D	E	L	V
Chloroplast[b]	P	I	N	I	L	E	D	F	T	K	P	L	S	L	S	F	**R**	L	F	G	N	I	L	A	D	E	L	I
Esch. coli	I	L	E	G	V	S	L	L	S	K	P	V	S	L	G	L	**R**	L	F	G	N	M	Y	A	G	E	L	I
			196[c]						203			206				210				214				219			

a sequence is identical to subunit-6 sequence of chinese hamster and mouse.
b Spinach chloroplast CF_OIV
c Numbers correspond to the amino acid sequence for *Esch. coli*

4.1.(ii) Formation of a methylated, covalently closed heteroduplex

For heteroduplex formation several indigenous *E. coli* enzymes were substituted for their less efficient viral analogues. The activity of DNA pol III holoenzyme in the presence of single-stranded binding protein was assayed. The annealing mix contained:

1.3 μg single-stranded template
30 ng phosphorylated oligonucleotide primer
6 mM $MgCl_2$;10 mM DTT;60 mM Tris-Cl pH 7.5

in a final volume of 25 μl. The mix was heated to 65°C for 5 mins and allowed to cool to 30°C over a period of 30 mins.

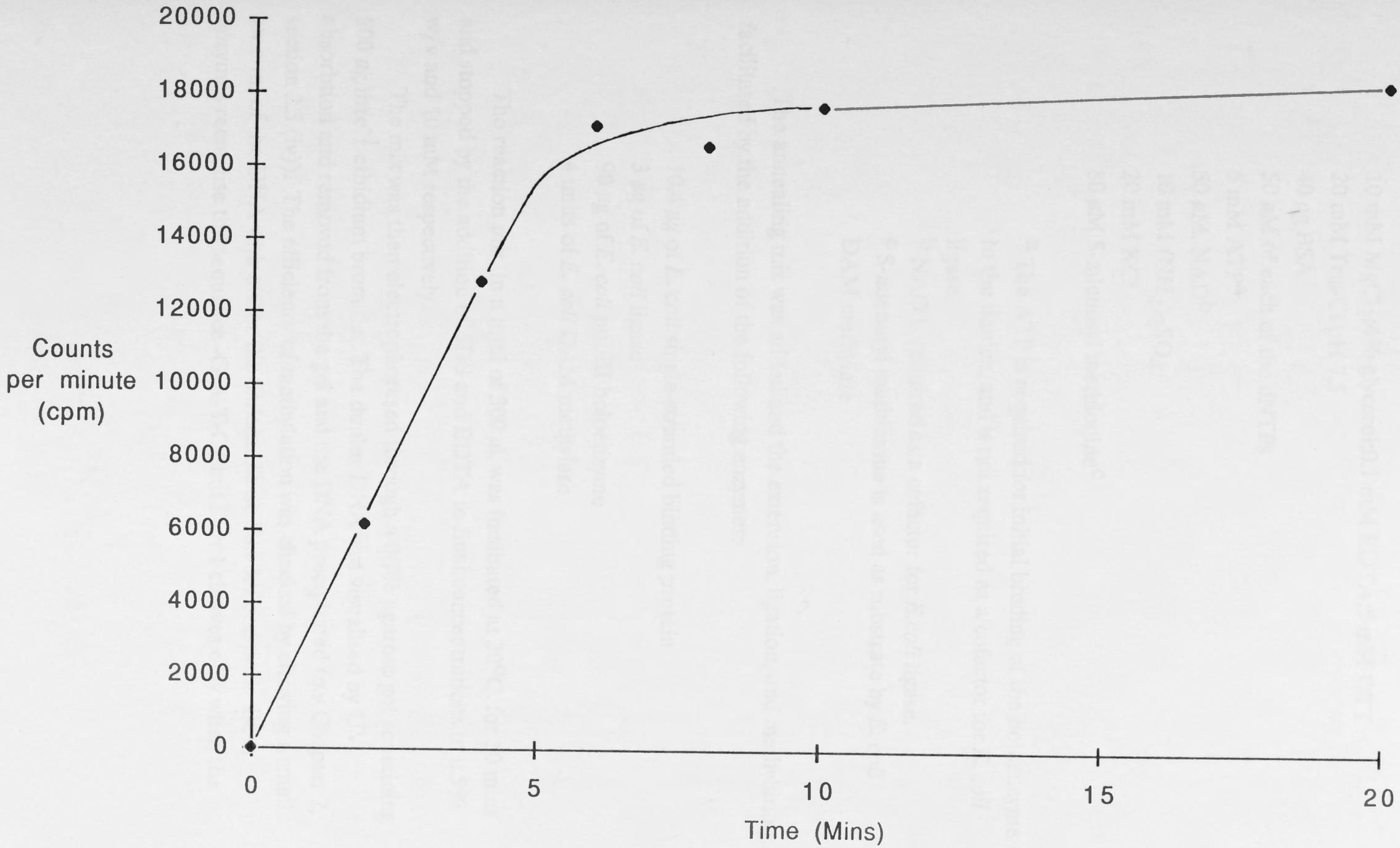
A premix solution was prepared containing:

10 mM $MgCl_2$;4% glycerol;0.1 mM EDTA;5 mM DTT
1 μg BSA
2.5 mM [^{3}H]-dNTP mix
5 mM ATP
10.4 μg *E. coli* single-stranded binding protein
20 mM Tris-Cl pH 7.5

The polymerisation was started after the addition of the annealing mix to a final volume of 250 μl, by adding 90 μg of DNA pol III holoenzyme at 30°C. 75 μl of the premix (from a total volume of 300 μl) was retained and counted. At timed intervals, 25 μl aliquots were removed and quenched by the addition of 200 μl stop solution containing 10% trichloroacetic acid and 100 mM Na_2HPO_4. These were then filtered, and the incorporated radiolabel counted. From the plateau of the graph (Fig 4.1), the complete polymerisation of the template was seen to be attained after 5 mins at 30°C.

Phosphorylated oligonucleotide (40 ng) carrying the single base substitution was annealed along with 20 ng of phosphorylated universal primer to 1.3 μg single-stranded phage template form of pAN461. Annealing was achieved by heating the annealing mix to 65°C for 5 min followed by cooling to 37°C over a period of 30 min. This partial duplex was then completed, covalently closed, and methylated in the following reaction mix (Final concentrations):

Fig 4.1 Assay for the activity of *E. coli* pol III holoenzyme in the presence of *E. coli* single-stranded binding protein. The assay mixture and conditions are detailed in section 4.1.(ii). Samples were taken and counted after 0, 2, 4, 6, 8, 10 and 20 min after the addition of 90 μg of pol III holoenzyme to the assay mixture. The samples were counted in a xylene-based scintillant on a Packard Tricarb 300 scintillation counter.


20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Counts per minute (cpm)
0
5
10
15
20
Time (Mins)

10 mM $MgCl_2$;4% glycerol;0.1 mM EDTA;5 mM DTT
20 mM Tris-Cl pH 7.5
40 μg BSA
50 μM of each of the dNTPs
5 mM ATP[a]
50 μM. NAD[b]
10 mM $(NH_4)_2SO_4$
20 mM KCl
80 μM S-adenosyl methionine[c]

[a] The ATP is required for initial binding of the holoenzyme to the duplex, and is not required as a cofactor for *E. coli* ligase.
[b] NAD is required as a cofactor for *E.coli* ligase.
[c] S-adenosyl methionine is used as substrate by *E. coli* DAM methylase

The annealing mix was added and the extension, ligation, and methylation facilitated by the addition of the following enzymes:

10.4 μg of *E. coli* single-stranded binding protein
3 μg of *E. coli* ligase
90 μg of *E. coli* pol III holoenzyme
4 units of *E. coli* DAM methylase

The reaction mix, in a total of 300 μl, was incubated at 30°C. for 30 mins and stopped by the addition of SDS and EDTA to final concentrations of 0.5% w/v and 10 mM respectively.

The mix was then electrophoresed through a 0.7% agarose gel containing 500 $\mu g.litre^{-1}$ ethidium bromide. The duplex DNA was visualised by UV absorbtion and removed from the gel and the DNA precipitated (*q.v.* Chapter 2, section 2.5.(iv)). The efficiency of methylation was checked by cleaving a small amount of the DNA with either the endonuclease *Sau* 3A or *Dpn* 1. Both enzymes recognise the sequence -G-A-T-C-, but *Dpn* 1 cleaves only when the

TABLE 4.2

SYNTHETIC OLIGONUCLEOTIDES USED IN THE PREPARATION AND SEQUENCE CONFIRMATION OF THE SUBUNIT-*A* SUBSTITUTION R210Q, AND FOR SEQUENCE ANALYSIS OF THE *uncB* GENE.

Preparation of the oligonucleotides is detailed in Chapter 2, section 2.7.(i). The base which is underlined signifies a base substitution from the normal sequence.

Synthetic oligonucleotide	Relevant amino-acid substitution	Notes
5'-CTCGGTTTGCAACTGTTCGG-3'	R210Q	Also used as sequencing primer for sequencing from nucleotide 638 in the *uncB* gene.
5'-GTAAAACGACGGCCAGT-3'	-	Phage M13 universal primer which anneals onto the viral strand approximately 50 bases 5' from the *Hin*d III restriction site in the mp9 polylinker. Used for sequencing from nucleotide -150 in the *uncB* gene.
5'-ATGGCACTGGGCGTATTTA-3'	-	Sequencing primer used for sequencing from nucleotide 475 in the *uncB* gene.
5'-GGGTCTGTTGTTCCTGGTTTTA-3'	-	Sequencing primer used for sequencing from nucleotide 154 in the *uncB* gene.

adenine moiety has been methylated. Agarose gel electrophoresis of the endonuclease-treated DNA confirmed a high efficiency of methylation (Data not shown).

4.1.(iii) Identification of phage carrying the mutation

The methylated DNA was used to transform strain K37 (*q.v.* Chapter 2, section 2.5.(iii)). Resultant plaques, between 50-400 per plate, were screened by plaque hybridisation utilising the differential-temperature hybridisation detailed in Chapter 2, section 2.7.(iii). A hybridisation temperature of 58^{o}C. was chosen and the chamber was incubated in the presence of the relevant [γ-^{32}P]-labeled oligonucleotide probe for 14 hrs.

Plaques of phage apparently carrying the mutation were visible after washing with 0.9 M NaCl;90 mM Na^{+} citrate for 2 min at 25^{o}C, although the signals from the mutants were more obvious after washes at higher temperatures. From a total of approximately 1000 plaques analysed, only 8 appeared to show a stronger hybridisation signal than background plaques. Phage was purified from each of these plaques and the DNA subsequenltly isolated. DNA Sequence analysis showed phage isolated from all eight plaques carried the single base substitution. One of these phage preparations was retained, the phage purified, and the base substitutions confirmed by sequence analysis. After confirmation of the correct base substitution, the oligonucleotide used to produce the relevant mutation was utilised as a sequencing primer. The phage preparation exhibited a single sequencing pattern from the mutant oligonucleotide primer, and was retained. The DNA sequence carrying the base substitution is shown in Fig 4.2. Using the *uncB* gene-specific primers listed in Table 4.2 the complete *uncB* gene was sequenced, and proved to differ from the normal sequence by only the single base substitution at nucleotide 629. The base replacement is highlighted in Fig 4.3, with the altered base underlined and the substituted amino acid in bold type.

4.1.(iv) Subcloning of the *Hin*d III-generated 4 kb fragment coding for the R210Q mutation.

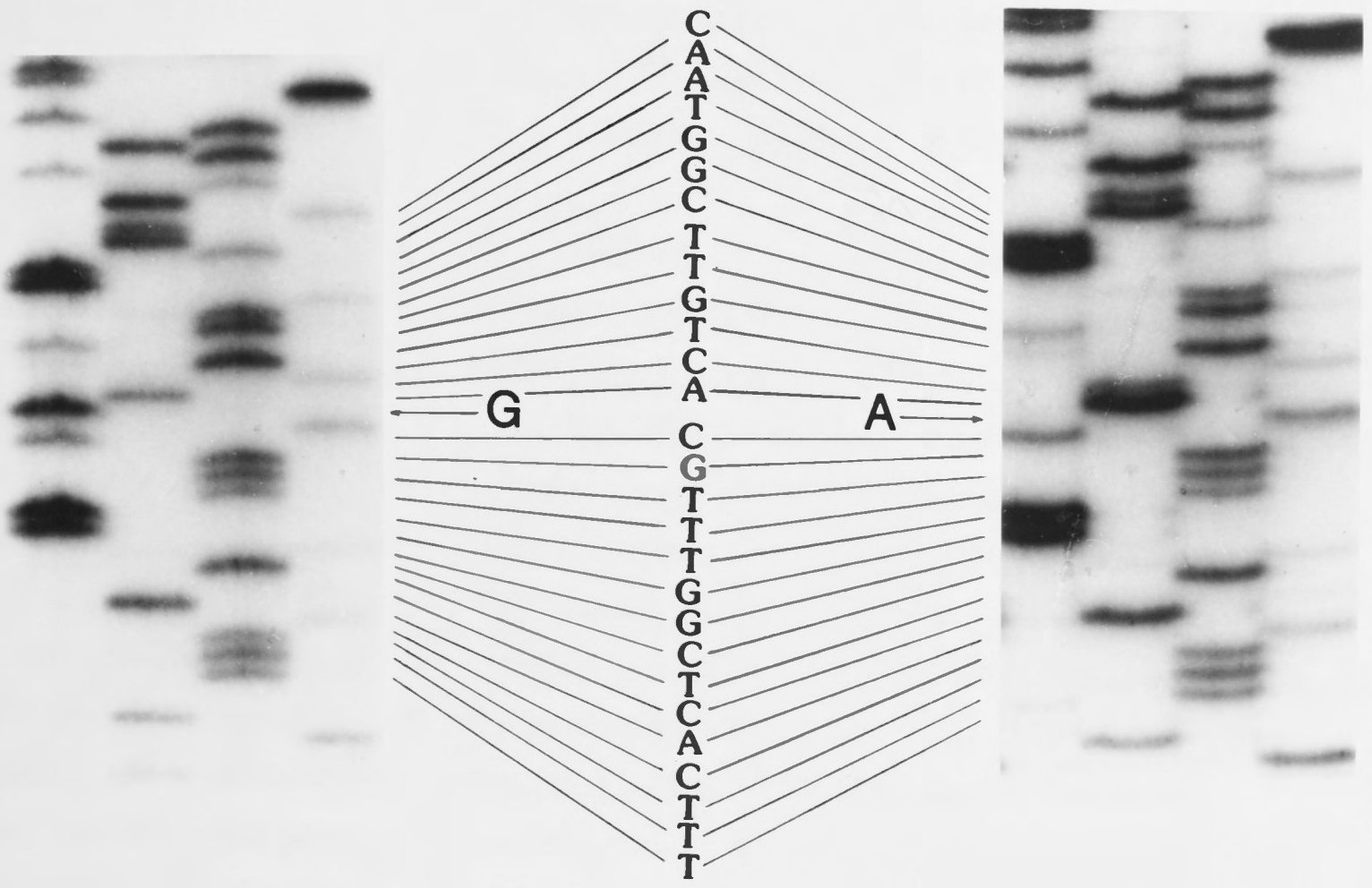
The phage replicative form derivative of pAN461 carrying the single base substitution was cleaved with nuclease *Hin*d III, and mixed with *Hin*d III-digested, dephosphorylated vector pACYC184. Formation of pACYC184 constructs carrying the 4.4 kb fragment from the mutant replicative form was attempted by ligating with T4 DNA ligase at 12^{o}C for 5 hrs. Strain AN2015(*uncH241*) was used as the recipient strain, as only colonies which

Fig 4.2 Sections of DNA sequencing gels showing the *unc540*(R210Q) nucleotide substitution. Samples of DNA from both normal and mutant were sequenced, and the sequencing reactions electrophoresed through 6% (w/v) polyacrylamide as described in Chapter 2, section 2.6. Sequencing gels may be read in the order G, A, T, C from left to right. The indented nucleotides indicate the nucleotides which have been substituted.

DNA SEQUENCE CHANGE IN UNCB540

CONTROL

MUTANT
UNCB540



TRIPLET CHANGE: CGA ⟶ CAA

R_{210} ⟶ Q

contained plasmids coding for a normal *uncH* gene from the 4.4 kb *Hin*d III-generated fragment from the phage replicative form would be able to grow on succinate as sole carbon source. Half of the ligation mix was used to transform competent cells from strain AN2015(*uncH*241) to growth on chloramphenicol-supplemented succinate minimal media, and half to chloramphenicol resistance on nutrient agar.

Although this subcloning appeared to be straightforward, no transformants were isolated which could grow using succinate as sole carbon source. This cloning experiment was repeated several times without success. Isolation of the 4.4 kb insert from low melting point agarose (*q.v.* Chapter 2, section 2.5.(iv)), and ligation with either phosphatased or nonphosphatased vector yielded similar negative results. Selection by insertional inactivation of the tetracycline resistance gene product was also attempted. The *Hind* III recognition site in the vector pACYC184 is located in the *tet* promoter, resulting in loss of tetracycline resistance on successful insertion at the *Hind* III site (Stuber and Bujard (1981)). Again, this failed to yield any of the desired constructs.

The original cloning of the 4.4 kb insert from the *Hind* III-generated chromosomal digest carried in the F-mobilizable vector pGM706 (Young *et al.*, 1978) into the p15A derivative pACYC184 (Chang and Cohen, 1978) produced the plasmid pAN51 (Downie *et al.*, 1980). This construct contained a small deletion of approximately 100 base pairs (Jans, 1984), close to the tetracycline promoter which, it was suggested, may decrease the level of transcription from the promoter (see also Chapter 4, section 4.3).

Strain AN1952 contains the vector pAN174. This vector is identical to pACYC184, except for the 100 base pair deletion (see above). Consequently, the vector pAN174 was isolated from strain AN1952. After cutting the vector with *Hind* III, the terminal phosphates were removed by alkaline phosphatase, and the vector ligated to the isolated 4.4 kb fragment carrying the base substitution coding for the R210Q substitution, over a 2 hour incubation period at room temperature by T4 DNA ligase. This ligation mix was used to transform strain AN2015(*uncH241*) to growth on solid succinate media supplemented with chloramphenicol. Approximately 20 transformant colonies were produced from competent cells transformed with 50 ng of ligated vector. Twelve colonies were purified, their plasmids isolated using the alkaline lysis procedure (Chapter 2, section 2.3.(iv)), and those plasmids screened for similar size and *Hind* III-generated restriction pattern to the plasmid pAN51.

Of the twelve plasmids preparations, all contained the 4.4 kb insert, but nine carried two copies of the vector pAN174. One purified colony containing

Fig 4.3 Nucleotide sequence of the *uncB* gene coding for the *E. coli* F_o-F_1 ATPase subunit-*a*, together with the amino acid sequence. The sequence shown corresponds to *uncB540* with the replaced nucleotide underlined at position 629 and the substituted amino acid printed in bold type.

```
 M  A  S  E  N  M  T  P  Q  D  Y  I  G  H  H  L  N  N  L
ATGGCTTCAGAAAATATGACGCCGCAGGATTACATAGGACACCACCTGAATAACCTT
         10         20         30         40         50
 Q  L  D  L  R  T  F  S  L  V  D  P  Q  N  P  P  A  T  F
CAGCTGGACCTGCGTACATTCTCGCTGGTGGATCCACAAAACCCCCCAGCCACCTTC
 60         70         80         90        100        110
 W  T  I  N  I  D  S  M  F  F  S  V  V  L  G  L  L  F  L
TGGACAATCAATATTGACTCCATGTTCTTCTCGGTGGTGCTGGGTCTGTTGTTCCTG
   120        130        140        150        160        170
 V  L  F  R  S  V  A  K  K  A  T  S  G  V  P  G  K  F  Q
GTTTTATTCCGTAGCGTAGCCAAAAAGGCGACCAGCGGTGTGCCAGGTAAGTTTCAG
      180        190        200        210        220       2
 T  A  I  F  I  V  I  G  F  V  N  G  S  V  K  D  M  Y  H
ACCGCGATTGAGCTGGTGATCGGCTTTGTTAATGGTAGCGTGAAAGACATGTACCAT
30         240        250        260        270        280
 G  K  S  K  L  I  A  P  L  A  L  T  I  F  V  W  V  F  L
GGCAAAAGCAAGCTGATTGCTCCGCTGGCCCTGACGATCTTCGTCTGGGTATTCCTG
  290        300        310        320        330        340
 M  N  L  M  D  I  L  P  I  D  L  L  P  Y  I  A  E  G  V
ATGAACCTGATGGATTTACTGCCTATCGACCTGCTGCCGTACATTGCTGAACATGTA
     350        360        370        380        390       40
 L  G  L  P  A  L  R  V  V  P  S  A  D  V  N  V  T  L  S
CTGGGTCTGCCTGCACTGCGTGTGGTTCCGTCTGCGGACGTGAACGTAACGCTGTCT
0        410        420        430        440        450
 M  A  L  G  V  F  I  L  I  L  F  Y  S  I  K  M  K  G  I
ATGGCACTGGGCGTATTTATCCTGATTCTGTTCTACAGCATCAAAATGAAAGGCATC
 460        470        480        490        500        510
 G  G  F  T  K  E  L  T  L  Q  P  F  N  H  W  A  F  I  P
GGCGGCTTCACGAAAGAGTTGACGCTGCAGCCGTTCAATCACTGGGCGTTCATTCCT
    520        530        540        550        560        570
 V  N  L  I  L  E  G  V  S  L  L  S  K  P  V  S  L  G  L
GTCAACTTAATCCTTGAAGGGGTAAGCCTGCTGTCCAAACCAGTTTCACTCGGTTTG
       580        590        600        610        620

 N  L  F  G  N  M  Y  A  G  E  L  I  F  I  L  I  A  G  L
CAACTGTTCGGTAACATGTATGCCGGTGAGCTGATTTTCATTCTGATTGCTGGTCTG
630        640        650        660        670        680

 L  P  W  W  S  Q  W  I  L  N  V  P  W  A  I  F  H  I  L
TTGCCGTGGTGGTCACAGTGGATCCTGAATGTGCCGTGGGCCATTTTCCACATCCTG
   690        700        710        720        730        740
 I  I  T  L  Q  A  F  I  F  M  V  L  T  I  V  Y  L  S  M
ATCATTACGCTGCAAGCCTTCATCTTCATGGTTCTGACGATCGTCTATCTGTCGATG
     750        760        770        780        790       8
 A  S  E  E  H
GCGTCTGAAGAACAT
00       810
```

the mutated plasmid construct with a single copy of the vector was retained, and the plasmid isolated. This plasmid, after partial cleavage with *Hind* III was mixed with plasmid pAN36, which had been fully digested with the endonuclease, and ligated with T4 DNA ligase. The ligation mixture was used to transform strain AN1273 (*uncG428*) and transformants were again selected by their ability to grow on solid succinate media supplemented with chloramphenicol.

From a ligation mix containing approximately 500 ng. partially cleaved mutant plasmid and 1 μg of fully cleaved pAN36, only 10 colonies from the transformation experiment resulted. Eight of the colonies were purified, and their plasmids isolated. Plasmids carrying the *unc* operon in its entirety were indicated by their similarity in size and *Hind* III-generated restriction pattern to plasmid pAN45. One colony carrying a mutant plasmid similar to pAN45 was retained, and the plasmid designated pAN378(*uncB540*).

Fig 4.4 shows the purified plasmid pAN378 with pAN45, cut with endonuclease *Hind* III. Plasmid pAN378 was used to transform strain AN887(*uncB⁻Mu::413*). Transformants were selected on nutrient agar containing chloramphenicol. One colony from the transformation experiment was purified and then retained . This strain was designated AN2608(pAN378, carrying *uncB540*), and will be referred to as strain AN2608(R210Q).

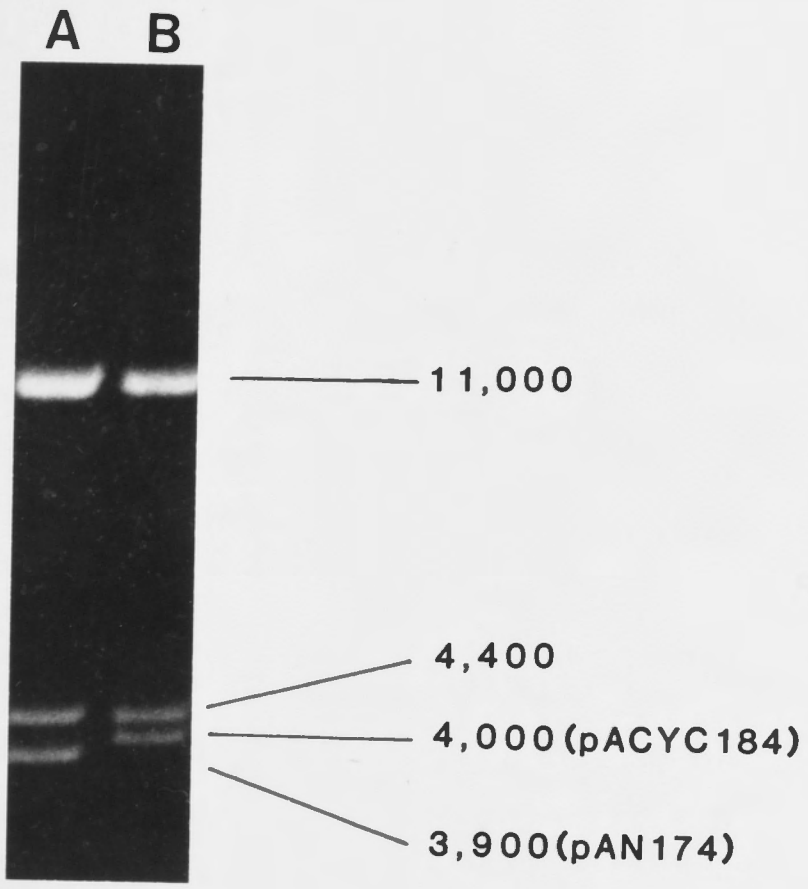
4.2. Characterisation of strain AN2608(R210Q)

4.2.(i) Growth characteristics of strain N2608(R210Q) and complementation analysis using plasmid pAN378(*uncB540*).

Strain AN2608(R210Q) was examined for its ability to grow on succinate minimal medium and for its growth yield on a limiting level of glucose (Table 4.3). Strain AN2608(R210Q) was unable to grow on solid succinate media in the presence of 0.06% casamino acids, or in succinate liquid media. The growth yield of this strain on 5 mM glucose was similar to that of the uncoupled control strain AN2584(pAN174/*uncB⁻Mu::413*).

Plasmid pAN378 was used to transform the chromosomal *unc* reference strains AN727(*uncB402*), AN943(*uncE429*), AN1440(*uncF469*), AN2015(*uncH*241), AN730(*uncA401*), AN818(*uncD409*) and AN802(uncC424), along with the strain carrying a Mu phage insert promoter distal to *uncB*, strain AN888(*uncB⁺Mu::416*). The strains transformed, their ability to grow on succinate as sole carbon source, and their growth yield on limiting (5 mM) glucose is listed in Table 4.3. All transformed strains carrying a chromosomal

Fig. 4.4. Agarose gel electrophoresis of plasmid DNA.



Samples of plasmid DNA were cleaved with endonuclease Hind III and subjected to agarose gel electrophoresis through a 1.0% gel as detailed in Chapter2, section 2.5.(iv). (A) 500 ng of plasmid pAN378(R210Q): (B) 500 ng plasmid pAN45. The gel shows the difference in size of the linearised vectors pAN174 (A) and pACYC184 (B). Sizes are shown in bases.

TABLE 4.3

COMPLEMENTATION ANALYSIS WITH pAN385(*uncB540*, R210Q).

Each strain was transformed to chloramphenicol resistance by plasmid pAN385. Growth yields were measured in media containing 5 mM glucose supplemented with 5% v/v Luria and 90 $\mu g.ml^{-1}$ chloramphenicol. The value shown is the average of at least three attempts. For further details of strains see either section 4.2.(i) or tables 2.1. and 2.2.

BACTERIAL STRAIN	pAN385(R210Q)		pAN436(*unc*$^+$)	
	GROWTH YIELD (5mM Glucose)	GROWTH ON SUCCINATE	GROWTH YIELD (5mM Glucose)	GROWTH ON SUCCINATE
AN727(*uncB402*)	141	-	228	+
AN943(*uncE429*)	222	+	229	+
AN1440(*uncF476*)	211	+	218	+
AN2015(*uncH241*)	224	+	227	+
AN730(*uncA401*)	184	+	189	+
AN1273(*uncG428*)	192	+	195	+
AN818(*uncD409*)	217	+	215	+
AN802(*uncC424*)	192	+	195	+
AN887(*uncB$^-$Mu::413*)	140	-	231	+
AN888(*uncB$^+$Mu::416*)	225	+	229	+

mutation were rescued with respect to their ability to grow on succinate and in their growth yield on limiting glucose, except for strain AN727 carrying the *uncB402* mutation. Strain AN888(*uncB*$^{+}$*Mu::416*) was also rescued by transformation with pAN378, as would be predicted from the complementation analysis of the other reference strains. This strain requires a copy of all the wild type *unc* genes except the *uncB* gene to be introduced into the cell to facilitate growth on succinate. Thus, the complementation analysis confirms that the phenotype of strain AN2608(R210Q) is not due to a mutation introduced into another gene by the oligonucleotide being inadvertently introduced into the phage replicative form by originally annealing at a second site on the single-stranded template.

4.2.(ii) Isolation and characterisation of the revertant strain AN2706

Approximately 10^8 cells of strain AN2608(R210Q) were plated out onto solid succinate minimal media containing chloramphenicol. After 36-48 hours, an estimated 120 colonies were visible, consistent with a single base reversion rate of $1:10^6$. Plasmids from 12 separate revertant colonies were prepared, and retransformed into strain AN887(*uncB*$^{-}$*Mu::413)*. Colonies which were then able to grow on succinate as sole carbon source were purified and retained. Growth yields for each of the revertant colonies were estimated, and all were similar to the growth yield for the coupled control strain AN1460 (pAN45/*uncB*$^{-}$*M*u::413).

Plasmids from eight of the revertant colonies were prepared and the 4.4 kb *Hin*d III-generated insert from each plasmid was subcloned back into M13 mp9. Sequence analysis showed that all eight plasmids carried the single base reversion of -CAA- to -CGA- at nucleotide 629, confirming a full reversion.

One colony containing a plasmid carrying the reversion was retained and designated AN2706(pAN433, *unc*$^{+}$).

4.2.(iii) ATPase activities

Subcellular fractions were prepared from strains AN2608(R210Q), the full revertant AN2706(R210Q210R), coupled control strain AN1460(pAN45/*uncB*$^{-}$*Mu::413*), and uncoupled control strain AN2584(pAN174/*uncB*$^{-}$*Mu::413*)(Table 4.4). The cytoplasmic ATPase levels in all strains assayed were negligible, with no specific activity calculated being above 0.1 μg Pi produced per min per mg protein. The membrane-bound ATPase activity for the uncoupled control strain AN2584(pAN174/*uncB*$^{-}$

TABLE 4.4

ATPase ACTIVITIES OF SUBCELLULAR FRACTIONS PREPARED FROM MUTANT STRAIN AN2608(R210Q), FULL REVERTANT STRAIN AN2706(R210Q210R), COUPLED CONTROL STRAIN AN1460(*unc*$^+$) AND UNCOUPLED CONTROL STRAIN(*unc*$^-$)

.

The four subcellular preparations were isolated simulataneously as described in Chapter 2, section 2.8.(i), and ATPase activities assayed as described in Chapter 2, section 2.8.(iii). Removal of the F_1-ATPase from the membranes was performed by dialysing the membranes against low ionic strength buffer in the absence of *p*-aminobenzamidine. Further details of strains are given in the text to Chapter 4, or in Table 2.1 and 2.2.

BACTERIAL STRAIN (Plasmid)	ATPase ACTIVITY (μmol.min^{-1}.mg protein^{-1})		
	CYTOPLASM	MEMBRANE	
		Pre-dialysis	Post-dialysis
AN2608(R210Q) (pAN378)	0.1	0.3	0.6
AN2706(R210Q210R) (pAN433)	<0.1	0.7	0.7
AN2584(unc$^-$) (pAN174)	<0.1	<0.1	<0.1
AN1460(unc$^+$) (pAN45)	0.1	1.9	1.9

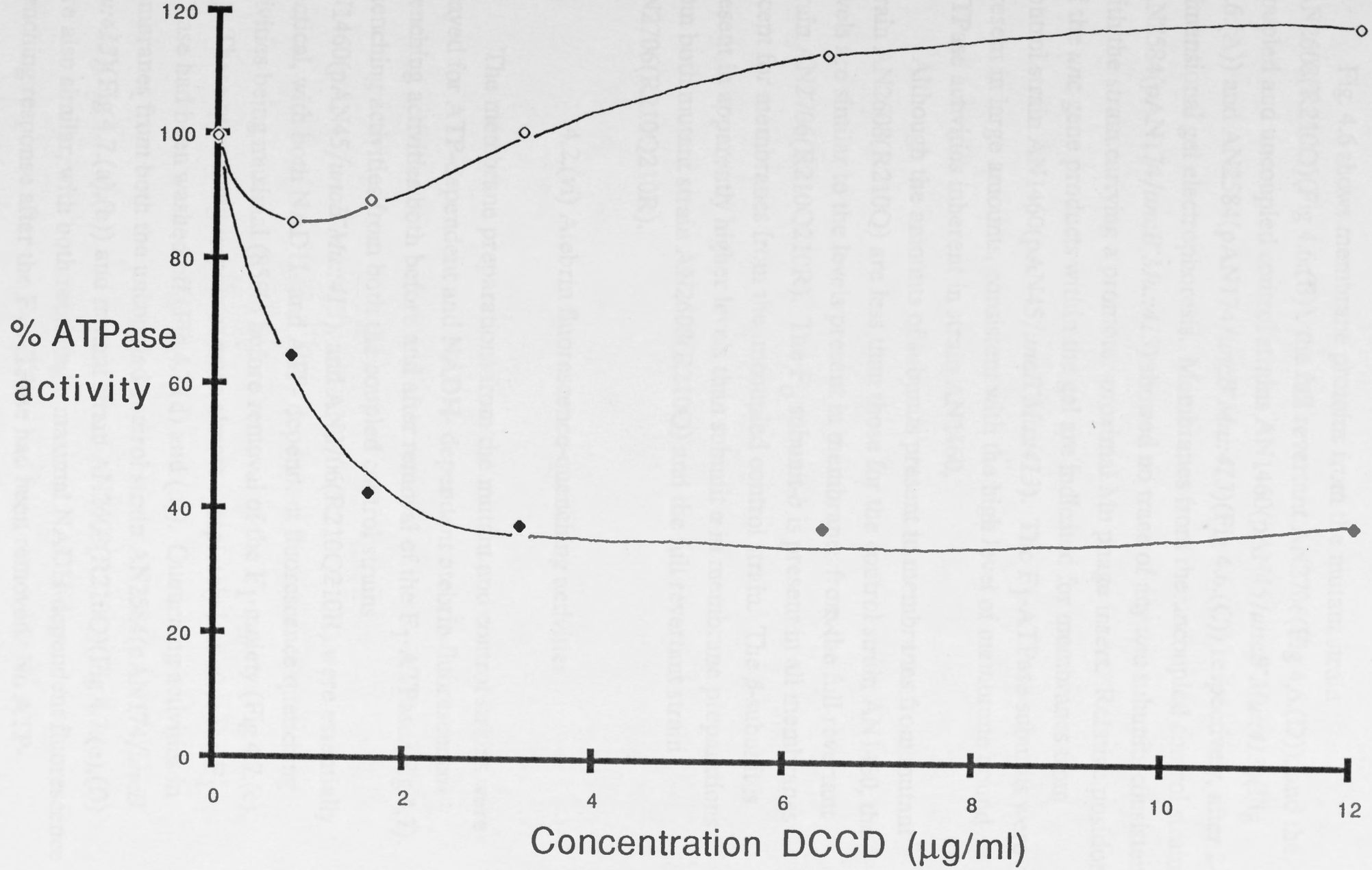
Mu::413) was also negligible. Consistent with the possibility that effective transcription from pAN174 was reduced with respect to the same promoter in pACYC184, the membrane-bound activity for the coupled control strain AN1460(pAN45/*uncB⁻Mu::413*) was higher than the comparable activity for the full revertant strain AN2706(R210Q210R) (Table 4.4).

The membrane-bound ATPase activity from the mutant strain AN2608(R210Q) was 0.3 μmol Pi.min.mg protein^{-1}, intermediate between the levels found in membranes from the coupled (AN2706) and uncoupled (AN2584) control strains, giving an activity of 40% of the coupled membranes. However, if the membranes from the mutant strain were dialysed against low ionic strength buffer in the absence of *p*-aminobenzamidine and reassayed, the ATPase activity increased to 0.6 μmol Pi.min.mg protein^{-1}, about 85% of the coupled control value. The dialysis treatment causes the F_1-ATPase to be released from the membranes. This was confirmed for the mutant strain AN2608(R210Q) by the lack of any appreciable ATPase activity on assaying the membranes after centrifugation of the dialysed preparation. Dialysis treatment did not affect the levels of ATPase activities in preparations assayed from the control or revertant strains (Table 4.4)

4.2.(iv) Sensitivity of membrane-bound ATPase activities to the inhibitor DCCD

ATPase activities were measured in the presence of differing concentrations of the inhibitor DCCD in membranes from the mutant strain AN2608(R210Q) and the two coupled control strains AN2706(R210Q210R), and AN1460(pAN45/*uncB⁻Mu::413*) (Fig 4.5). Membranes from both coupled strains were maximally inhibited by approximately 65%, whilst the mutant strain AN2608(R210) exhibited a maximal inhibition of 20% when assayed in the presence of the lower concentrations of DCCD. However, in higher concentrations (>4 μg.ml^{-1} DCCD per 300 μg membrane protein), the activity was actually stimulated by approximately 20% of levels assayed in the absence of the inhibitor.

Fig 4.5 Inhibition of ATPase activity by DCCD. Membranes (0.3 mg of protein) were incubated at 30°C in 5 ml of the assay mixture together with the indicated levels of DCCD. The mixture was sampled at intervals, the rate determined for each DCCD concentration and the percent inhibition was calculated (*q.v.* Chapter 2, section 2.8.(iii)). Symbols: ◆, membranes from coupled control strain AN2706(R210Q210R); ◇ ,membranes from mutant strain AN2608(R210Q). Inhibition of ATPase activity in membranes from coupled control strain AN1460(pAN45/*uncB-Mu::413*) was similar to the inhibition noted in membranes from strain AN2706(R210Q210R).


120
100
80
% ATPase
activity
60
40
20
0
0
2
4
6
8
10
12
Concentration DCCD (µg/ml)

4.2.(v) 2-dimensional gel electrophoresis of membrane proteins from mutant strain AN2608(R210Q) and control strains.

Fig. 4.6 shows membrane proteins from the mutant strain AN2608(R210Q)(Fig 4.6.(B)), the full revertant AN2706(Fig 4.6.(D)), and the coupled and uncoupled control strains AN1460(pAN45/*uncB⁻Mu::413*)(Fig 4.6.(A)) and AN2584(pAN174/*uncB⁻Mu::413*)(Fig 4.6.(C)) respectively, after 2-dimensional gel electrophoresis. Membranes from the uncoupled control strain AN2584(pAN174/*uncB⁻Mu::413*) showed no trace of any *unc* subunits consistent with the strain carrying a promoter-proximal Mu phage insert. Relative positions of the *unc* gene products within the gel are indicated for membranes from control strain AN1460(pAN45/*uncB⁻Mu::413*). The F_1-ATPase subunits were present in large amounts, consistent with the high level of membrane bound ATPase activities inherent in strain AN1460.

Although the amounts of subunits present in membranes from mutant strain AN2608(R210Q) are less than those for the control strain AN1460, the levels are similar to the levels present in membranes from the full revertant strain AN2706(R210Q210R). The F_o subunit-*b* is present in all membranes except for membranes from the uncoupled control strain. The β-subunit is present in apparently higher levels than subunit α in membrane preparations from both mutant strain AN2608(R210Q) and the full revertant strain AN2706(R210Q210R).

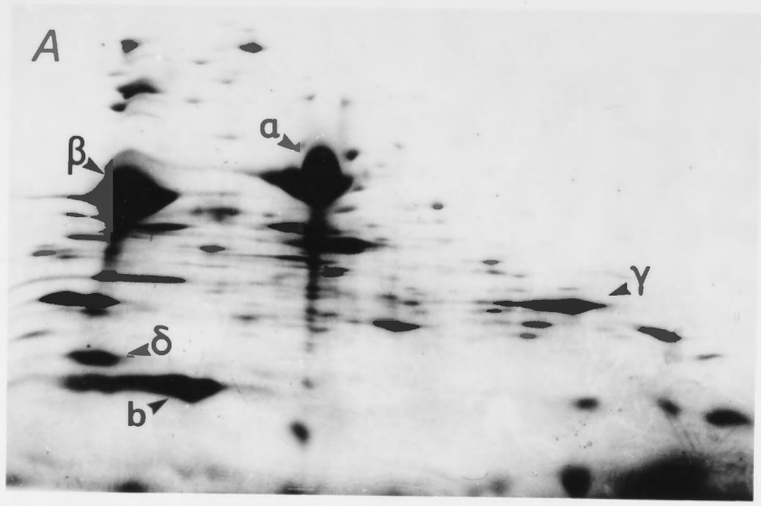
4.2.(vi) Atebrin fluorescence-quenching activities

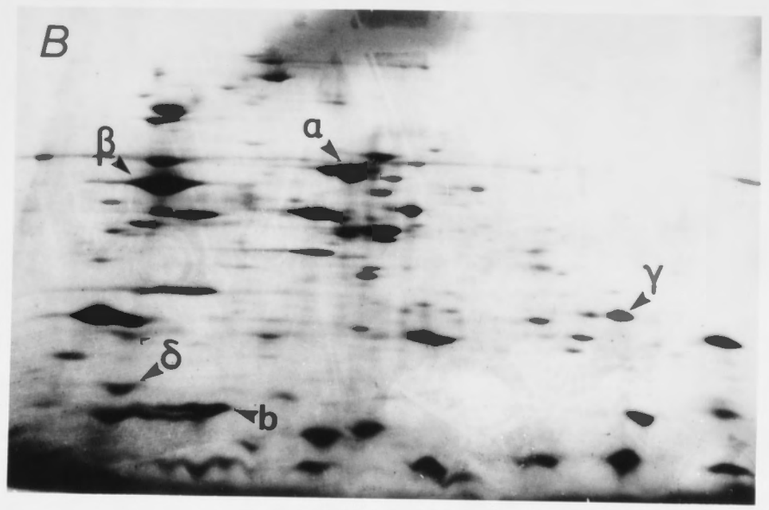
The membrane preparations from the mutant and control strains were assayed for ATP-dependent and NADH- dependent atebrin fluorescence-quenching activities both before and after removal of the F_1-ATPase (Fig 4.7). Quenching activities from both the coupled control strains AN1460(pAN45/*uncB⁻Mu::413*), and AN2706(R210Q210R) were essentially identical, with both NADH- and ATP- dependent fluorescence quenching activities being maximal (85%) before removal of the F_1-moiety (Fig 4.7.(c), (g)). These quenching responses were almost completely lost after the F_1-ATPase had been washed off (Fig 4.7.(d) and (h)). Quenching activities in membranes from both the uncoupled control strain AN2584(pAN174/*uncB⁻Mu::413*)(Fig 4.7.(a),(b)) and mutant strain AN2608(R210Q)(Fig 4.7.(e),(f)) were also similar, with both retaining a maximal NADH-dependent fluorescence quenching response after the F_1-ATPase had been removed. No ATP-

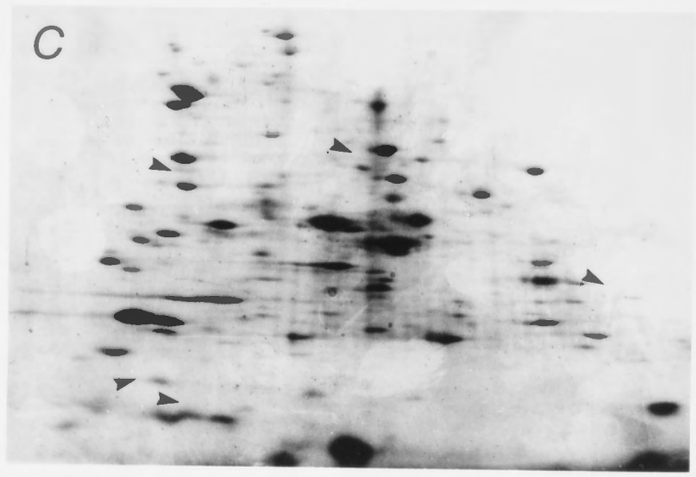
Fig 4.6 Two dimensional gel electrophoresis of membrane preparations. Samples (1 ml containing approximately 20 mg protein) of membranes were extracted three times with 5 ml of acetone. The dried residues were solubilised in approximately 800 µl of lysis buffer (Chapter 2, section 2.9.(i)) and 50 µl used for electrophoresis. In the first dimension, ampholines whosed pH's ranged from 5 to 7 and from 3.5 to 10 were present in 1.2% and 0.8% (w/v), respectively. In the second dimension, an acrylamide gradient of 22.5% to 7.5% (w/v) was used. The arrows labelled α, β, γ, δ and *b* identify the corresponding subunits of the *E. coli* F_oF_1-ATPase. Unlabelled arrows indicate the normal positions of the absent subunits. Due to their strongly lipophilic nature, the intramembranous subunits -*a* and -*c* do not focus well and are therefore not identified on the gels. A: membranes from the coupled control strain AN1460(pAN45/*uncB*$^-$*Mu::413*) B: membranes from the mutant strain AN2608(R210Q) C: membranes from the uncoupled control strain AN2584(pAN174/*uncB*$^-$*Mu::413*) D: membranes from the full revertant strain AN2706(R210Q210R).

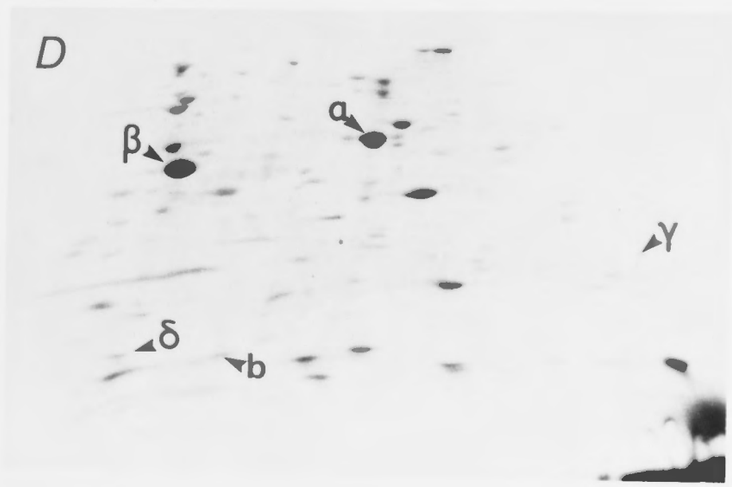
First dimension (Isoelectric focusing)

Second dimension (Electrophoresis)









dependent quenching activity could be noted in membranes from either the uncoupled control strain AN2584 or the mutant strain AN2608(R210Q).

4.2.(vii) Production of the control strain AN2680(pAN436/*uncB⁻Mu::413*)

To verify the results obtained with the full revertant, a plasmid was constructed which contained genes coding for all the *unc* structural genes and was identical to pAN45 except for the substitution of the vector pACYC184 by the deleted vector pAN174. This was achieved by mixing nuclease *Hin*d III digested pAN36 with nuclease *Hin*d III partially cleaved pAN51. These digests were ligated, and used to transform strain AN1273(*uncG428*). Plasmids were isolated from colonies which were able to grow on succinate solid media in the presence of chloramphenicol. A plasmid which was similar in size to pAN45 and showed a small deletion in the vector band after cleavage with nuclease *Hin*d III when analyzed by electrophoresis was retained and designated pAN436. Strain AN887(*uncB⁻Mu::413*) was transformed by plasmid pAN436, and a colony which was isolated on solid media containing succinate as carbon source and chloramphenicol as selective drug was purified and designated strain AN2680(pAN436/*uncB⁻*Mu::413).

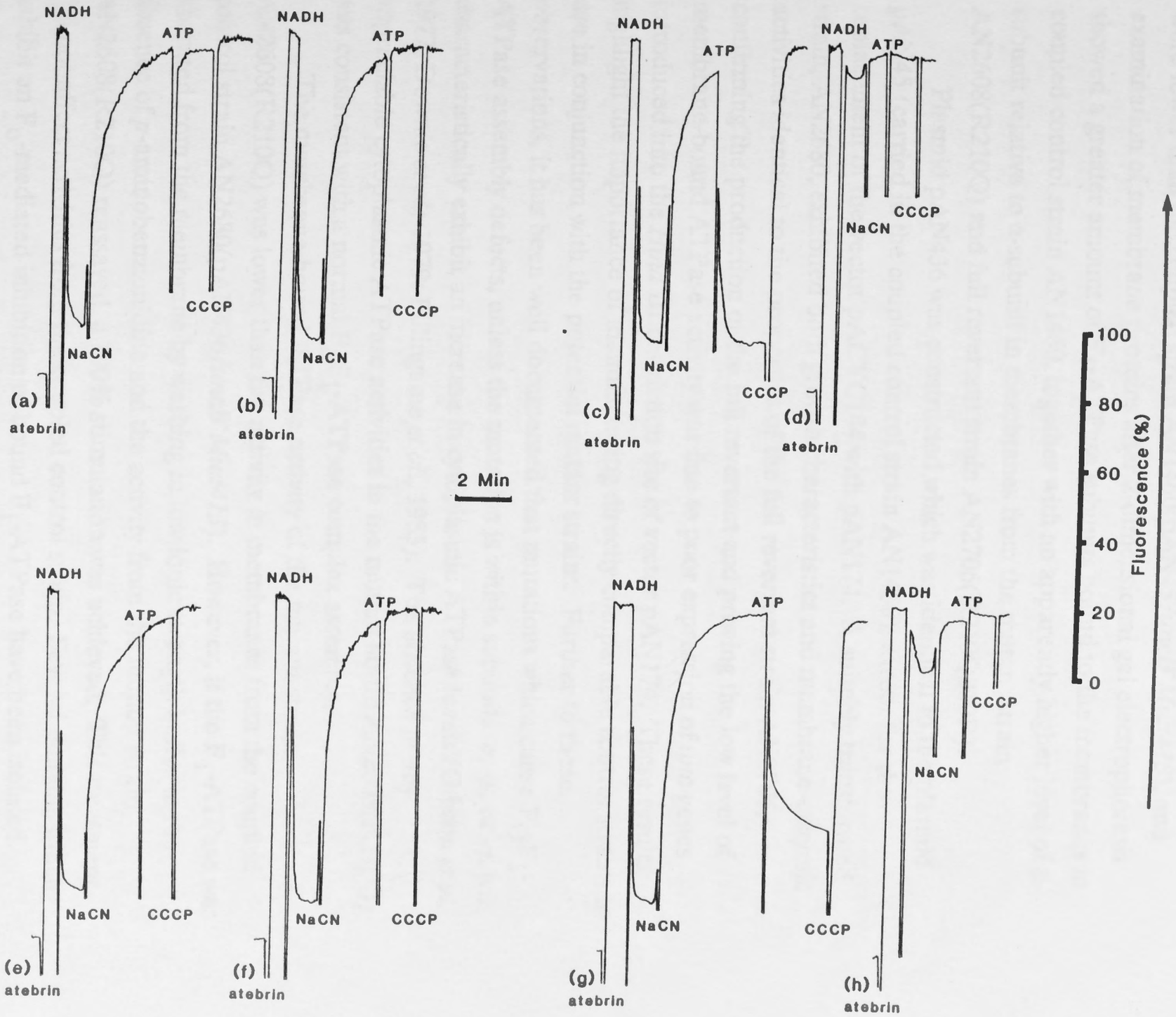
This strain exhibited similar growth characteristics to the coupled control strain AN1460(pAN45/*uncB⁻Mu::413*), and to the full revertant strain AN2706(R210Q210R). Membranes were prepared from strain AN2709(pAN436/*uncB⁻Mu::413*). The ATPase activities were identical to the full revertant strain AN2706. No increase in activity was noted after dialysis. DCCD sensitivities of membrane-bound ATPase activities were also identical to the sensitivities tabulated from the revertant, as were the NADH- and ATP-dependent fluorescence quenching activities both before and after removal of the F_1-ATPase.

Significantly, the disparate level of the two major F_1-ATPase subunits, α and β, was also apparent in membranes from this control strain, AN2709(pAN436/*uncB⁻Mu::413*).

4.3. Discussion

A plasmid carrying the single base substitution encoding the R210Q substitution in subunit-*a* of the *E. coli* F_oF_1-ATPase was constructed (pAN378). The substitution, in the proposed helix IV of subunit-*a*, causes a low growth yield of the mutant strain AN2608(R210Q) on limiting concentrations of glucose and

Fig 4.7 Atebrin fluorescence-quenching in membranes prepared from strains of *E. coli*. Atebrin fluorescence-quenching activities were assayed as detailed in Chapter 2, section 2.8.(ii). Atebrin was added to give a final concentration of 4 μM, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM and carbonylcyanide m-chlorophenylhydrazone (CCCP) to 2 μM. (a) membranes from uncoupled control strain AN2584pAN174/*uncB*$^{-}$*Mu::413*); (b) stripped membranes from uncoupled control strain AN2584(pAN174/*uncB*$^{-}$*Mu::413*); (c) membranes from coupled control strain AN1460(pAN45/*uncB*$^{-}$*Mu::413*); (d) stripped membranes from coupled control strain AN1460(pAN45/*uncB*$^{-}$*Mu::413*); (e) membranes from mutant strain AN2608(R210Q); (f) stripped membranes from mutant strain AN2608(R210Q); (g) membranes from the full revertant strain AN2706(R210Q210R); (h) stripped membranes from full revertant strain AN2706(R210Q210R).


NADH
ATP
NaCN
CCCP
atebrin
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
2 Min
100
80
60
40
20
0
Fluorescence (%)

an inability of the mutant strain to grow on succinate minimal medium. Both properties are consistent with those of a mutant strain in which phosphorylation is uncoupled from electron transport. Membrane-bound ATPase levels in the mutant strain AN2608(R210Q) and full revertant strain AN2706(R210Q210R) were lower than the coupled strain AN1460(pAN45/*uncB⁻Mu::413*), and examination of membrane proteins after 2-dimensional gel electrophoresis showed a greater amount of F_1-ATPase subunits bound to the membranes in coupled control strain AN1460, together with an apparently higher level of β-subunit relative to α-subunit in membranes from the mutant strain AN2608(R210Q) and full revertant strain AN2706(R210Q210R).

Plasmid pAN436 was constructed which was identical to the plasmid pAN45 (carried in the coupled control strain AN1460), except for the replacement of the vector pACYC184 with pAN174. A suitably transformed strain, AN2680, exhibited both growth characteristics and membrane-enzymic activities identical to the properties of the full revertant strain AN2706, confirming the production of the full revertant and proving the low level of membrane-bound ATPase activity was due to poor expression of *unc* genes introduced into the *Hind* III restriction site of vector pAN174. These results highlight the importance of manufacturing directly comparable control strains to use in conjunction with the potential mutant strains. Further to these observations, it has been well documented that mutations which cause F_OF_1-ATPase assembly defects, unless the mutation is within subunits -α, -β, or -γ, will characteristically exhibit an increase in cytoplasmic ATPase levels (Gibson *et al.*, 1977; Downie *et al.*, 1979; Fillingame *et al.*, 1983). The absence of any appreciable cytoplasmic ATPase activities in the mutant strain AN2608(R210Q) was consistent with a normal F_0F_1-ATPase complex assembly.

The membrane-bound ATPase activity of the mutant strain AN2608(R210Q) was lower than the activity in membranes from the coupled control strain AN2680(pAN436/*uncB⁻Mu::413*). However, if the F_1-ATPase was liberated from the membrane by washing in low ionic strength buffer in the absence of *p*-aminobenzamidine and the activity from the mutant strain AN2608(R210Q) reassayed, a 100% stimulation was achieved. This treatment did not affect activities from the coupled control strain. Several mutants which exhibit an F_0-mediated inhibition of bound F_1-ATPase have been isolated. Strain AN955 carrying the chromosomal mutation *uncE410* (P64L)(Fimmel *et al.*, 1983) maintains only 15% of the membrane-bound ATPase activity noted in the coupled control strain, but after removal of the F_1-ATPase from the membrane and reassaying, its activity increases by five fold. Similarly, strain MM164 carrying the *uncE107*(D61N) mutation (Fillingame *et al.*, 1984) has an

inhibited membrane-bound ATPase activity which, on release, increases by approximately 100%.

Membrane-bound ATPase activities from the mutant strain were not inhibited in the presence of DCCD and were slightly stimulated at high concentrations of the inhibitor, unlike the ATPase activities from the coupled control strains which were maximally inhibited by approximately 65%. Membranes from the mutant strain AN2608(R210Q) lacked ATP-dependent atebrin fluorescence-quenching activity and were proton impermeable even when the F_1-ATPase was removed, retaining a maximal NADH-dependent quenching response. In the original model, Arg-210 was proposed to be the residue that interacted with Asp-61 of subunit-*c* in forming the proton pore. The properties of strain AN2608(R210Q) are consistent with this proposition, and are very similar to the properties exhibited by the mutant *uncE107*(D61N) as published by Fillingame *et al.*(1984).

The method used originally for mutant production was the double-priming procedure detailed in Chapter 3.3, utilising the single-stranded template prepared from phage isolated after transformation of strain K37 with the phage replicative form pAN461. Unfortunately, after using the double priming technique, although the efficiency of transformation was high, no mutants could be detected by plaque hybridisation. A series of dot blot hybridisations were also attempted without any success, indicating a mutation rate of $<0.1\%$. Many reasons for possible low yields in mutagenesis experiments have been suggested(Zoller and Smith, 1983; Smith, M., 1985). One possibility is that the recipient strain is being transformed with the single-stranded template carrying no annealed oligonucleotide. Enrichment for the heteroduplex was attempted after electrophoresis through an agarose gel. The heteroduplex migrates at a slower rate than the single-stranded form, and can be located by running the gel in the presence of 500 μg.litre^{-1} ethidium bromide. Extraction was facilitated by binding the DNA to DE81 paper

Another problem may be the 3'-5' exonucleolytic proof-reading activity , or the strand displacement (helicase) activity inherent in DNA polymerase 1 large fragment (Smith, M., *et al.,* 1979). These activities may be responsible for removal of the substituted base(s).

Perhaps the biggest problem in mutant production using a heteroduplex system occurs after transforming the recipient strain of *E. coli*. The host has a mismatch repair system, responsible for locating and correcting base pair mismatches within the genome(Loeb and Kunkel, 1982). Kramer *et al.* (1984), have shown that the repair system is directed to the nascent strand of DNA, targeted by its transient undermethylation. As the newly synthesised strand of

the heteroduplex has been formed *in vitro*, the duplex will be hemi-methylated, and the host mismatch repair system can easily discern between the two strands, favouring repair of the mutant, unmethylated strand.

Several new methods for specific mutant production have attempted to tackle the mismatch repair problem by selecting for the mutated strand (Kramer, 1984; Kunkel, T.A. (1985); Carter *et al.*, 1985). One elegant method utilises the incorporation of thionucleotides in to the mutant strand during heteroduplex formation. Nicks in the duplex are then produced in the parent strand by certain restriction endonucleases which fail to cleave phosphorothioate DNA. These nicks are recognised by Exonuclease III, which can then digest away the parent strand, leaving the single-stranded mutant which can be used as a template *in vitro* to produce a mutant homoduplex (Nakayama and Eckstein, 1986).

The problem of mismatch repair may also be circumvented by methylating the newly synthesised strand *in vitro*. The *E. coli* enzyme, DAM methylase, recognises the four base sequence 5'-G-A-T-C-3' in a DNA duplex and catalyses the methylation of the adenine moiety at the N-6 atom (Herman and Modrich, 1982). Once the newly synthesised strand is methylated, the mismatch repair system is incapable of discerning which of the two bases in any mismatch is the normal one (Pukkila *et al.*, 1983), increasing the possibility of targeting the repair of the wild type sequence. This method was applied in the production of the R210Q substitution.

Finally, *E. coli* enzymes pol III holoenzyme, single-stranded binding protein (SSB, DNA-binding protein), and ligase were also utilised in formation of the heteroduplex (A kind gift from Dr.N.E.Dixon). The enzyme pol III holoenzyme is primarily responsible for DNA replication in the *E. coli* cell (McHenry and Kornberg, 1981) and has a 5'-3' polymerase activity approximately 50 times that of DNA polymerase 1 large fragment, incorporating one kilobase of nucleotides in two seconds (O'Donnell and Kornberg, 1985a). It also remains bound to the single-stranded DNA until polymerisation is completed (O'Donnell, M.E. and Kornberg 1985a), unlike pol 1(see Kornberg 1980 pp.101-166), facilitating ligation by *E. coli* ligase, and has no appreciable repair activity (O'Donnell,M.E. (1985b)). Single-stranded binding protein has the same function as phage T4 gene 32 protein (Alberts and Frey, 1970; Kornberg, 1980), which has been used successfully in mutagenesis experiments to prevent formation of any secondary structures in the single-stranded template DNA that may cause the polymerase to stall or fall off (Craik *et al.*, 1985). It coats the single-stranded DNA molecule, producing a regular, extended structure recognised as substrate by pol III holoenzyme (McHenry, C., 1981).

Thus, the base substitution encoding the R210Q replacement was introduced by a modified version of the double priming mutagenesis procedure, using an enrichment step for the heteroduplex, a methylation step, and utilising several indigenous *E. coli* DNA manipulating enzymes. These modifications were found to be necessary, as no mutants were produced using the standard double priming procedure. The level of mutant production after utilising the modified procedure was still extremely low, $<1\%$.

Several reasons for obtaining such a low yield of mutants have already been suggested, but the yield was so low it was possible that the mutated gene product, not the mutagenesis, was responsible, having a strongly deleterious affect on the cell. Zoller and Smith (1983), state that genes cloned in to phage M13 may be expressed at high levels, possibly due to the high copy number (approx. 200 copies per cell) of phage replicative form in infected cells. This premise is mainly based on the results of Winter *et al.* (1982) which show that on cloning of the gene encoding tyrosyl-tRNA synthetase in to M13 mp93 the enzyme is expressed to produce $>50\%$ of the total cell protein. However, the authors suggest that the high level of expression may be affected by the synthetase promoter, inherent in the cloned DNA fragment, coupled with the high copy number, not necessarily by the phage *lac* promoter. The 4.4 kb *Hin*d III fragment in pAN461 does not contain the major *unc* promoter.

Attempts to subclone the 4.4 kb fragment carrying the base replacement coding for the R210Q substitution in to the multicopy plasmid vector pACYC184 proved to be difficult with many plasmid constructs containing large deletions, suggesting that the potential clone was unstable. As discussed (section 4.1.(iv)), Downie *et al.*, (1980) isolated a spontaneous deletion in the vector pACYC184 when subcloning the wild type 4.4 kb fragment, and designated the resultant vector pAN174. Utilising this vector, it was possible to subclone the intact mutated fragment, and consequently to produce the plasmid pAN378 which carried the *uncB540* allele and all of the remaining *unc* structural genes. The effect on expression of the small deletion was shown by comparing the membrane bound ATPase activities from two isogenic strains AN1460(pAN45/*uncB⁻Mu::413*), and AN2680(pAN436/*uncB⁻Mu::413*). Both plasmids are identical except pAN436 contains the vector pAN174 and pAN45 contains vector pACYC184. ATPase activities in membranes from strain AN1460 were approximately three times as high as the levels in membranes from AN2680. As the two strains are isogenic, the disparity in membrane bound ATPase levels must be due to the differences in transcription levels of the *unc* genes.

Stuber and Bujard (1981), in their analysis of transcriptional signals in plasmid pACYC184, identified two promoters P1 and P2 at the beginning of the *tet* region which initiate transcription in opposite directions, with P2 being the promoter for the *tet* gene. These two promoters share a region of 20 base pairs within the RNA polymerase binding site, and the efficiencies of the two promoters are similar. Restriction pattern analysis after cleavage with nuclease *Eco* R1 showed that plasmids pAN45, pAN436, and pAN378 all carried their respective vectors in similar orientations with promotion of the *unc* genes facilitated in the direction of the promoter, P1. Previous restriction analysis with endonuclease *Hinf* 1 (Jans, 1984) had located the deletion within a 600 base pair region from the *Hind* III site, a region containing promoter P1 and part of the *tet* resistance gene. The P1 promoter lies approximately 230 base pairs away from the transcription initiation codon of *tet* (Sutcliffe, 1978), thus it is feasible that the deletion had affected promotion from P1 without loss of the *tet* structural gene, which is retained by vector pAN174.

Porter *et al.* (1983), had shown, using the promoter detection plasmid pRZ5255, that the *unc* operon has several potential promoters within the *uncI* reading frame. This evidence is in agreement with von Meyenberg *et al.* (1982) and more recently with Kanazawa *et al.* (1984) who localised a weak promoter approximately 90 base pairs upstream from *uncB*, showing, using maxicells, that this promoter was responsible for expression of subunit-*a* in plasmids carrying IS1 insertions upstream from the putative weak promoter, effectively preventing any expression from the major *unc* promoter. This weak promoter is present on the 4.4 kb *Hind* III-generated fragment used for mutant production (pAN461) and although the efficiency of this promoter is still debatable (Kanazawa *et al.*, 1984) it is possible that the reason for the low level of *unc* gene promotion from plasmids pAN436 and pAN378 is due to the destruction of the *tet* promoter in the vector, necessitating promotion from the weak promoter in *uncI*.

Problems associated with small deletions in and around the *unc* promoter region have been documented by several groups originating with the analysis of plasmids carrying the *E. coli* chromosomal origin of replication, *oriC*. Plasmids carrying *oriC* have been isolated (Yasuda *et al.*, 1977; Miki *et al.*, 1978; von Meyenberg *et al.*, 1978), locating the origin between the *uncB* and *asnA* genes around 83 minutes on the chromosomal map (Bachmann, 1983). None of these plasmids could be stably inherited under nonselective conditions until Ogura *et al.* (1980) formed a construct from one of these original plasmids pMCR115 and the mini-F pSC138, producing plasmid pXX11, which carried *oriC*, *oriV* (mini-F origin of replication), *asnA* and a large piece of the *unc* operon.

Stable maintenance of pXX11 was affected in Hfr mafA (Maintaining Autonomous F plasmid) strains. These mutant strains are incapable of stably supporting autonomous replication of F plasmids (Wada *et al.*, 1976). Thus, pXX11 can replicate in this strain by utilising the *oriC* replication origin, and is believed to be maintained under nonselective conditions by the partition mechanism of the mini-F plasmid. High copy number mutants of the strain carrying pXX11 were isolated on the basis of colony size, and analysis of the plasmids isolated from these strains indicated spontaneous insertions and deletions within the plasmids localised to a 0.7 kb region designated *cop* for copy number mutants.

Interestingly, the parent plasmid pXX11 complemented the chromosomal mutation *uncB402*, whilst all the *cop*$^-$ plasmids failed to complement the *uncB* mutation, prompting Ogura *et al.*(1980) to suggest that the *cop*$^-$ and *uncB* region were in close proximity.

Yamaguchi and Yamaguchi (1983), also produced *cop*$^-$ mutants by constructing plasmid pKY16 by self-ligating a 9.2 kb nuclease *Eco* R1-generated fragment from the *E. coli* chromosome. This plasmid carried *oriC*, *asnA* and *unc* genes *I, B, E, F,* and *H* and was found to be unstable in the original selection strain NK1054 (a*snA, asnB, recA1* (Yamaguchi *et al.*, 1982)). A 2.5 kb *Hae* II-generated fragment from plasmid pKY16 was cloned in to the high copy number pBR322 derivative pKY135, and the resultant plasmid, pKY159 carried none of the *oriC* region, but still contained the *unc* promoter region and *unc* genes *I, B, E* and approximately 70% of *uncF*. Plasmid pKY159 could only be isolated in *E. coli* strain NK1037 (*Hfr*, *dnaA46*, *recA1*), causing growth inhibition and was only maintained in a low copy number (This low copy number may have been due to the temperature sensitive *dnaA* mutation. This gene product is required for *colE1* and *oriC* replication). When strains not carrying the *dnaA* chromosomal mutation were transformed with plasmid pKY159, the only viable strains harboured the plasmid with either spontaneous deletions or insertions within the 2.5 kb insert. Amongst other possibilities, the authors postulated that over expression of F_0 subunits may produce a lethal effect, possibly due to dissipation of the transmembrane gradient.

Further to these observations, Kanazawa *et al.* (1984), working with the same plasmid, pKY159, concluded that the deleterious effect produced by this plasmid was due to overproduction of subunit-*a*. This conclusion was based on a series of engineered deletions and insertions within the 2.5 kb Hae II fragment.

von Meyenberg *et al.* (1985), reported that when a 10-fold overexpression of subunit-*a* alone was induced from the strong phage lambda promoter pR, a dissipation of the membrane potential resulted, claiming this physiological

response could be attributed to the protonophoric activity of subunit-*a* on integration into the membrane. The authors claim the activity is due to the formation of an abnormal transmembranous pore produced by subunit-*a* multimers which form after accumulation of the subunits in the cytoplasm on induction of the *uncB* gene. Interestingly, this protonophoric activity is sensitive to DCCD. Thus, if the *uncB540* gene-product was affected such that the subunit-*a*-subunit-*a* binding-affinity was increased, the incidence of multimer formation would also be increased. Thus, assuming the abnormal intramultimeric proton pore was unaffected, the levels of the *uncB540* gene-product expressed from the undeleted vector may be sufficient to form the multimeric protonophore which could then dissipate the membrane potential. Lower levels of subunit-*a* formation such as are expressed from the deleted vector may be tolerated. The loss of an arginine residue would also affect the dynamic equilibrium of molecular insertion into the membrane, which suggests that the multimers may form in the membrane preventing integration into an assembly.

This hypothesis is consistent with the results discussed above and with the problems encountered on production and cloning of the 4.4 kb fragment carrying the base substitution coding for the R210Q substitution.

However, these are only speculations, but given the problems encountered with the production of plasmid pAN378, the deleted vector pAN174 was used when problems were experienced with subcloning from any mutant derivatives of pAN461.

CHAPTER 5

A SPECIFIC REQUIREMENT FOR ARGININE AT POSITION 210 OF THE *E. coli* F_oF_1-ATPASE SUBUNIT-*a*: CHARACTERISATION OF THE R210K MUTATION AND OF A SECOND-SITE PARTIAL REVERTANT

5.1 Introduction

The single amino acid substitution of R210Q in subunit-*a* prevents proton translocation through the F_O-sector (*q.v.* Chapter 4). All eight revertants isolated and sequenced had regained the original nucleotide sequence (*q.v.* Section 4.2.(ii)), suggesting the requirement for arginine at position 210 of subunit-*a* was absolute. To test whether this was the case, or whether conservation of a potentially positively-charged residue was sufficient, Arg-210 of subunit-*a* was replaced by lysine.

5.2. Production and characterisation of the subunit-*a* substitution R210K

5.2.(i) Mutagenesis

Potential mutants were prepared using the standard double-priming procedure detailed in Chapter 3, section 3.3 with single-stranded template prepared from cells transformed by phage M13 replicative form pAN461 and screened using the differential-temperature hybridisation method. The relevant [γ-^{32}P]-labeled oligonucleotide was used to probe the filters. Phage carrying the possible base substitutions were isolated and the nucleotide sequence around the mutation site determined. Those phage which contained the substitutions were purified and the substitutions verified after isolation and sequencing of the phage DNA. After confirmation of the correct base substitutions, the oligonucleotide used in the mutagenesis experiment was utilised as a sequencing primer. Phage which exhibited both the expected base substitutions and a single sequencing pattern from the oligonucleotide primer were retained. The sequencing pattern of phage DNA from one of the mutant phage is shown in Figure 5.1. The oligonucleotide used to produce the desired substitutions is listed in Table 5.1 with the substituted bases underlined.

5.2.(ii) Production of plasmid pAN430(R210K)

The mutant pAN461 derivative was mixed with pAN36 and pAN174 and the mixture was cleaved with nuclease *Hin*d III and ligated with T4 DNA ligase. Competent cells of strain AN1273(*uncG428*) were transformed by the ligation mix and colonies containing plasmids potentially carrying the substituted bases in the 4.4 kb *Hin*d III-generated fragment selected by plating the transformed cells on to solid succinate minimal media supplemented with chloramphenicol. Twelve colonies were selected, the strains purified, and their plasmids isolated.

TABLE 5.1

SYNTHETIC OLIGONUCLEOTIDES USED IN THE PREPARATION AND SEQUENCE CONFIRMATION OF THE SUBUNIT-*A* SUBSTITUTION R210K, AND FOR SEQUENCE ANALYSIS OF THE *uncB* GENE.

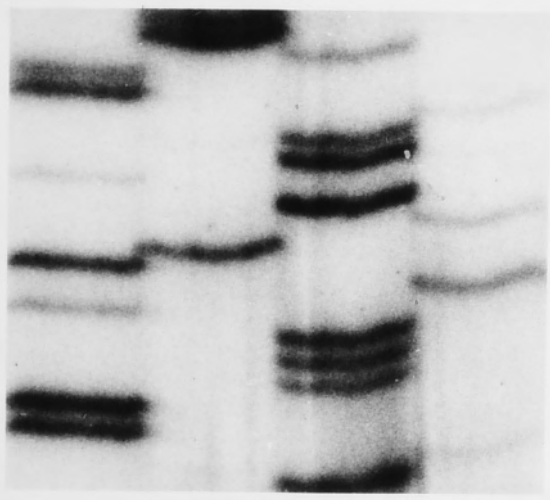
Preparation of the oligonucleotides is detailed in Chapter 2, section 2.7.(i). The base which is underlined signifies a base substitution from the normal sequence.

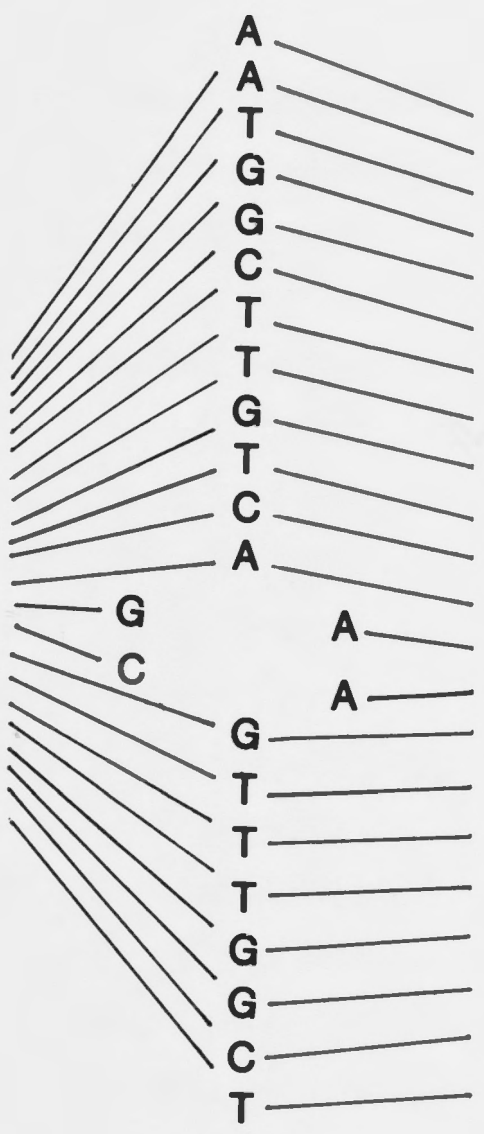
Synthetic oligonucleotide	Relevant amino-acid substitution	Notes
5'-CTCGGTTTGAAACTGTTCGG-3'	R210K	Also used as sequencing primer for sequencing from nucleotide 638 in the *uncB* gene.
5'-GTAAAACGACGGCCAGT-3'	-	Phage M13 universal primer which anneals onto the viral strand approximately 50 bases 5' from the *Hind* III restriction site in the mp9 polylinker. Used for sequencing from nucleotide -150 in the *uncB* gene.
5'-ATGGCACTGGGCGTATTTA-3'	-	Sequencing primer used for sequencing from nucleotide 475 in the *uncB* gene.
5'-GGGTCTGTTGTTCCTGGTTTTA-3'	-	Sequencing primer used for sequencing from nucleotide 154 in the *uncB* gene.

Fig 5.1. Sections of DNA sequencing gels showing the *uncB569*(R210K) nucleotide substitution. Samples of DNA from both normal and mutant were sequenced and the sequencing reactions electrophoresed through 6% (w/v) polyacrylamide as described in Chapter 2, section 2.6. Sequencing gels may be read in the order G, A, T, C from left to right. The indented nucleotides indicate the nucleotides which have been substituted.

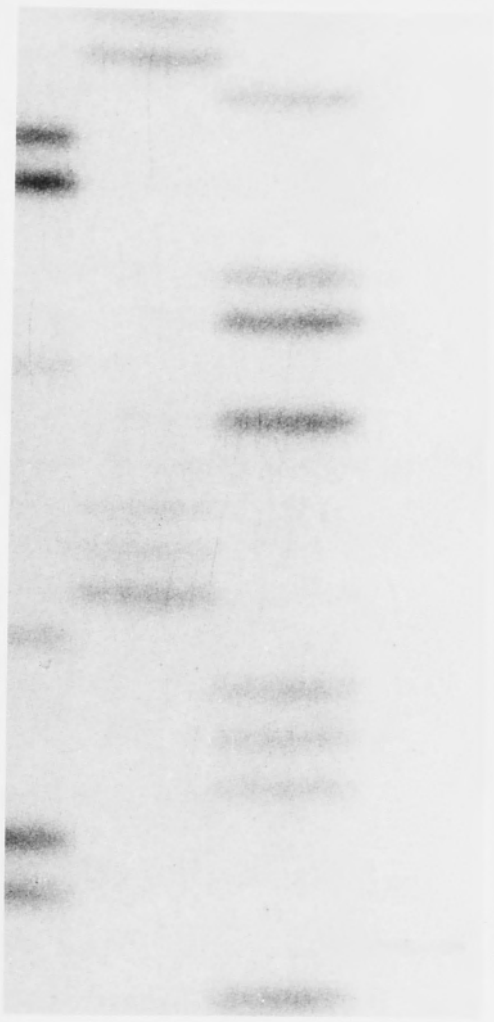
DNA SEQUENCE CHANGE IN UNCB569

NORMAL





MUTANT



TRIPLET CHANGE

CGA ⟶ AAA

R210K

All the plasmids carried pAN174 as vector. One colony carrying a mutant plasmid similar in size and *Hin*d III-cleaved restriction pattern to pAN45, but containing the vector pAN174, was retained and the plasmid designated pAN430. Plasmid pAN430 was then used to transform the strain AN887(*uncB⁻Mu::413)* to chloramphenicol resistance. One colony from the transformation experiment was purified and retained. This strain was designated AN2690(pAN430, carrying *uncB569*), and will be referred to as strain AN2690(R210K).

5.3. Characterisation of strain AN2690(R210K)

5.3.(i) Growth characteristics of strain AN2690(R210K): complementation analysis using plasmid pAN430(*uncB569*), and resistance to the antibiotic, neomycin.

Strain AN2690(R210K) was examined for its ability to grow on succinate minimal medium and for its growth yield on a limiting level of glucose (Table 5.2). Strain AN2690(R210K) was unable to grow on solid or in liquid succinate media. The growth yield of this strain on 5 mM glucose was lower than that of the uncoupled control strain AN2584(pAN174/*uncB⁻Mu::413*). Plasmid pAN430 was used to transform the chromosomal *unc* reference strains AN727(*uncB402*), AN943(*uncE429*), AN1440(*uncF469*), AN2015(*uncH*241), AN730(*uncA401*), AN818(*uncD409*) and AN802(*uncC424*), along with the strain carrying a Mu phage insert promoter distal to *uncB*, strain AN888(*uncB⁺Mu::416*). The strains transformed, their ability to grow on succinate as sole carbon source, and their growth yield on limiting (5 mM) glucose are listed in Table 5.2. The *uncB* reference strain AN727 and the *uncB⁻Mu* strain AN887 were unable to utilise succinate after transformation to chloramphenicol resistance by plasmid pAN430, consistent with the R210K subsitution in subunit-*a* uncoupling oxidative phosphorylation. However, the *uncB⁺Mu* strain AN888, the *uncA* reference strain AN730, the *uncD* strain AN818, and the *uncC* strain AN802 were also unable to grow on succinate after similar transformations with pAN430. Thus, the R210K mutation was dominant when introduced into these strains. To confirm that the phenotype produced on transformation by plasmid pAN430 was due to the R210K substitution, and not to any inadvertently produced mutations during the mutagenesis, in addition to the routine checking of the single-site annealing of the base-substituted oligonucleotide by using it as a sequencing primer, the complete mutagenesis, sub-cloning, and complementation analysis was repeated.

TABLE 5.2

COMPLEMENTATION ANALYSIS WITH pAN430(R210K) AND pAN435(R210K,L259M).

Growth yields were measured in media containing 5mM glucose supplemented with 5% v/v Luria and 90 $\mu g.ml^{-1}$ chloramphenicol. The value shown is the average of at least three attempts. For further details of plasmids and relevant strains see either section 5.2 (ii) or 5.4 (ii) or see Tables 2.1. and 2.2.

BACTERIAL STRAIN	pAN430(R210K)		pAN435(R210K,L259M)		pAN436(*unc*$^+$)	
	GY[a]	Succ[b]	GY	Succ	GY	Succ
AN727(*uncB402*)	112	-	139	-	224	+
AN943(*uncE429*)	142	+	171	+	231	+
AN1440(*uncF476*)	145	+	172	+	219	+
AN2015(*uncH241*)	130	+	174	+	230	+
AN730(*uncA401*)	133	-	142	-	184	+
AN1273(*uncG428*)	113	+	169	+	195	+
AN818(*uncD409*)	140	-	152	-	216	+
AN802(*uncC424*)	115	-	160	c	201	+
AN887(*uncB$^-$Mu::413*)	124	-	148	+	230	+
AN888(*uncB$^-$Mu::416*)	147	-	168	+	227	+

a GY: Growth yield in 5 mM glucose measured in Klett units.
b Succ: The ability of cells to utilise succinate as sole carbon source.
c Slow growth on succinate.

TABLE 5.3

ATPase ACTIVITIES OF SUBCELLULAR FRACTIONS PREPARED FROM MUTANT STRAIN AN2690(R210K), PARTIAL REVERTANT STRAIN AN2708(R210K,L259M), COUPLED CONTROL STRAIN AN2680(*unc*$^+$) AND UNCOUPLED CONTROL STRAIN AN2584(*unc*$^-$)

The four subcellular preparations were isolated simulataneously as described in Chapter 2, section 2.8.(i), and ATPase activities assayed as described in Chapter 2, section 2.8.(iii). Removal of the F_1-ATPase from the membranes was performed by dialysing the membranes against low ionic strength buffer in the absence of *p*-aminobenzamidine. Further details of strains are given in the text to Chapter 5, or in Table 2.1 and 2.2.

BACTERIAL STRAIN (Plasmid)	ATPase ACTIVITY (μmol.min^{-1}.mg protein^{-1})		
	CYTOPLASM	MEMBRANE	
		Pre-dialysis	Post-dialysis
AN2690(R210K) (pAN430)	0.1	0.8	0.7
AN2708(R210K,L259M) (pAN435)	0.1	0.7	0.7
AN2584(unc$^-$) (pAN174)	0.1	<0.1	<0.1
AN2680(unc$^+$) (pAN436)	<0.1	0.7	0.7

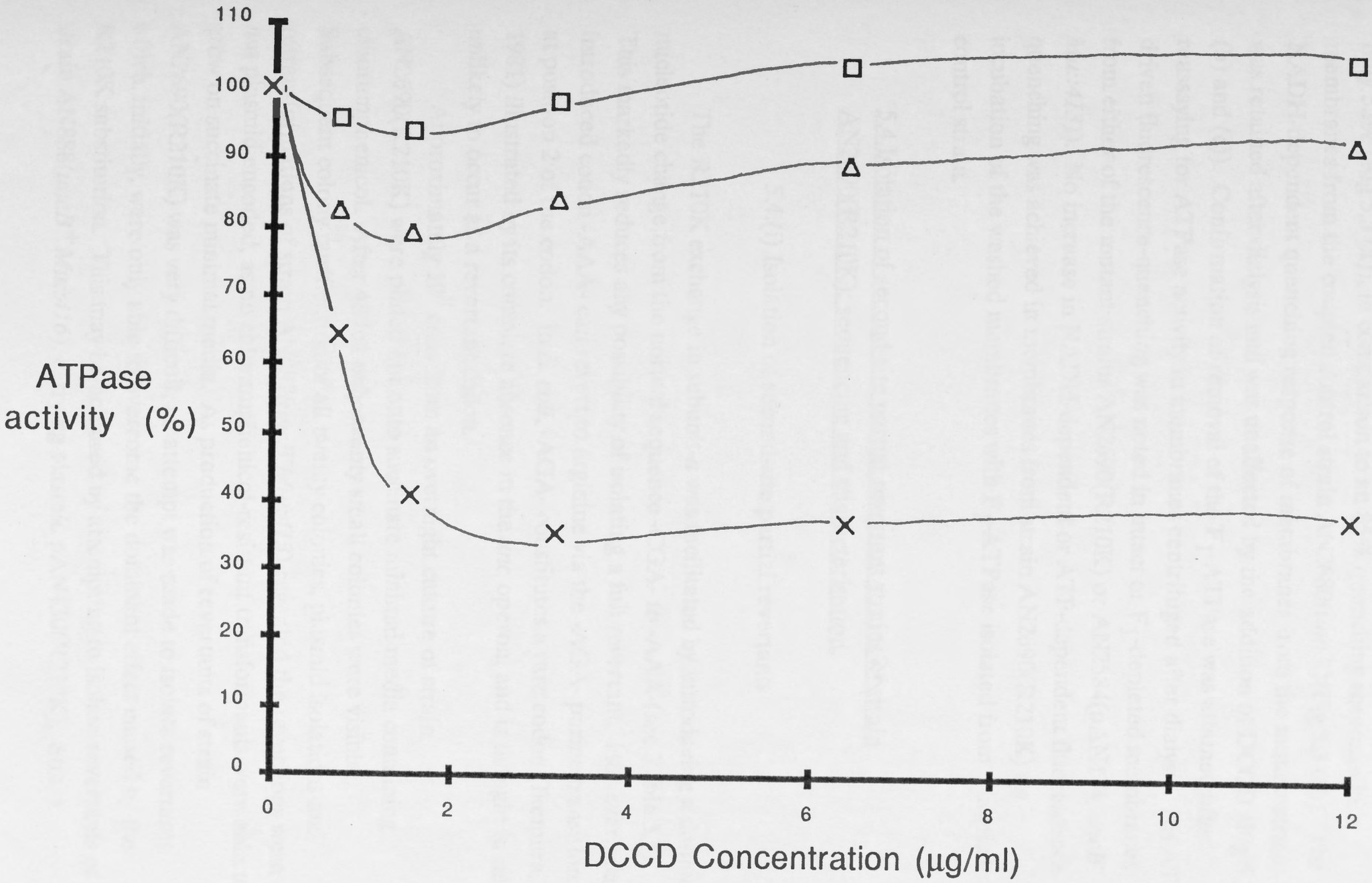
As mentioned above, the growth yield of strain AN2690(R210K) on limiting glucose was lower than the uncoupled control strain AN2584(pAN174/*uncB⁻Mu::413*). One possible explanation for this phenomenon was that membranes from strain AN2690(R210K) were more permeable to passive proton translocation, and that the strain was reliant on proton-pumping by ATP hydrolysis to maintain a normal membrane potential. Neomycin, an aminoglycoside antibiotic, is believed to be taken up through the plasma membrane of bacteria such as *E. coli* by utilising the membrane potential (Bryan and van den Elden, 1977), and consequently, mutant strain AN2690(R210K), and the two control strains AN2584(pAN174/*uncB⁻Mu::413)* and AN2680(pAN436/*uncB⁻Mu::413*) were all tested for their resistance to neomycin. After spreading approximately 10^8 cells of each of the three strains separately onto glucose minimal agar, a small filter was treated with 500 μg of neomycin sulphate and laid onto the centre of the plates. The zone of growth inhibition was measured after 24 hrs and found to be consistently smaller for plates containing cells of strain AN2690(R210K). In a series of four experiments, the diameter of the zone of inhibition varied from 22-29 mm with cells from strain AN2690(R210K, 32-41 mm with cells from the uncoupled control strain AN2584(pAN174/*uncB⁻Mu::413*), and 36-40 mm with cells from the coupled control strain AN2680(pAN436/*uncB⁻Mu::413*). Thus, strain AN2690(R210K) was less susceptible to inhibition of growth by the antibiotic, neomycin, than either of the two control strains, AN2584(pAN174/*uncB⁻Mu::413)* or AN2680(pAN436/*uncB⁻Mu::413*).

5.3.(ii) Membrane-enzymic properties of strain AN2690(R210K)

Subcellular fractions were prepared from the mutant strain AN2690(R210K) and the two control strains AN2584(pAN174/*uncB⁻Mu::413)* and AN2680(pAN436/*uncB⁻Mu::413*). Membrane-bound ATPase activities were similar for membranes from the coupled control strain AN2680(*unc*$^+$) and the mutant strain AN2690(R210K)(Table 5.3). No increase in activity was noted when the activities of any of the three strains were reassayed after removal of the F_1-ATPase by washing in the absence of *p*-aminobenzamidine in a low ionic-strength buffer. Cytoplasmic levels of ATPase in all strains were negligibile (Table 5.3). Membrane-bound ATPase activities of the mutant strain AN2690(R210K) were virtually unaffected by the presence of the inhibitor DCCD, although activities were slightly stimulated in high concentrations of the inhibitor (Fig 5.2).

Fig 5.2 Inhibition of ATPase activity by DCCD. Membranes (0.3 mg of protein) were incubated at 30°C in 5 ml of the assay mixture together with the indicated levels of DCCD. The mixture was sampled at intervals, the rate determined for each DCCD concentration and the percent inhibition was calculated (*q.v.* Chapter 2, section 2.8.(iii)).

Symbols: × , membranes from coupled control strain AN2680(pAN436/*uncB*$^{-}$*Mu::413*); □ , membranes from mutant strain AN2690(R210K); △ , membranes from the partial revertant strain AN2708(R210K,L259M).


ATPase activity (%)
110
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
DCCD Concentration (µg/ml)

NADH- and ATP- dependent atebrin fluoresence-quenching activities of washed and intact membranes are shown in Fig 5.3. The level of NADH-dependent quenching achieved in membranes from strain AN2690(R210K) was only 52% (Fig 5.3 (a)), in comparison to an 85% quenching response in membranes from the coupled control strain AN2680(*unc*$^+$)(Fig 5.3 (d)). The NADH-dependent quenching response of membranes from the mutant strain was retained after dialysis and was unaffected by the addition of DCCD (Fig 5.3 (b) and (c)). Confirmation of removal of the F_1-ATPase was attained after reassaying for ATPase activity in membranes centrifuged after dialysis. No ATP-driven fluorescence-quenching was noted in intact or F_1-depleted membranes from either of the mutant strains AN2690(R210K) or AN2584(pAN174/*uncB*$^-$*Mu::413*)). No increase in NADH-dependent or ATP-dependent fluorescence-quenching was achieved in membranes from strain AN2690(R210K) on incubation of the washed membranes with F_1-ATPase isolated from a normal control strain.

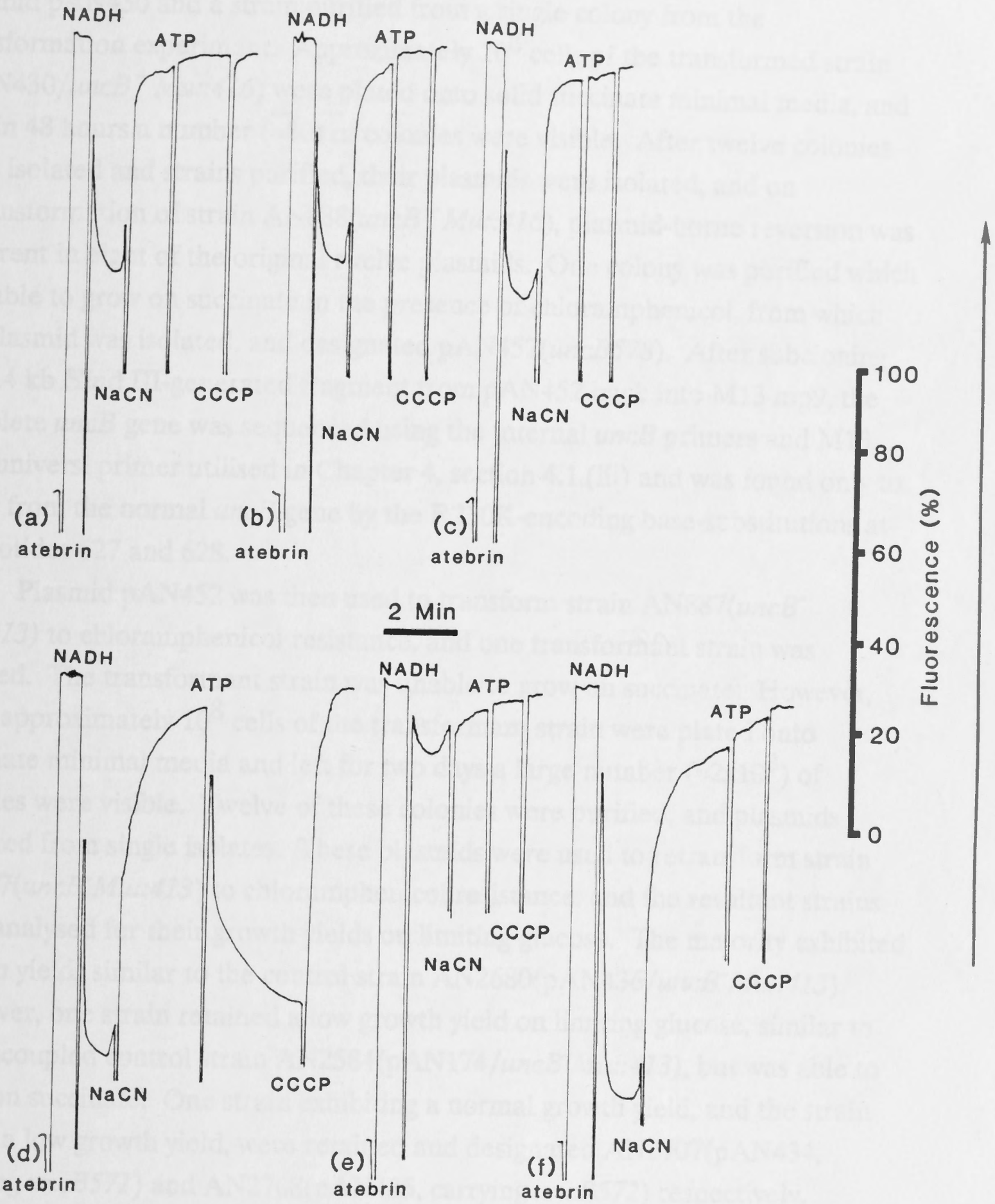
5.4.Isolation of second-site partial revertant strains of strain AN2690(R210K): sequencing and characterisation.

5.4.(i) Isolation of second-site partial revertants

The R210K exchange in subunit-*a* was facilitated by introducing a double nucleotide change from the normal sequence -CGA- to -AAA-(see Table 5.1). This markedly reduces any possibility of isolating a full revertant. However, the introduced codon -AAA- can revert to arginine via the -AGA- purine transition at position 2 of the codon. In *E. coli*, -AGA- constitutes a rare codon (Ikemura, 1981) illustrated by its complete absence in the *unc* operon, and is thought to be unlikely to occur as a revertant codon.

Approximately 10^8 cells from an overnight culture of strain AN2690(R210K) were plated out onto succinate minimal media containing chloramphenicol. After 48 hrs only twenty small colonies were visible. Subsequent colony purification of all twenty colonies, plasmid isolations and retransformations of strain AN887(*uncB*$^-$*Mu::413*) revealed the reversions were not plasmid encoded, as no chloramphenicol-resistant transformants were able to grow on succinate minimal media. As production of revertants of strain AN2690(R210K) was very difficult, an attempt was made to isolate revertants which, initially, were only able to overcome the dominant effect caused by the R210K substitution. This may be achieved by attempting to isolate revertants of strain AN888(*uncB*$^+$*Mu::416*), carrying plasmid pAN430(R210K). Strain

Fig 5.3 Atebrin fluorescence-quenching in membranes prepared from strains of *E. coli*. Atebrin fluorescence-quenching activities were assayed as detailed in Chapter 2, section 2.8.(ii). Atebrin was added to give a final concentration of 4 μM, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM and carbonylcyanide m-chlorophenylhydrazone (CCCP) to 2 μM. (a) membranes from mutant strain AN2690(R210K); (b) stripped membranes from mutant strain AN2690(R210K); (c) stripped membranes from mutant strain AN2690(R210K) assayed after a 10 min preincubation in 50 μM (Final) DCCD; (d) membranes from coupled control strain AN2680(*unc*$^+$); (e) stripped membranes from coupled control strain AN2680(*unc*$^+$); (f) stripped membranes from coupled control strain AN2680(*unc*$^+$) assayed after a 10 min preincubation in 50 μM (Final) DCCD.


NADH
ATP
NaCN
CCCP
(a)
atebrin
(b)
atebrin
(c)
atebrin
2 Min
(d)
atebrin
(e)
atebrin
(f)
atebrin
100
80
60
40
20
0
Fluorescence (%)

AN888(*uncB*$^+$*Mu::416*) was transformed to chloramphenicol resistance by plasmid pAN430 and a strain purified from a single colony from the transformation experiment. Approximately 10^8 cells of the transformed strain (pAN430/*uncB*$^+$*Mu::416*) were plated onto solid succinate minimal media, and within 48 hours a number (≈80) of colonies were visible. After twelve colonies were isolated and strains purified, their plasmids were isolated, and on retransformation of strain AN888(*uncB*$^+$*Mu::416*), plasmid-borne reversion was apparent in eight of the original twelve plasmids. One colony was purified which was able to grow on succinate in the presence of chloramphenicol, from which the plasmid was isolated, and designated pAN452(*uncB578*). After subcloning the 4.4 kb *Hin*d III-generated fragment from pAN452 back into M13 mp9, the complete *uncB* gene was sequenced using the internal *uncB* primers and M13 mp9 universl primer utilised in Chapter 4, section 4.1.(iii) and was found only to differ from the normal *uncB* gene by the R210K-encoding base-substitutions at nucleotides 627 and 628.

Plasmid pAN452 was then used to transform strain AN887(*uncB*$^-$ *Mu::413)* to chloramphenicol resistance, and one transformant strain was purified. The transformant strain was unable to grow on succinate. However, when approximately 10^8 cells of the transformant strain were plated onto succinate minimal media and left for two days a large number (≈$2x10^3$) of colonies were visible. Twelve of these colonies were purified, and plasmids removed from single isolates. These plasmids were used to retransform strain AN887(*uncB*$^-$*Mu::413*) to chloramphenicol resistance, and the resultant strains were analysed for their growth yields on limiting glucose. The majority exhibited growth yields similar to the control strain AN2680(pAN436/*uncB*$^-$*Mu::413*). However, one strain retained a low growth yield on limiting glucose, similar to the uncoupled control strain AN2584(pAN174/*uncB*$^-$*Mu::413)*, but was able to grow on succinate. One strain exhibiting a normal growth yield, and the strain giving a low growth yield, were retained and designated AN2707(pAN434, carrying *uncB571*) and AN2708(pAN435, carrying *uncB572*) respectively.

5.4.(ii) Sequence analysis of plasmids pAN*434(uncB571*) and pAN435(*uncB572*)

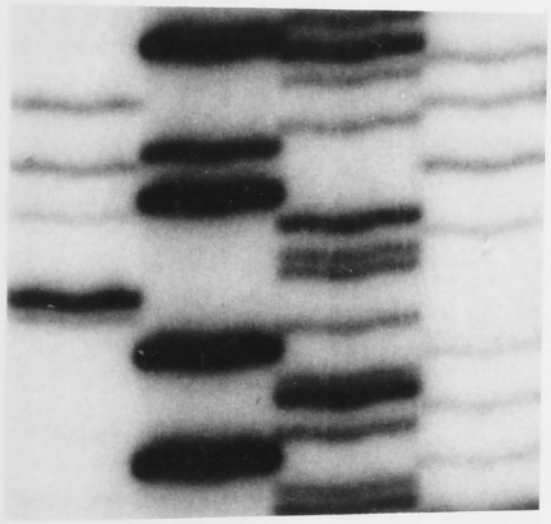
The 4.4 kb *Hin*d III-generated fragment was subcloned from both plasmids pAN434 and pAN435 in to phage M13 mp9 and the nucleotide sequence *of* the *uncB* alleles determined using the internal *uncB* primers and M13 mp9 universl primer detailed in Table 5.1. Plasmid pAN435 contained a single base change from the published sequence in *uncB,* at nucleotide 774,

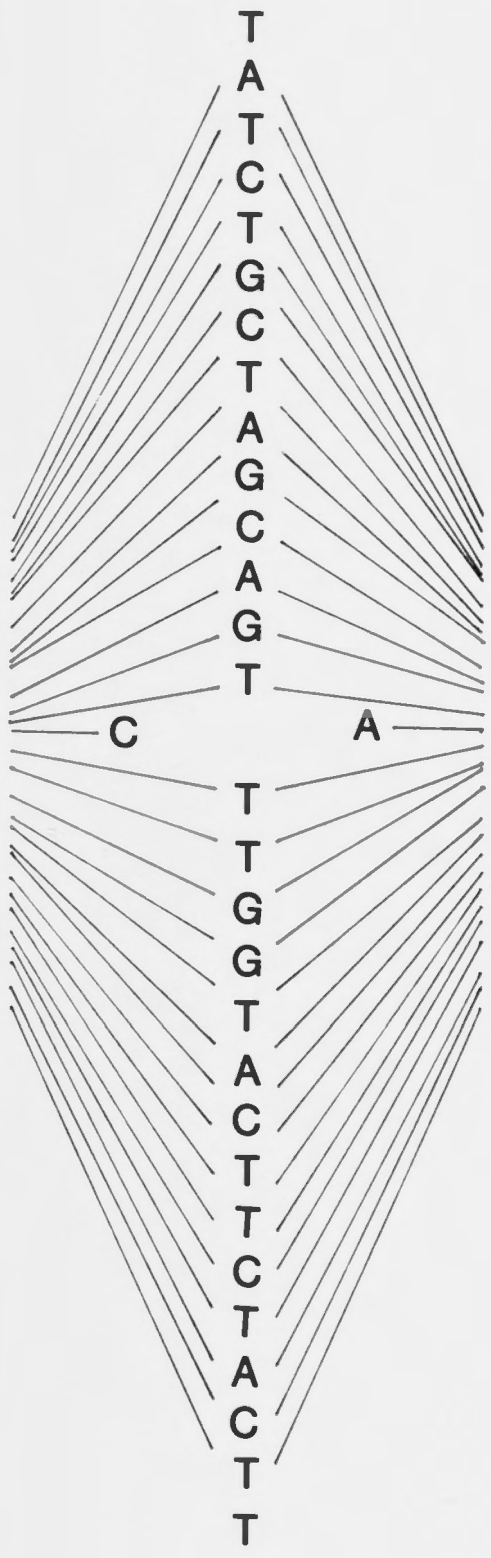
Fig 5.4. Sections of DNA sequencing gels showing the *uncB572*(R210K,L259M) base substitution at nucleotide 775. Samples of DNA from both normal and mutant were sequenced and the sequencing reactions electrophoresed through 6% (w/v) polyacrylamide as described in Chapter 2, section 2.6. Sequencing gels may be read in the order G, A, T, C from left to right. The indented nucleotides indicate the nucleotide which has been substituted.

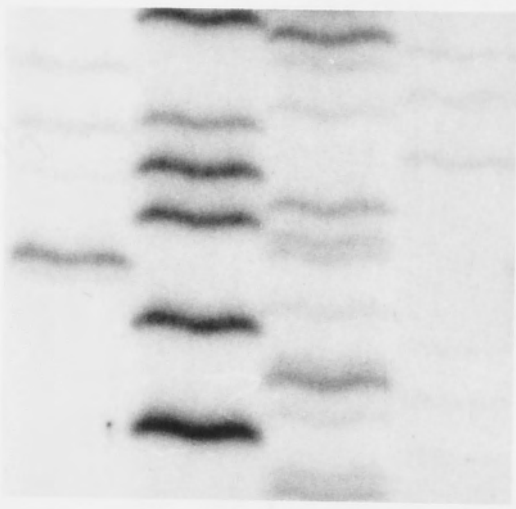
DNA SEQUENCE CHANGE IN UNCB572

NORMAL

MUTANT







TRIPLET CHANGE CTG ⟶ ATG

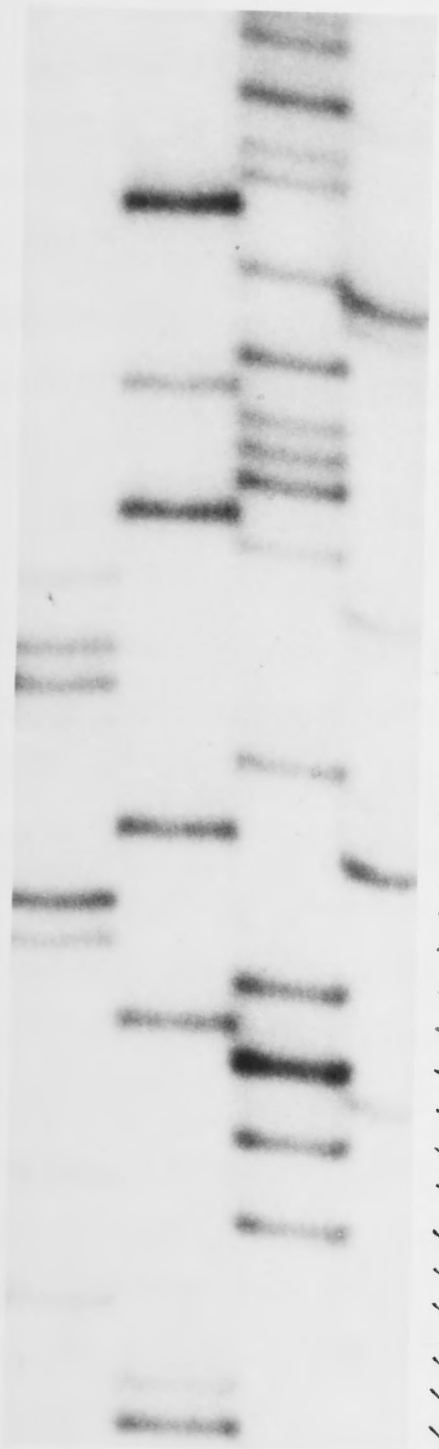
L259M

Fig 5.5. Sections of DNA sequencing gels showing the five base pair deletion in the *uncB571* allele. Samples of DNA from both normal and mutant were sequenced and the sequencing reactions electrophoresed through 6% (w/v) polyacrylamide as described in Chapter 2, section 2.6. Sequencing gels may be read in the order G, A, T, C from left to right. The indented nucleotides indicate the five nucleotides which have been deleted from *uncB571*.

DNA SEQUENCE CHANGE IN UNCB571

NORMAL

MUTANT



T
T
G
T
C
T
T
A
G
T
C
C
T
A
T
T
T
A
T
G

C
G
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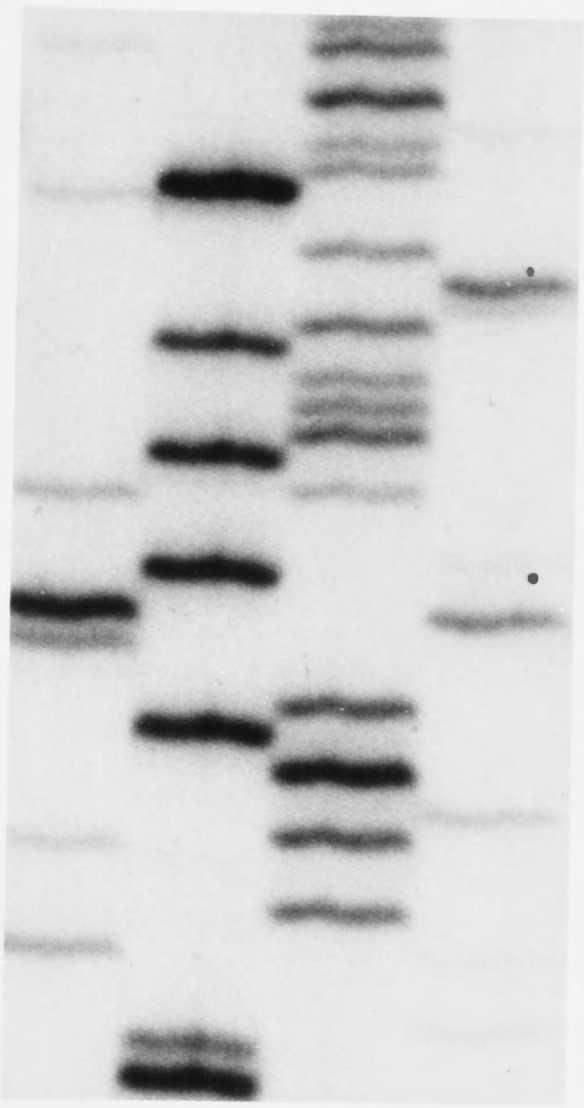
C
G
C
A
A



5 BASE PAIR DELETION

resulting in a missense mutation substituting Leu-259 by a methionine residue (Fig 5.4.). Plasmid pAN434 proved to carry a 5 base-pair deletion in *uncB* at position 463-467 inclusively, (Fig 5.5). To confirm the validity of the 5 base pair deletion, plasmid pAN434 was reisolated from strain AN2707, and the subcloning and sequencing repeated.

5.4.(iii) Growth and membrane-enzymic properties of the second -site partial revertant strain AN2708(R210K,L259M).

Complementation analysis with pAN435 was attempted (Table 5.2). Intriguingly, although able to transform both Mu polar strains AN888(*uncB*$^+$*Mu::416*) and AN887(*uncB*$^-$*Mu::413)* to growth on succinate, plasmid pAN435 was unable to rescue growth in strain AN727(*uncB402)*. No complementation was achieved with either the *uncD* or *uncA* mutations after transformation with plasmid pAN435, and growth in the *uncC* background was weak.

Subcellular fractions were prepared from the mutant strain AN2708(R210K,L259M), the coupled (AN2680(u*nc*$^+$)) and uncoupled (AN2584*(unc*$^-$)) control strains (Table 5.3). Membrane-bound ATPase activities from the mutant strain AN2708(R210K,L259M) and the coupled control strain AN2680(pAN436/*uncB*$^-$*Mu::413*) were identical, with no increase noted on reassaying the activities after removal of the F_1-ATPase. The cytoplasmic levels of ATPase activity were negligible in each of the strains. Membrane-bound ATPase activities in strain AN2708(R210K,L259M) were slightly inhibited when assayed in the presence of DCCD (Fig 5.2). Fluorescence-quenching activities of membranes from the mutant strain AN2708(R210K,L259M) were also attempted. The NADH-dependent fluorescence-quenching activity was 76%, in comparison to 85% for activities of membranes from coupled control strain AN2680(pAN436/*uncB*$^-$*Mu::413*). However, although the activity was not as high as the activity in the coupled control membranes, it was greater than the 52% NADH-dependent quenching activity in membranes from strain AN2690(R210K). When the F_1-sector was removed from membranes of the mutant strain AN2708(R210K,L259M) by dialysis, the NADH-dependent activity was lowered to 37%. Again, this response was not noted in stripped membranes from mutant strain AN2690(R210K), which was measured at 54%. An ATP-dependent fluorescence-quenching response of 12% was apparent in membranes from the mutant strain AN2708, which was lost after removal of the F_1-ATPase (Data not shown).

5.5 Reassessment of parent strain AN887(*uncB Mu::413*) and of mutant strain AN2690(R210Q)

The 5 base pair deletion of nucleotides 463-467 in the *uncB* gene, present in plasmid pAN434 (Fig 5.5), is predicted by the nucleotide sequence of *uncB* to result in a truncated gene-product, $M_r \approx 19{,}500$, with a termination codon 120 base-pairs from the deletion site. This was a puzzling result as plasmid pAN435 transformed strain AN887(*uncB⁻Mu::413*) to growth on succinate. However, when the same plasmid was used to transform strain AN727(*uncB402*), the resultant strain was unable to utilise succinate as sole carbon source. This result demonstrated that strain AN887(*uncB⁻Mu::413*) must synthesise a sufficient level of subunit-*a* to maintain adequate levels of F_0F_1-ATPase complexes for oxidative phosphorylation in strains which contain all *unc* genes except *uncB*. The original characterisation of AN887 as a strain containing a Mu phage insert promoter proximal to *uncB*, was facilitated by analyses of growth characteristics and membrane-enzymic properties of partial diploid strains carrying the chromosomal *uncB⁻Mu::413* allele with a selection of F-plasmids carrying mutations in separate *unc* genes. The partial diploid strain AN914 carried the chromosomal *Mu::413* allele and the *uncB402* allele on an F-plasmid. This strain was apparently unable to grow on succinate, but membranes from strain AN914 were recorded as possessing 20% ATP-dependent fluorescence-quenching activity and as having a similar level of ATP-dependent transhydrogenase activity to the coupled control strain. It may be possible that the bacteriophage Mu inserted promoter-distal of *uncB* in strain AN887 and reduced levels of transcription of the *uncB* gene. Consequently, this complication necessitates that strain AN887 cannot be used as a parent strain for characterising the effects of amino-acid substitutions in subunit-*a*, and strain AN727(*uncB402*) was chosen as the recipient strain. As the two subunit-*a* mutations detailed in Chapters 3 and 4 (K167Q,K169Q; R210Q), and the R210K substitution detailed in this Chapter were characterised on plasmids which had transformed strain AN887, it was possible that the low level of subunit-*a* synthesised by the chromosomal copy of *uncB* from strain AN887 may have affected the properties of these strains. Thus, to confirm that the phenotype of these strains were unaffected by this level of normal subunit-*a*, plasmids pAN385(K167Q,K169Q), pAN378(R210Q) and pAN430(R210K) were used to transform strain AN727 to chloramphenicol resistance and growth characteristics and membrane properties of the resultant transformant strains were determined. The growth characteristics and membrane-enzymic properties of the resultant strains were identical to properties already documented for the same plasmids in strain AN887. The

AN887. The strains were designated AN2770(pAN385/K167Q,K169Q), AN2735(pAN378/R210Q) and AN2760(pAN430/R210K). Two suitable control strains were also produced by transformation of strain AN727 with either pAN436, or pAN174. The coupled control strain was designated AN2709(*uncB402,* carrying pAN436), and the uncoupled control strain AN2736(*uncB402*, carrying pAN174).

5.6 Discussion

The mutation R210K in subunit-*a* resulted in the strain AN2690(R210K) being unable to couple phosphorylation to respiration . The same phenotype is displayed by strain AN2608(R210Q)(Chapter 5) and the inability to isolate any partial revertants of strain AN2608, together with the effects noted for the R210K substitution, indicates that Arg-210 in subunit-*a* cannot be replaced by any other amino acid residue without the loss of oxidative phosphorylation. This is also in accord with the complete conservation of this residue in the subunit-*a* equivalents sequenced in all other species to date.

There are several interesting characteristics of mutant strain AN2690(R210K). Growth yields on limiting glucose are lower than the uncoupled control strain AN2584(*unc*$^-$) and the strain is less sensitive to the antibiotic neomycin than either of the control strains. The observation that strain AN2690(R210K) was less susceptible to growth inhibition by the antibiotic, and that membranes from the same strain were leaky to protons as judged by NADH-dependent fluorescence-quenching suggest that the proton electrochemical gradient across the plasma membrane of strain AN2690(R210K) is partially dissipated. Membrane-bound ATPase activities from the mutant strain AN2760(R210K) were unaffected by the presence of inhibitor, as was the NADH-dependent fluorescence-quenching response in both intact and F_1-depleted membranes. The DCCD-resistant passive proton permeability indicated by the fluorescence-quenching activities can be interpreted in one of two ways:

(i) The DCCD is still able to bind to Asp-61 of subunit-*c*, but the passive flux does not require this residue as an integral member, relying on a separate, less efficient, pore which is generated through subtle conformational changes within the complex ring of F_o helices. Passive proton permeability is unaffected whether the F_1-ATPase is bound or removed. This apparent uncoupling also suggests that the protons are utilising a different transmembranous pathway..

(ii) The DCCD is unable to bind to Asp-61 as presence of the lysine residue in helix IV of subunit-*a* causes a slight rearrangement of the helical faces such that the aspartate residue is no longer accessible from the lipid phase and is facing inside the ring of *c*-subunit helices.

It appears most likely that the first of the two explanations is correct. However, it would be of interest to treat membranes from this strain with the inhibitor, isolate the DCCD-binding proteolipid and confirm whether the DCCD has bound to the aspartate, an experiment which is being attempted presently in this laboratory.

Interestingly, the R210K mutation is dominant in *uncA,* and *uncD* strains. Several attempts were made to isolate partial revertant strains from both AN730(*uncA401*) and AN818(*uncD409*) carrying either pAN430 or pAN452 with no success. Both of the mutant strains AN730, or AN818 synthesise mutant subunits which are able to bind to F_O subunits, or be integrated in to nonfunctional assemblies. Thus, although these strains can be rescued by the introduction of normal subunits from a suitable plasmid, the growth yield of the transformed strain is intermediate between fully coupled or uncoupled, reflecting the mixing of subunits synthesised from the chromosome and from the plasmid. Both α and β subunits expressed from plasmid pAN430 or pAN452, although normal, must be unable to mix with ATPase subunits expressed by the chromosome. If the integration of subunit-*a* is the first event in complex assembly, and the rate limiting step, followed immediately by binding of the major subunits, the lack of complementation may be explained on the basis of increasing the rate of subunit-*a* integration into the membrane, thereby sequestering the major subunits immediately after synthesis and preventing the mixing of subunits required for complementation. Assuming that the newly synthesised gene-product is in dynamic equilibrium between an aqueous form and an intramembranous form, the substitution of lysine for arginine may affect this equilibrium resulting in a more immediate integration.

The recipient strain AN887(*uncB⁻Mu::413*) was deduced to express a small amount of subunit-*a*, a conclusion based on the ability of strain AN2707(pAN434/*uncB402*) to grow on succinate. This strain carried pAN434, which after sequencing analysis, proved to contain a five base deletion in *uncB*, producing a truncated subunit-*a,* but was still capable of transforming strain AN887(*uncB⁻Mu::413*) to growth on succinate. This deletion was not produced during the subcloning procedure as the plasmid isolation, subcloning and sequence analysis were repeated, confirming the deletion. Strain AN727(*uncB402*) was chosen as the recipient strain. This mutant was

characterised as an intramembranous subunit mutation originally by Butlin, (Butlin *et al.*, 1973), producing a significant level of ATPase activity in the cytoplasm and some on the membrane. However, the membranes exhibit no ATP-dependent fluorescence-quenching activity and the maximal NADH-dependent quenching response is retained after removal of the F_1-sector. The membrane-bound ATPase activity is resistant to DCCD. As shown in Table 5.3 the levels of membrane-bound and cytoplasmic ATPase activities are similar, being 0.2 μmol. Pi.min^{-1}.mg protein^{-1}. Fillingame *et al.* (1983), isolated the *uncB402* operon on a lambda transducing phage, which was then used in an *in vitro* transcription-translation system. After gel electrophoresis, a prominent product was noted with an $M_r \approx 18{,}000$, believed to be the truncated subunit-*a*. These data show strain AN727 is a competent recipient strain in which to examine the effects of specific *uncB* mutants.

As it was not possible to produce partial revertants of strain AN2690(*uncB*$^-$*Mu::413*/R210K) which were able to grow on succinate, the problem was partly circumvented by transforming the strain AN888(*uncB*$^+$*Mu::416)* with plasmid pAN430(R210K), isolating a revertant strain which was able to suppress the dominant effect of the R210K substitution, isolating the plasmid which facilitated the suppression, retransforming strain AN887(*uncB*$^-$*Mu::413*), and then isolating revertant strains which were able to compensate specifically for the R210K substitution. However, although this method successfully produced a strain which was able to grow on succinate when the strain carried a plasmid coding for the R210K substitution (AN2708(R210K,L259M), the plasmid also carried another substitution which had originally enabled the plasmid to rescue strain AN888(*uncB*$^+$*Mu::416*) to growth on succinate. It is tempting to conclude that the unidentified mutation would not affect the membrane-enzymic activities, as the activities of membranes from strain AN2690 and strain AN887 carrying plasmid pAN452 were similar. Leu-259 is predicted to lie intramembranously, in helix V, in close proximity to both Arg-210 of subunit-*a* and Asp-61 of subunit-*c*. It is possible that the methionine may interact with the proton pore responsible for the passive proton flux in strain AN2760(R210K), perturbing the low level activity of translocation either indirectly, by causing a conformational change, or directly, by short-circuiting the proton flux via a hydrogen-bond between the sulphur of methionine and an amine of any residue involved in the proton flux which lay within 0.37 nm. This type of bond is the weakest form of hydrogen-bond found in proteins and has been noted for Met-180 in chymotrypsin (Birktoft and Blow, 1972). As leucine is similar in bulk to methionine the latter seems more likely. By preventing the partial dissipation of the membrane potential, a low level of

normal copies of subunit-*a* from the chromosome of strain AN887(*uncB*$^{-}$ *Mu::413)* may be able to form enough functional complexes to facilitate oxidative phosphorylation. Although unlikely, it is possible that the difference in phenotype between strain AN2690(R210K) and strain 2708(R210K,L259M) is not produced by the L259M substitution but is caused by another mutation in another gene. The L259M may be a silent mutation formed spontaneously. However, the complementation analysis with pAN435 did not highlight any mutations in a separate gene.

CHAPTER 6

THE SUBSTITUTIONS E196A, K203Q, OR K203I IN SUBUNIT-*a* OF THE F_oF_1-ATPASE DO NOT AFFECT THE COUPLING OF ELECTRON TRANSPORT TO OXIDATIVE PHOSPHORYLATION: FURTHER REFINEMENT OF THE SECONDARY STRUCTURE OF SUBUNIT-*a*

6.1. Introduction

The working model for F_o-mediated proton translocation implicated several residues along one face of the putative subunit-*a* amphipathic helix IV as being involved in a transmembrane hydrogen-bonded network (*q.v.* Chapter 1, section 1.5.(i)). Substitution of one of these residues, Arg-210, has been shown to prevent motion of protons through the F_o (*q.v.* Chapters 4 and 5). Both Lys-203, and Glu-196 are predicted to be intrinsic to the "proton wire". Table 6.1 shows the amino acid sequences for the subunit-*a* homologues with residues corresponding to *E.coli* subunit-*a* residues Glu-196 and Lys-203, with conserved residues underlined (For relevant references for Table 6.1 see legend to Table 4.1). The residue equivalent to Lys-203 in the subunit-*a* analogue subunit-*6* from other species is lysine, arginine or glutamine, whilst the glutamine residue at position-196 is conserved in the homologues at the equivalent position (the cyanobacterium *Synechococcus 6301* analogue (Lys) and spinach chloroplast CF_oIV (Asn) are the exceptions)..

As the comparable residue to Lys-203 in the human mitochondrial ATPase subunit-*6* is a glutamine, it may be possible for the amide group to compensate for the ε-amino from the lysine residue by hydrogen-bonding with the next residue in the proton wire, suggesting that it is the potential for forming hydrogen-bonds that is important at this position, and the presence of an ionisable side-group, such as the ε-amino group, may not be compulsory for proton translocation. This theory was challenged by substituting Lys-203 by either the polar amino-acid glutamine or the bulky, hydrophobic residue isoleucine. The highly conserved Glu-196 was replaced by alanine, which, like glutamate, is predicted to have a high propensity for involvement in helix formation (Chou and Fasman, 1978). By substituting Glu-196 with alanine, the affect of the loss of a charged residue at position -196 on proton translocation could be studied without producing any adverse affect on helix formation, and thus structure assembly. All substitutions required only a single base-change from the original codon.

6.2 Production of the subunit-*a* substitutions E196A, K203Q, and K203I

6.2.(i) Mutagenesis

Mutants were prepared using the standard double-priming procedure detailed in Chapter 3, section 3.3 with single-stranded template prepared from cells transformed by phage replicative form pAN461, and screened using the

TABLE 6.1

COMPARISON OF THE AMINO-ACID SEQUENCES OF THE PUTATIVE HELIX IV OF SUBUNIT-*A* (ATPase *6*) FROM SEVERAL SPECIES

Human	I	I	**E**	T	I	S	L	L	I	**Q**	P	M	A	L	A	V	R	L	T	A	N	I	T	A	G	H	L	L
Bovine	I	I	**E**	T	I	S	L	F	I	**Q**	P	M	A	L	A	V	R	L	T	A	N	I	T	A	G	H	L	L
Rat[a]	I	I	**E**	T	I	S	L	F	I	**Q**	P	M	A	L	A	V	R	L	T	A	N	I	T	A	G	H	L	L
X. laevis	I	I	**E**	T	I	S	L	F	I	**R**	P	L	A	L	G	V	R	L	T	A	N	L	T	A	G	H	L	L
D. melanogaster	C	I	**E**	T	I	S	N	I	I	**R**	P	G	T	L	A	V	R	L	T	A	N	M	I	A	G	H	L	L
T. glabrata	V	I	**E**	L	L	S	Y	V	A	**R**	A	F	S	L	G	L	R	L	S	A	N	I	F	S	G	H	L	L
S. cerevisiae	I	I	**E**	T	L	S	Y	I	A	**R**	A	I	S	L	G	L	R	L	G	S	N	I	L	A	G	H	L	L
A. nidulans	L	I	**E**	F	I	S	Y	L	S	**R**	N	V	S	L	G	L	R	L	A	A	N	I	L	S	G	H	M	L
N. crassa	L	I	**E**	F	I	S	Y	L	A	**R**	N	I	S	L	G	L	R	L	A	A	N	I	L	S	G	H	M	L
Synechococcus.6301	P	F	**K**	I	L	E	D	F	T	**K**	P	L	S	L	S	F	R	L	F	G	N	I	L	A	D	E	L	V
Chloroplast[b]	P	I	**N**	I	L	E	D	F	T	**K**	P	L	S	L	S	F	R	L	F	G	N	I	L	A	D	E	L	I
Esch. coli	I	L	**E**	G	V	S	L	L	S	**K**	P	V	S	L	G	L	R	L	F	G	N	M	Y	A	G	E	L	I
			196[c]							203		206				210				214				219				

a Sequence is identical to subunit-6 sequence of chinese hamster and mouse.
b Spinach chloroplast CF_OIV
c Numbers correspond to the amino acid sequence for *Esch. coli*

differential-temperature hybridisation method. The relevant [γ-^{32}P] labeled oligonucleotide was used to probe the filters. Phage carrying the possible base substitution(s) were isolated and the nucleotide sequence around the mutation site determined. Those phage which contained the substitution(s) were purified and the substitution(s) verified after isolation and sequencing of the phage DNA. After confirmation of the correct base substitution(s), the oligonucleotide used to produce the relevant mutation was utilised as a sequencing primer. Phage which exhibited both the expected base substitution(s) and a single sequencing pattern from the relevant oligonucleotide primer were retained. Sequencing patterns of phage DNA from one of each of the mutations produced are shown in figures 6.1 and 6.2. The oligonucleotides used to produce the desired substitutions are listed in Table 6.2 with each substituted base being underlined. The base replacements are highlighted in Fig 6.3, with the altered bases underlined, and the substituted amino acids in bold type.

6.2.(ii) Production of plasmids pAN413(E196A), pAN415(K203Q) and pAN437(K203I)

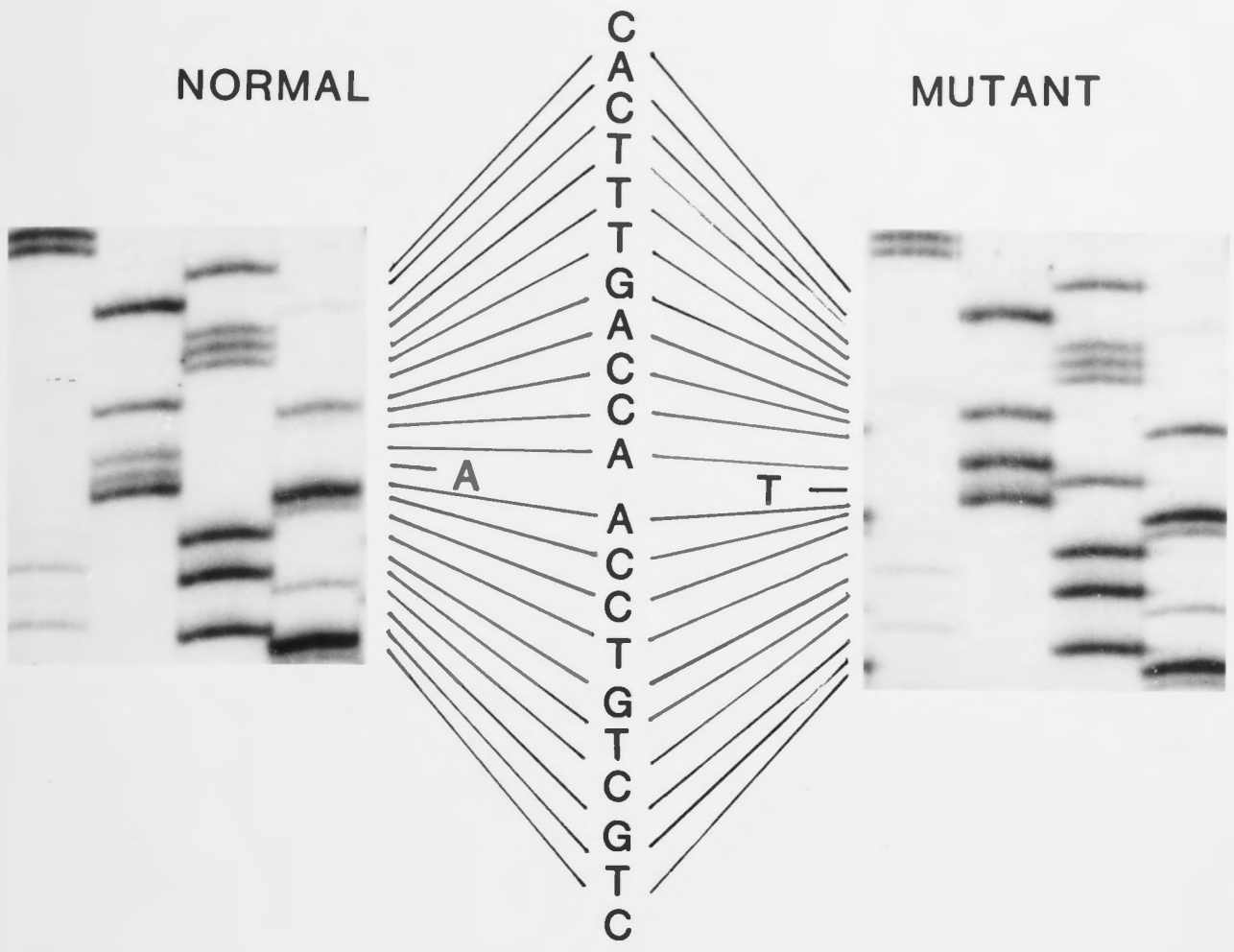
The mutant pAN461 derivatives were cleaved with nuclease *Hin*d III, mixed with partially cut pAN36 and ligated with T4 DNA ligase. Competent cells of strain AN1273(*uncG428*) were transformed by each of the ligation mixes and cells carrying recombinant plasmids containing the fragment incorporating the base substitutions, selected by plating the transformed cells on to solid succinate minimal media supplemented with chloramphenicol.

From these three separate transformation experiments, only one yielded colonies which could grow on succinate media. Twelve strains containing plasmid constructs from the pAN461 derivative carrying the E196A-encoding base substitution were purified and their plasmids isolated. Four of these plasmids contained more than one vector, but one preparation containing a plasmid identical in size and *Hin*d III-generated restriction pattern to pAN45 carrying a single copy of vector pACYC184 was retained. This plasmid was designated pAN413(E196A, *uncB566*).

To successfully subclone the two independent Lys-203 substitutions it was necessary to fully cleave the mutant derivatives of pAN461, pAN36 and the deleted vector pAN174 with nuclease *Hin*d III, and ligate the mix at 20^{o}C for 5 hrs with T4 DNA ligase. After transforming strain AN1273(*uncG428*) with each of the ligation mixes, twelve colonies which were able to utilise succinate as their sole carbon source in the presence of chloramphenicol were purified from each transformation mix and their plasmids isolated. *Hin*d III-generated restriction

Fig 6.1. Sections of DNA sequencing gels showing the *uncB567*(K203Q) and *uncB573*(K203I) nucleotide substitutions. Samples of DNA from both normal and mutant were sequenced, and the sequencing reactions electrophoresed through 6% (w/v) polyacrylamide as described in Chapter 2, section 2.6. Sequencing gels may be read in the order G, A, T, C from left to right. The indented nucleotides indicate the nucleotides which have been substituted.

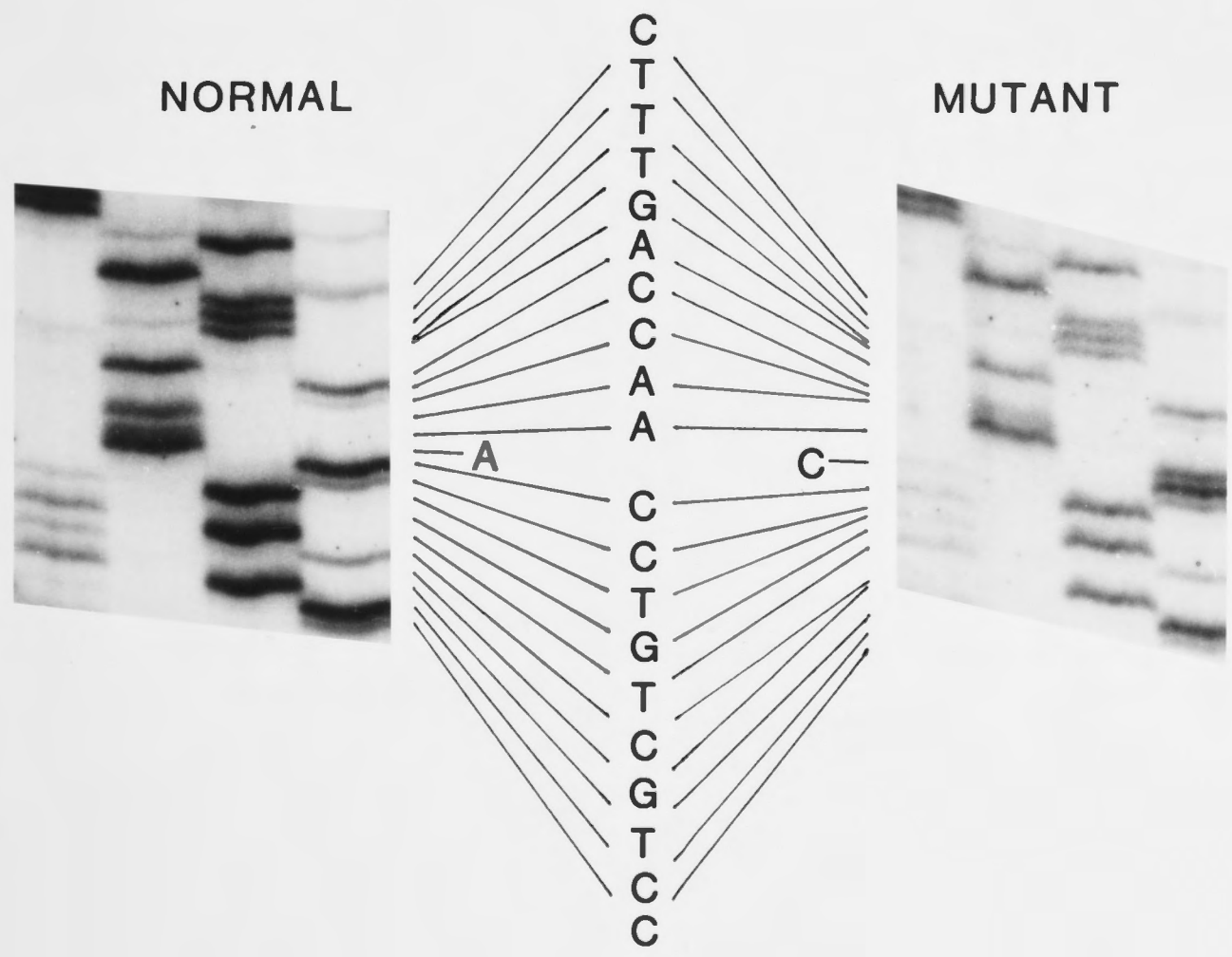
DNA SEQUENCE CHANGE IN UNCB573



TRIPLET CHANGE AAA ⟶ ATA

K203I

DNA SEQUENCE CHANGE IN UNCB567



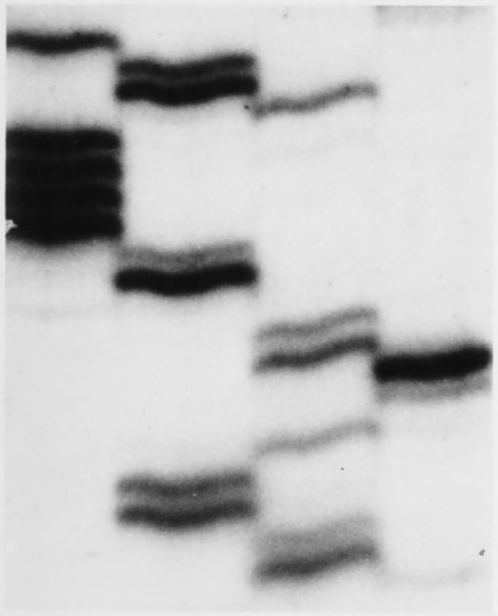
TRIPLET CHANGE AAA ⟶ CAA

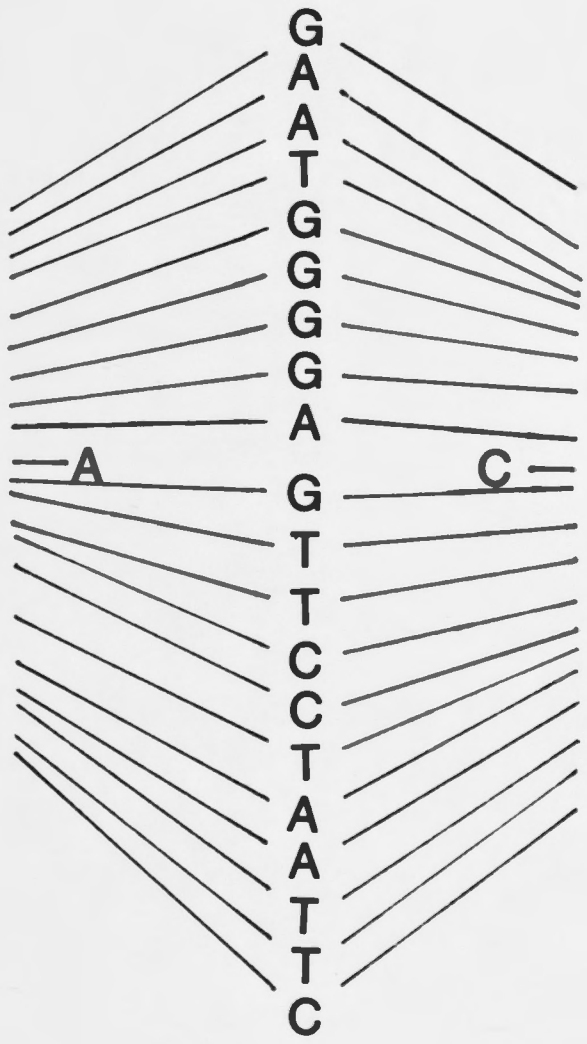
K203Q

Fig 6.2 Sections of DNA sequencing gels showing the *uncB 566*(E196A) nucleotide substitution. Samples of DNA from both normal and mutant were sequenced, and the sequencing reactions electrophoresed through 6% (w/v) polyacrylamide as described in Chapter 2, section 2.6. Sequencing gels may be read in the order G, A, T, C from left to right. The indented nucleotides indicate the nucleotide which has been substituted.

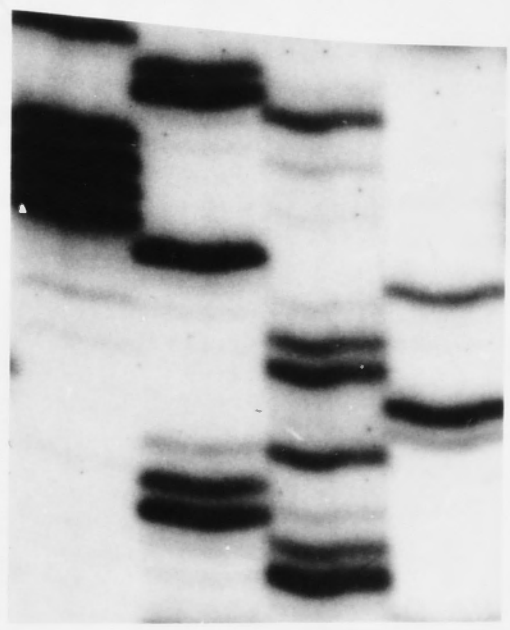
DNA SEQUENCE CHANGE IN UNCB566

NORMAL





MUTANT
UNCB566



TRIPLET CHANGE GAA ⟶ GCA

E196A

Fig 6.3 Nucleotide sequence of the *uncB* gene coding for the *E. coli* F_0F_1-ATPase subunit-*a*, together with the amino acid sequence. The sequence shown incorporates all the base substitutions in *uncB566*(E196A), *uncB567*(K203Q) and *uncB573*(K203I) with the replaced nucleotides underlined at positions 587, 607 and 608 respectively and the substituted amino acids printed in bold type.

```
 M  A  S  E  N  M  T  P  Q  D  Y  I  G  H  H  L  N  N  L
ATGGCTTCAGAAAATATGACGCCGCAGGATTACATAGGACACCACCTGAATAACCTT
         10         20         30         40         50
 Q  L  D  L  RL  T  F  S  L  V  D  P  Q  N  P  P  A  T  F
CAGCTGGACCTGCGTACATTCTCGCTGGTGGATCCACAAAACCCCCCAGCCACCTTC
 60         70         80         90         100        110
 W  T  I  N  I  D  S  M  F  F  S  N  N  L  G  L  L  F  L
TGGACAATCAATATTGACTCCATGTTCTTCTCGGTGGTGCTGGGTCTGTTGTTCCTG
   120        130        140        150        160        170
 V  L  F  R  S  V  A  K  K  A  T  S  G  V  P  G  K  F  Q
GTTTTATTCCGTAGCGTAGCCAAAAAGGCGACCAGCGGTGTGCCAGGTAAGTTTCAG
      180        190        200        210        220        2
 T  A  I  F  I  V  I  G  F  V  N  G  S  V  K  D  M  Y  H
ACCGCGATTGAGCTGGTGATCGGCTTTGTTAATGGTAGCGTGAAAGACATGTACCAT
30         240        250        260        270        280
 G  K  S  K  L  I  A  P  L  A  L  T  I  F  V  W  V  F  L
GGCAAAAGCAAGCTGATTGCTCCGCTGGCCCTGACGATCTTCGTCTGGGTATTCCTG
  290        300        310        320        330        340
 M  N  L  M  D  I  L  P  I  D  L  L  P  Y  I  A  E  G  V
ATGAACCTGATGGATTTACTGCCTATCGACCTGCTGCCGTACATTGCTGAACATGTA
     350        360        370        380        390        40
 L  G  L  P  A  L  R  N  N  P  S  A  D  V  N  V  T  L  S
CTGGGTCTGCCTGCACTGCGTGTGGTTCCGTCTGCGGACGTGAACGTAACGCTGTCT
0         410        420        430        440        450
 M  A  L  G  V  F  I  L  I  L  F  Y  S  I  Q  M  Q  G  I
ATGGCACTGGGCGTATTTATCCTGATTCTGTTCTACAGCATCAAAATGAAAGGCATC
 460        470        480        490        500        510
 G  G  F  T  K  E  L  T  L  Q  P  F  N  H  W  A  F  I  P
GGCGGCTTCACGAAAGAGTTGACGCTGCAGCCGTTCAATCACTGGGCGTTCATTCCT
    520        530        540        550        560        570

 V  N  L  I  L  A  G  V  S  L  L  S  QI  P  V  S  L  G  L
GTCAACTTAATCCTTGCAGGGGTAAGCCTGCTGTCCCTACCAGTTTCACTCGGTTTG
       580        590        600        610        620

 R  L  F  G  N  M  Y  A  G  E  L  I  F  I  L  I  A  G  L
CGACTGTTCGGTAACATGTATGCCGGTGAGCTGATTTTCATTCTGATTGCTGGTCTG
630        640        650        660        670        680
 L  P  W  W  S  Q  W  I  L  N  V  P  W  A  I  F  H  I  L
TTGCCGTGGTGGTCACAGTGGATCCTGAATGTGCCGTGGGCCATTTTCCACATCCTG
   690        700        710        720        730        740
 I  I  T  L  Q  A  F  I  F  M  V  L  T  I  V  Y  L  S  M
ATCATTACGCTGCAAGCCTTCATCTTCATGGTTCTGACGATCGTCTATCTGTCGATG
      750        760        770        780        790        8
 A  S  E  E  H
GCGTCTGAAGAACAT
00         810
```

pattern analysis of the plasmid preparations showed that all constructs containing the 4.4 kb base-substituted insert, carried the vector pAN174. A plasmid similar in size to pAN45 carrying the deleted vector and either of the two substitutions was retained and designated pAN415(K203Q, *uncB567*) or pAN437(K203I, *uncB573*)

These three plasmid preparations were used to independently transform strain AN727(*uncB402*) to chloramphenicol resistance. Colonies were isolated on nutrient agar containing chloramphenicol, and purified. Strains were designated as follows: AN2768(pAN413, carrying *uncB566*); AN2712(pAN437, carrying *uncB573*); AN2773(pAN415, carrying *uncB567*). For ease of reference, these strains will be referred to as AN2768(E196A); AN2712(K203I); AN2773(K203Q).

6.3. Growth characteristics and membrane properties of strains AN2768(E196A), AN2712(K203I) and AN2773(K203Q)

6.3.(i) Growth characteristics

Mutant strains were assayed along with their respective coupled and uncoupled control strains for their ability to utilise succinate minimal medium for growth, and for their growth yields on limiting levels of glucose (Tables 6.3 and 6.4). All three mutant strains were able to grow using succinate as sole carbon source and their growth yields were similar to their respective coupled control strains. To test the possibility that the substitutions may affect the stability of the complex, growth yields of mutant and control strains were also performed in limiting glucose at 42^{o}C. No difference in the yields was noted at the higher temperature.

6.3.(ii) ATPase activities

Subcellular fractions were prepared from the mutant and control strains. The ATPase activities of the cytoplasmic fractions in mutant and coupled control strains were negligible, whereas the cytoplasmic fraction from uncoupled control strain AN2736(pAN174/*uncB402*) had an activity of approximately 0.2 μmol.min^{-1}.mg protein^{-1}. Membrane-bound ATPase activities of the mutant strains were the same as the activities from the respective coupled control strains (Tables 6.3 and 6.4). ATPase activities after removal of the F_1-ATPase from the F_o-moiety by dialysis against a low ionic strength buffer in the absence of *p*-aminobenzamidine were not increased in preparations of any mutant or control

TABLE 6.2

SYNTHETIC OLIGONUCLEOTIDES USED IN THE PREPARATION AND SEQUENCE CONFIRMATION OF THE SUBUNIT-*A* SUBSTITUTIONS E196A, K203I AND K203Q.

Preparation of the oligonucleotides is detailed in Chapter 2, section 2.7.(i). The underlined base signifies a base substitution from the normal sequence.

Synthetic oligonucleotide	Relevant amino acid substitution	Notes
5'-TTAATCCTTG<u>C</u>AGGGGTAAG-3'	E196A	-
5'-CTGCTGTCCA<u>T</u>ACCAGTTTCA-3'	K203I	-
5'-CTGCTGTCC<u>C</u>AACCAGTTTCA-3'	K203Q	-
5'-GGGTCTGTTGTTCCTGGTTT-3'		Sequencing primer used for sequencing from nucleotide 475 in the *uncB* gene.
5'-GTAAAACGACGGCCAGT-3'		Phage universal primer which anneals onto the viral strand approximately 50 bases 5' from the *Hin*d III restriction site in the mp9 polylinker.

TABLE 6.3

GROWTH PROPERTIES AND MEMBRANE ENZYMIC ACTIVITIES OF MUTANT STRAINS AN2712(K203I), AN2773(K203Q), COUPLED AND UNCOUPLED CONTROL STRAINS.

Growth yields were measured as turbidities (Klett units) after growth on media containing 5mM glucose supplemented with 5% luria and chloramphenicol. The value shown is the average of at least three attempts. Properties of membrane preparations were determined as described in Chapter 2. Inhibition of ATPase activities were determined in 12 µg/ml DCCD. For further details of strains see either section 6.2.(ii) or tables 2.1 and 2.2.

RELEVANT PROPERTY	STRAIN			
	AN2712 (K203I)	AN2773 (K203Q)	AN2709 (*unc*$^+$)	AN2736 (*unc*$^-$)
Growth on succinate	+	+	+	-
Growth yield(Klett units)	226	223	228	143
ATPase activity ($\mu mol.min^{-1}.mg\ protein^{-1}$):				
Cytoplasmic	<0.1	<0.1	0.1	0.2
Membrane, Pre-dialysis	0.7	0.7	0.7	0.2
Membrane, Post-dialysis	0.7	0.7	0.7	0.2
DCCD inhibition of ATPase activity(%):	65	64	68	-
NADH-dependent atebrin fluorescence-quenching activity:				
% in intact membranes	82	82	84	85
% after removal of F_1-ATPase	14	16	18	84
ATP-dependent atebrin fluorescence-quenching activity:				
% in intact membranes	67	66	70	0
% after removal of F_1-ATPase	0	0	0	0

strain. Membrane-bound ATPase activities from mutant strains and coupled control strains exhibited similar levels of inhibition in the presence of DCCD (Table 6.3, Fig 6.4). However, the maximal inhibition was only attained in membranes from mutant strain AN2768(E196A) at relatively higher concentrations of the inhibitor (Fig 6.4).

6.3.(iii) Atebrin fluorescence-quenching activities.

The membrane preparations from the mutant and control strains were assayed for ATP- and NADH-dependent atebrin fluoresence-quenching activities both before and after removal of the F_1-ATPase. Quenching activities of membranes from the mutant strains AN2712(K203I), AN2773(K203Q) and the coupled control strain AN2709(pAN436/*uncB402*) were identical, with both NADH- and ATP-dependent fluorescence-quenching responses being lost after removal of the F_1-moiety (Table 6.3).

However, although the NADH-dependent fluorescence-quenching activities were similar in intact membranes from mutant strain AN2768(E196A) and coupled control strain AN2774(pAN45/*uncB402*), the ATP-dependent quenching response appeared to be extremely variable and biphasic (Fig 6.5). When assayed immediately after addition of membranes to the Hepes buffer the quenching response was low, approximately 20-30%, and appeared to be uniphasic (Fig 6.5 (a)). When the membrane preparation was preincubated in the buffer for a period of two minutes a second, slower, quenching response was noted. The rate of this second response increased with further incubation, until a quench similar in magnitude to the ATP-dependent quenching activity in membranes from the coupled control strain was achieved (Fig 6.5 (b-d)). However, the activity remained biphasic. Preincubation did not affect the quenching response in membranes from the coupled control strain AN2774(*unc*$^+$).

If the quenching activities of membranes from the mutant strain were assayed in Hepes buffer containing a concentration of the protease inhibitor *p*-aminobenzamidine similar to the concentration of the inhibitor in the membrane preparation (1 mg.ml^{-1}), the second phase of the quenching response was absent, and could not be regained by any period of preincubation. This treatment did not affect the quenching response in membranes from the coupled control strain AN2774(*unc*$^+$)(Fig 6.5 (f)).

A significant level of NADH-dependent quenching activity was retained in membranes from the mutant strain AN2768(E196A) after the F_1-ATPase had been removed by dialysis (Fig 6.5 (g)). The biphasic ATP-dependent quenching

TABLE 6.4

GROWTH PROPERTIES AND ATPASE ACTIVITIES OF MUTANT STRAIN AN2768(E196A), COUPLED AND UNCOUPLED CONTROL STRAINS.

Growth yields were measured as turbidities (Klett units) after growth on media containing 5 mM glucose supplemented with 5% luria and chloramphenicol. The value shown is the the average of at least three attempts. The subcellular preparations from the mutant and respective control strains were isolated simulataneously, and assayed as described in Chapter 2. Removal of the F_1-ATPase from the membranes was performed by dialysing the membranes against low ionic strength buffer in the absence of *p*-aminobenzamidine. For further details of strains see either section 6.2.(ii) or tables 2.1 and 2.2.

Bacterial strain (Plasmid)	Plasmid encoded amino acid substitution	Growth on succinate	Growth yield on 5mM glucose (Klett units)	ATPase activities (µmol.min-1.mg protein-1) Cytoplasmic	Membrane Pre-dialysis	Membrane Post dialysis
AN2768(pAN413)	E196A	+	228	<0.1	2.9	2.8
AN2774(pAN45)[a]	+	+	235	<0.1	3.1	2.9
AN2736(pAN174)[b]	-	-	144	0.2	0.2	0.2

[a] Coupled control strain
[b] Uncoupled control strain

Fig 6.4 Inhibition of ATPase activity by DCCD. Membranes (0.3 mg of protein) were incubated at 30°C in 5 ml of the assay mixture together with the indicated levels of DCCD. The mixture was sampled at intervals, the rate determined for each DCCD concentration and the percent inhibiton was calculated (*q.v.* Chapter 2, section 2.8.(iii)). Symbols: ◆, membranes from coupled control strain AN2774(pAN45/*uncB402*) ◇ ,membranes from mutant strain AN2768(E196A).

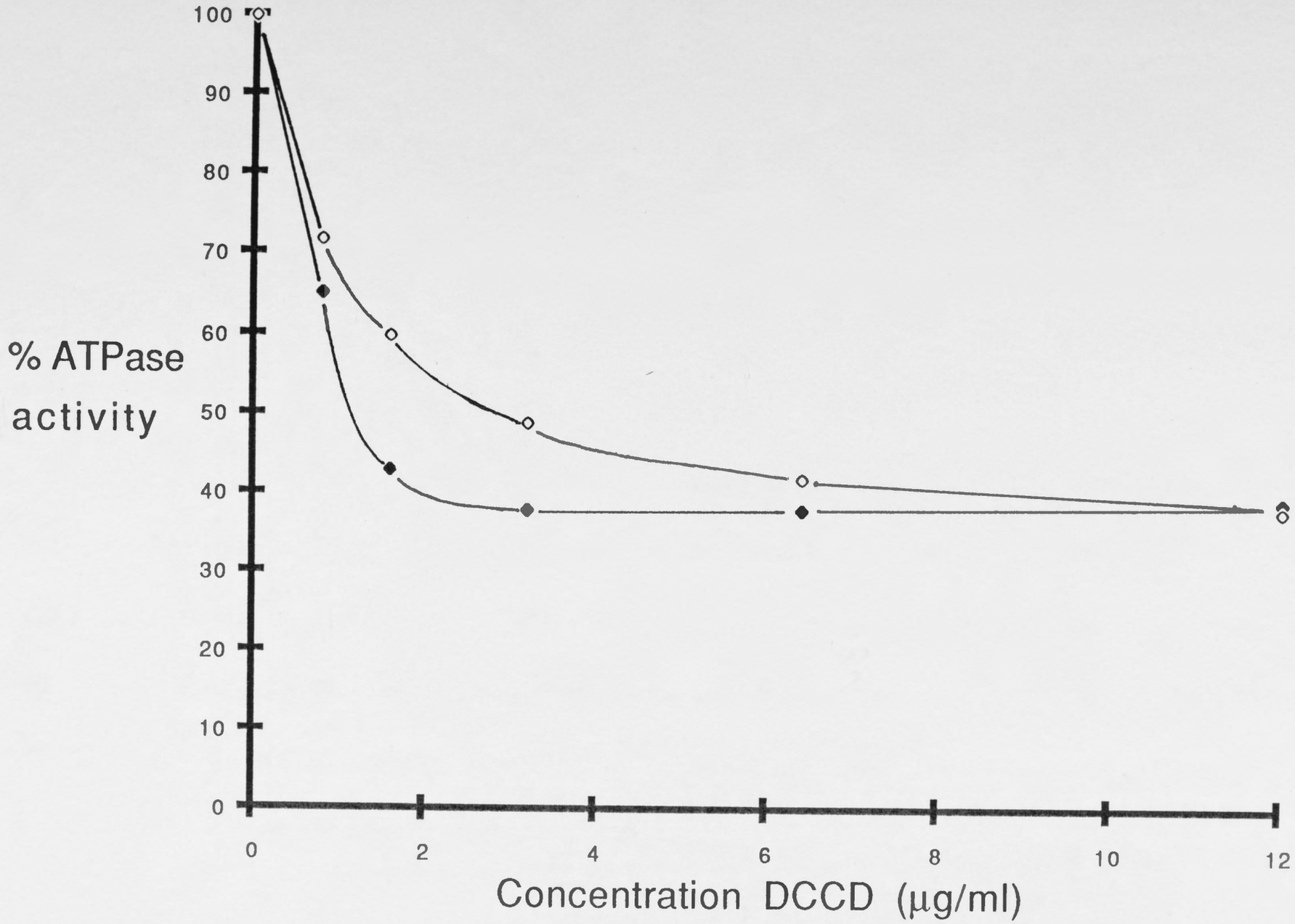
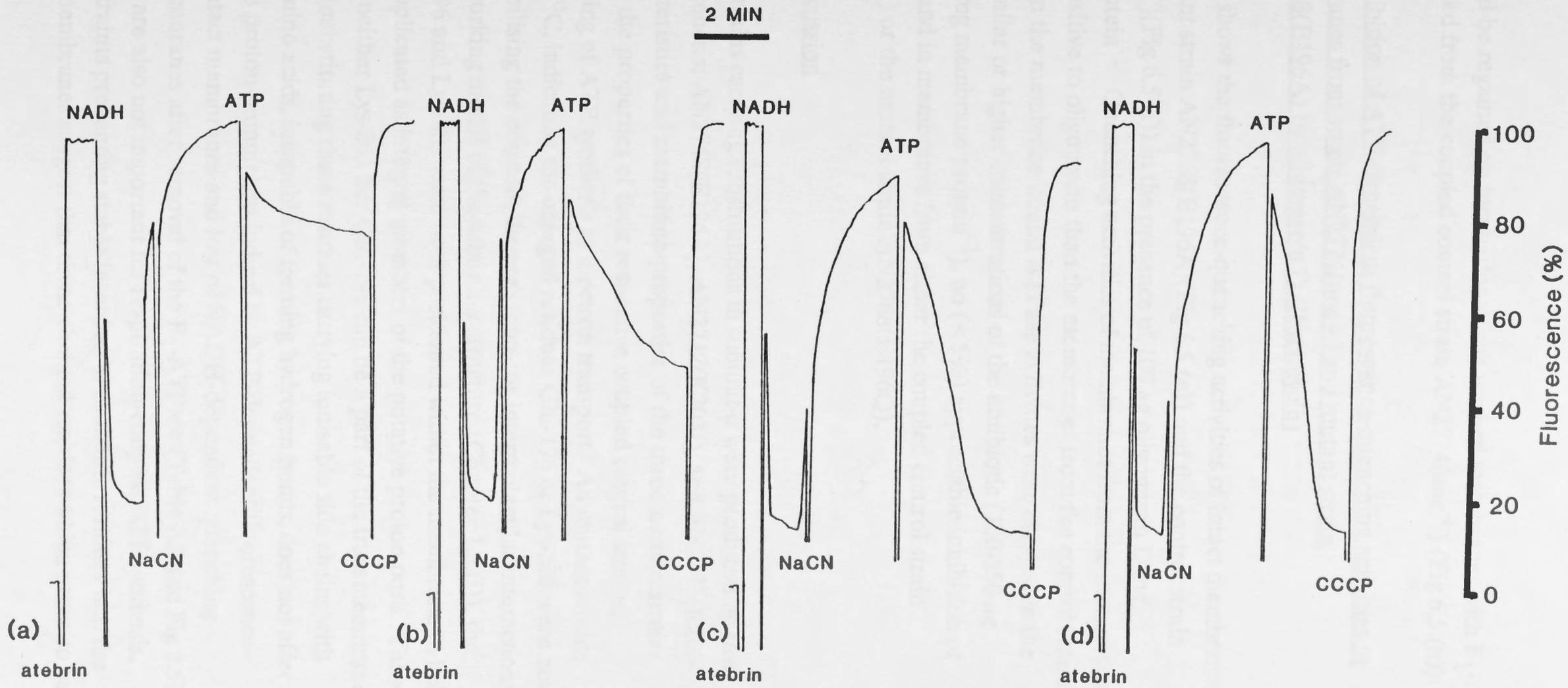

% ATPase activity
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Concentration DCCD (µg/ml)

Fig 6.5 (a-d) Atebrin fluorescence-quenching in membranes prepared from strain AN2768(E196A). Atebrin fluorescence-quenching activities were assayed as detailed in Chapter 2, section 2.8.(ii). Atebrin was added to give a final concentration of 4 μM, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to 2 μM. (a) membranes assayed immediately after addition to the assay buffer;(b) membranes assayed after a preincubation of 2 min in assay buffer; (c) membranes assayed after a preincubation of 4 min; (d) membranes after a ten minuted preincubation in assay buffer.


2 MIN
NADH
ATP
NADH
ATP
NADH
ATP
NADH
ATP
100
80
60
40
20
0
Fluorescence (%)
NaCN
CCCP
NaCN
CCCP
NaCN
CCCP
NaCN
CCCP
(a)
atebrin
(b)
atebrin
(c)
atebrin
(d)
atebrin

response could be regained on incubation of the washed membranes with F_1-ATPase isolated from the coupled control strain AN2774(*unc*$^+$) (Fig 6.5 (h))

6.4 Inhibition of ATP-dependent fluorescence-quenching activities in membranes from strain AN2774(unc+) and mutant strain AN2768(E196A) by oligomycin D (Rutamycin)

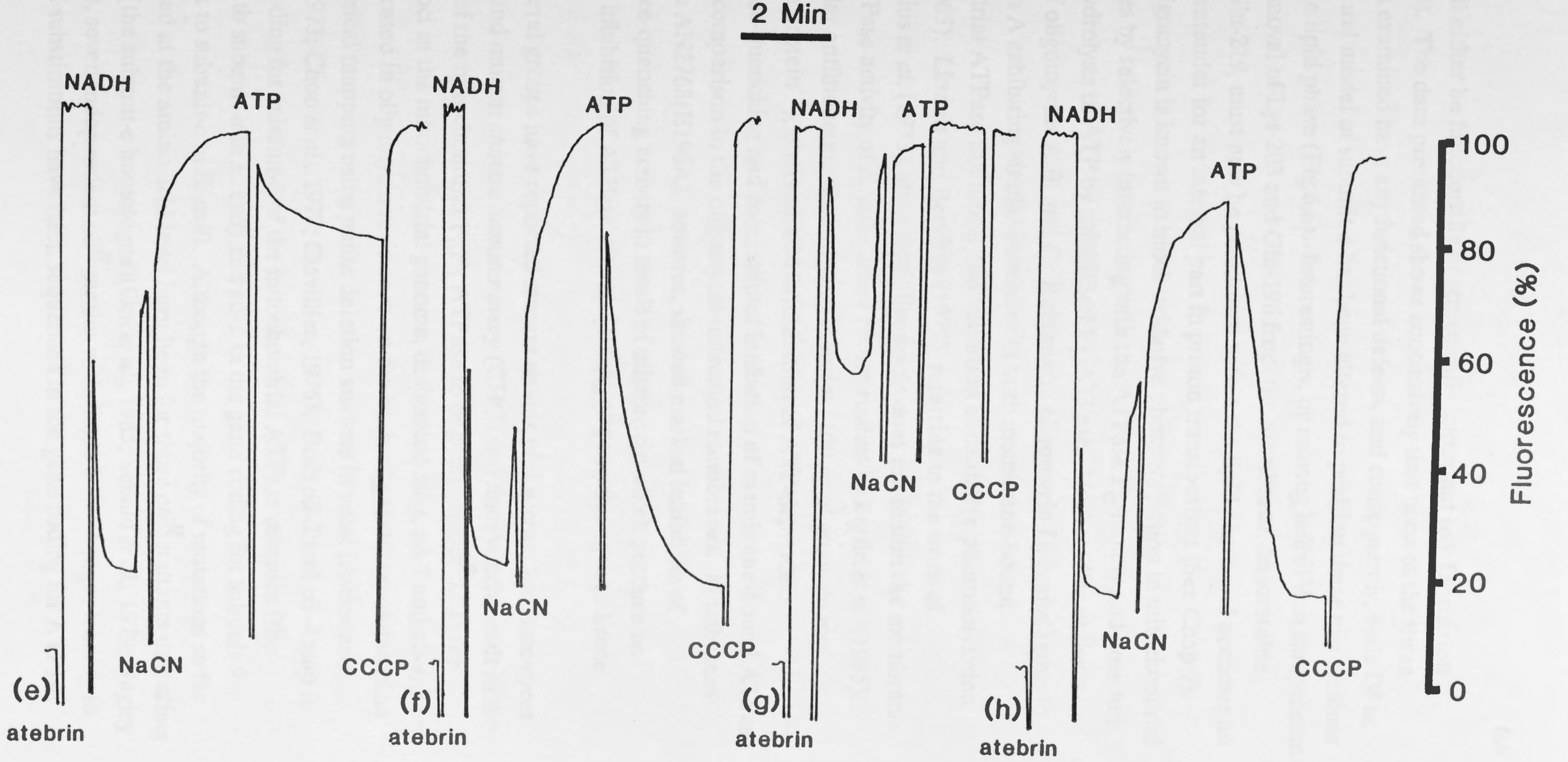
Fig 6.5 shows the fluorescence-quenching activities of intact membranes from the mutant strain AN2768(E196A)(Fig 6.5 (e)) and the control strain AN2774(*unc*$^+$)(Fig 6.5 (f)) in the presence of 100 μg oligomycin D.mg membrane protein^{-1}. Quenching activities of membranes from the mutant strain were more sensitive to oligomycin than the membranes from the control strain. However, when the membrane bound ATPase activities were assayed in the presence of similar or higher concentrations of the antibiotic (100-150 μg oligomycin D.mg membrane protein^{-1}), no (<5%) appreciable inhibition of activity was found in membranes from either the coupled control strain AN2774(*unc*$^+$) or the mutant strain AN2768(E196Q).

6.5. Discussion

Three strains carrying substitutions in subunit-*a* were produced by site-directed mutagenesis; AN2768(E196A), AN2712(K203I), and AN2773(K203Q). Growth characteristics and membrane-properties of the three mutant strains were similar to the properties of their respective coupled control strains, retaining coupling of ATP synthesis to electron transport. All mutant strains grew well at 42^{o}C, indicating the charged residues Glu-196 or Lys-203 were not involved in stabilising the complex through intra- or intermolecular interactions.

In the working model of the subunit-*a* structure (Chapter 1.5.(i)), the residues Glu-196 and Lys-203 were both positioned within the membrane in helix IV and were implicated as integral members of the putative proton pore. These data show that neither Lys-203 nor Glu-196 can be a part of the transmembrane proton pore, as substituting these residues carrying ionisable side chains with hydrophobic amino acids, incapable of forming hydrogen bonds, does not affect transmembrane proton-pumping as judged by ATP-dependent fluorescence-quenching in intact membranes and loss of NADH-dependent quenching activities in membranes after removal of the F_1-ATPase (Table 6.2 and Fig 6.5). These residues are also not important for respiration-coupled ATP synthesis. Energetic constraints preventing stable insertion of ionisable residues into the lipid phase of membranes suggest that those charged residues which prove to be

Fig 6.5 (e-h) Atebrin fluorescence-quenching in membranes prepared from strains of *E. coli*. Atebrin fluorescence-quenching activities were assayed as detailed in Chapter 2, section 2.8.(ii). Atebrin was added to give a final concentration of 4 μM, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to 2 μM. (e) membranes from strain AN2768(E196A) assayed in the presence of 100 μg oligomycin D. mg membrane protein $^{-1}$; (f) membranes from coupled control strain AN2774(*unc*$^{+}$) assayed in the presence of 100 μg oligomycin D. mg membrane protein $^{-1}$; (g) stripped membranes from mutant strain AN2768(E196A); (h) stripped membranes from mutant strain AN2768(E196A) assayed after a 15 min preincubation in the assay buffer with 100 μg of F_1-ATPase purified from strain AN2774(*unc*$^{+}$).


2 Min
NADH
ATP
NADH
ATP
NADH
ATP
NADH
ATP
100
80
60
40
20
0
Fluorescence (%)
NaCN
CCCP
NaCN
CCCP
NaCN
CCCP
NaCN
CCCP
(e)
atebrin
(f)
atebrin
(g)
atebrin
(h)
atebrin

buried will either be functionally or structurally important (*q.v.*Discussion Chapter 3). The data presented shows conclusively that none of the three mutations examined have any functional defects, and consequently, helix IV of the structural model of subunit-*a* has been altered to position these two residues outside the lipid phase (Fig 6.6). Interestingly, by moving helix IV to compensate for the removal of Lys-203 and Glu-196 from the membrane, an ionisable residue, Glu-219, must now be positioned within the lipid phase and becomes an obvious contender for an integral part in proton translocation (See Chap 7).

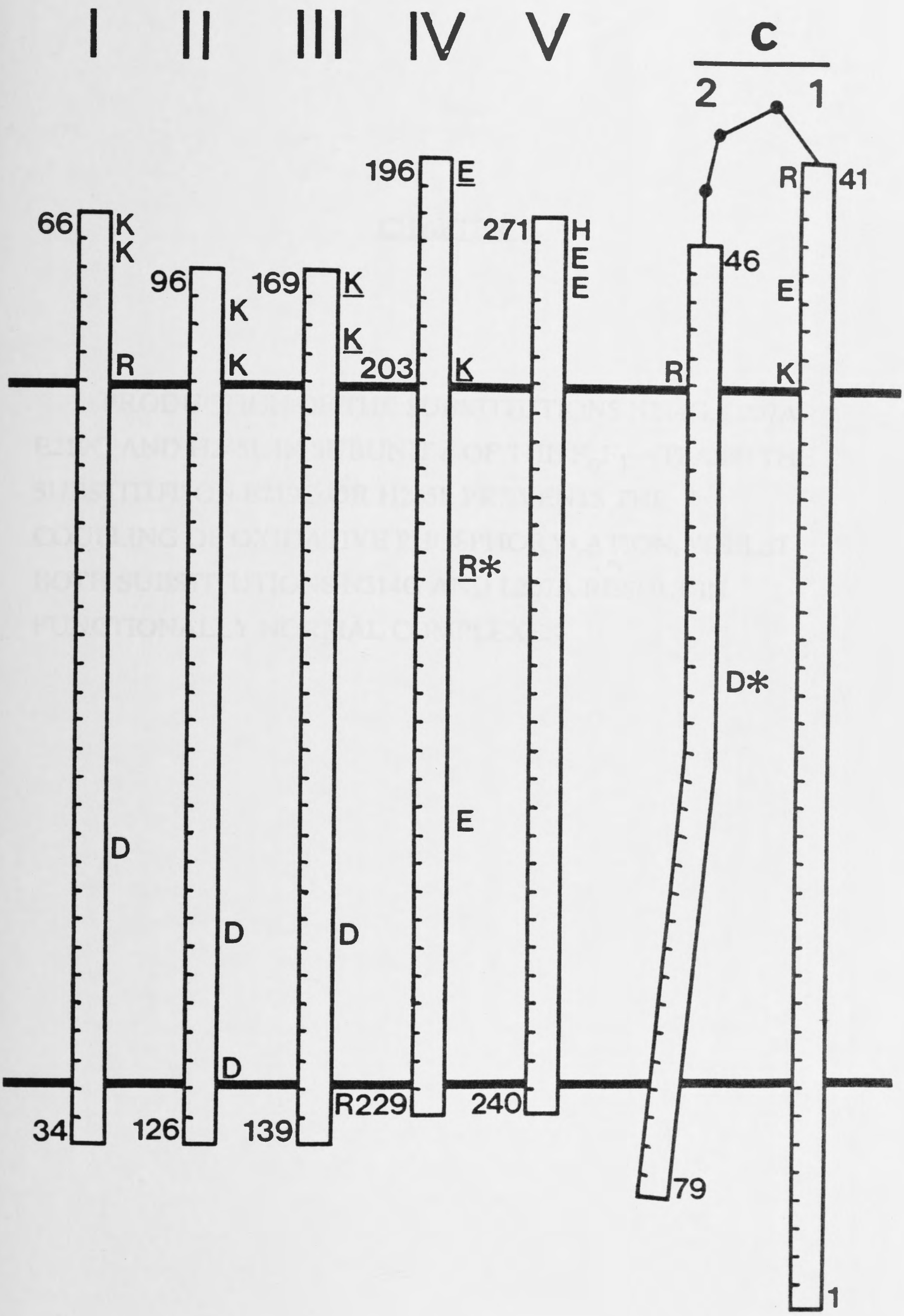
Oligomycin is known to block oxidative phosphorylation in mitochondrial membranes by selectively interacting with the ATPase F_o-moiety and does not prevent hydrolysis of ATP by solubilised F_1-ATPase. Most studies utilise a mixture of oligomycins A,B, and C. Rutamycin, oligomycin D, is analogous to oligomycin A exhibiting similar potencies in both membrane-bound mitochondrial ATPase inhibition and inhibition of oxidative phosphorylation (Lardy, 1965). Linnett and Beechey (1979), referring to the work of Nieuwenhius *et al.* (1973), state that oligomycin does not inhibit the membrane-bound ATPase activity of *E. coli*. More recent studies by Perlin *et al.* (1985), using similar antibiotic:membrane protein ratios (100 μg oligomycin.mg membrane protein^{-1}), oberved a 30% inhibition of ATP-dependent fluorescence-quenching and concomitant inhibition of membrane-bound ATPase activity in comparison to the oligomycin-untreated membranes. Membranes from strain AN2768(E196A), however, showed marked inhibition of fluorescence-quenching activity in levels of oligomycin which produce no detectable inhibition of ATPase activity (100 μg oligomycin.mg membrane protein^{-1}).

Several groups have reported mutant strains of the yeast *Saccharomyces cerevisiae*, and mutant *chinese hamster ovary* (CHO) cell lines which result in the resistance of the mitochondrial F_oF_1 ATPase to oligomycin (*oli*^{R}). Three potential loci in the mitochondrial genome, designated *oli-1*, *oli-2* and *oli-4*, have been implicated in oligomycin resistance on the basis of genetic recombination and on physical mapping using petite deletion analysis in yeast (Avner and Griffiths, 1973; Choo *et al.*, 1977; Clavillier, 1976). Both *oli-2* and *oli-4* map in the gene coding for subunit-*6* of the mitochondrial ATPase complex (the equivalent to subunit-*a* in *E. coli*) and *oli-1* in the gene coding for subunit-*9* (equivalent to subunit-*c* in *E. coli*). Although the majority of mutations so far characterised at the amino acid level have been for those *oli*^{R} mutants that affect subunit-*9*, (the subunit-*c* homologue)(Ooi *et al.*, 1985; Sebald *et al.*, 1979; Nagley *et al.*, 1986), seven independent *oli*^{R} strains with single base changes leading to amino-acid substitutions have been sequenced in the gene coding for ATPase

subunit-*6* in yeast mitochondria (the subunit-**a** homologue)(John, U.P. and Nagley, P. 1986). Interestingly, of these seven, four substitutions concern Ser-175 which corresponds to Ser-199 in subunit-*a* of the *E. coli* F_0F_1-ATPase, and two involve substitutions of Ile-171, corresponding to Leu-195 in the *E. coli* homologue. Both residues are in close proximity to Glu-196 in the *E. coli* subunit-*a*. None of these mutants are markedly affected in oxidative phosphorylation.

Breen *et al.* (1986), produced two mutant *CHO* cell lines which were resistant to oligomycin. Sequence analysis identified an identical single base substitution in the gene coding for the ATPase subunit-*6* in both mutant cell lines, predicting the nonconservative amino-acid change Glu-145 to lysine. This residue, essential for oligomycin sensitivity in *CHO* mitochondrial ATPase corresponds to Glu-196 in subunit-*a* of the *E. coli* F_0F_1-ATPase. Although the mutation caused resistance to oligomycin, the cell lines carrying the oli^R had comparable ATPase activities to the normal control strain with similar levels of both ATP-Pi exchange and growth in galactose-containing medium where approximately 98% of the cells energy is derived from oxidative phosphorylation (Reitzer *et al.*, 1979). Thus, the discovery that a mutation at position-145 in *CHO* cell lines that does not affect the coupling of phosphorylation with electron transport is consistent with the data shown for strain AN2768(E196A) which carries a mutation in the comparable position. However, the substitution of Glu-196 by Ala in subunit-*a* of the *E. coli* ATPase results in a complex which is more sensitive to the antibiotic than the normal complex. As is detailed above, membranes from this mutant strain exhibit inhibition of ATP-dependent fluorescence-quenching activity without a concomitant inhibition of membrane-bound ATPase activity. No appreciable increase in NADH-dependent quenching was noted on assaying the dialysed membranes after treatment with the antibiotic, suggesting the inhibition may not be due to the oligomycin preventing proton translocation by interacting with the F_0-moiety, as occurs in mitochondrial ATPase inhibition (Lardy, 1980). Rutamycin or *p*-aminobenzamidine may marginally perturb the integrity of the structure of the complex in the absence of glutamate at position -196 of subunit-*a*. However, although the exact role of Glu-196 in the F_0F_1-ATPase remains unclear, a glutamate residue at position 196 is not required for oxidative phosphorylation.

Fig 6.6. Diagrammatic representation of the five predicted intramembranous helices of subunit-*a* and the two intramembranous helices of subunit-*c*. The number of the residue at both N- and C-terminals of each putative helix are given. The thick horizontal line towards the top of the page represents the membrane/cytoplasm border, whilst the lower thick line represents the membrane/periplasm border. Every ionisable residue predicted to be within the membrane is marked and all residues which have been substituted are underlined. An asterisk next to a residue denotes the residue is involved in proton translocation. Helix IV has been repositioned with respect to the membrane, with residues Lys-203 and Glu-196 being placed outside the membrane in accord with results documented in Chapter 6.


I
II
III
IV
V
c
2
1
66
K
K
R
96
K
K
169
K
K
203
196
E
K
R*
271
H
E
E
R
46
R
41
E
K
D*
D
D
D
D
E
R229
240
34
126
139
79
1

CHAPTER 7

PRODUCTION OF THE SUBSTITUTIONS N214G, L207A, E219Q AND H245L IN SUBUNIT-*a* OF THE F_oF_1-ATPASE: THE SUBSTITUTION E219Q OR H245L PREVENTS THE COUPLING OF OXIDATIVE PHOSPHORYLATION, WHILST BOTH SUBSTITUTIONS N214G AND L207A RESULT IN FUNCTIONALLY NORMAL COMPLEXES

7.1 Introduction

Data outlined in the previous three chapters have aided in the structural realignment of subunit-*a* (See Fig 6.6). Removal of Glu-196 and Lys-203 from the membrane, and relocation of Glu-219 intramembranously as a component of the amphipathic helix IV suggests a functional role for this residue, possibly as an essential member of the proton pore. Although a glutamate residue is not conserved at the comparable position in any mitochondrial F_oF_1-ATPase subunit-*6* from species sequenced to date, a histidine residue is conserved (Cain and Simoni, (1986)). Further analysis of the subunit-*a* primary sequence from *E. coli*, revealed a histidine residue at position 245, whereas in mitochondrial sequences a single glutamate is conserved in a similar position. Thus, perhaps the glutamate and histidine are involved in a functionally important charge pair.

This possibility was suggested by Cain and Simoni (1986), after isolating two independent H245Y mutations produced by hydroxylamine mutagenesis. The H245Y substitution was transferred to the chromosome and the resultant strain(BC2001) exhibited an impaired growth yield on limiting glucose. Membrane preparations of strain BC2001 were impermeable to protons as judged by ATP- and NADH- dependent 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence-quenching, and membrane-bound ATPase activities were similar to the levels documented for the coupled control strain, apparently also exhibiting similar levels of sensitivity to the inhibitor DCCD.. A second paper (Vik and Simoni, 1987) confirmed the normal assembly of an F_oF_1-ATPase complex containing the subunit-*a* substitution H245Y, as judged by immunoprecipitation of the complex with antibodies to F_1-ATPase, and determining the relative proportions of F_o subunits bound to the complex.

To investigate the possible Glu-219/His-245 charge-pair in subunit-*a*, and to assess whether the phenotypic effects produced on substitution of His-245 were due to loss of the histidine *per se* or to the introduction of a tyrosine residue, the amino-acid residue His-245 in subunit-*a* was replaced by the hydrophobic residue leucine, and Glu-219 by the polar residue, glutamine.

Two other subunit-*a* substitutions were also attempted. These were the substitutions of Leu-207 by Ala, and Asn-214 by Gly. An asparagine residue and a leucine residue at comparable positions to Asn-214 and Leu-207 of the *E. coli* F_oF_1-ATPase subunit-*a* are found in the primary sequence of the mitochondrial ATPase-*6* subunits from diverse species, chloroplast *CF_oIV* from spinach, and the subunit-*a* homologue in the cyanobacterium *Synechococcus 6301*. Table 7.1 shows the amino acid sequences for the subunit-*a* homologues, with residues corresponding to *E.coli* subunit-*a* residues Leu-207, Asn-214 and Glu-219

TABLE 7.1

COMPARISON OF THE AMINO-ACID SEQUENCES OF THE PUTATIVE HELIX IV OF SUBUNIT-*A* (ATPase *6*) FROM SEVERAL SPECIES

Human	I	I	E	T	I	S	L	L	I	Q	P	M	A	**L**	A	V	R	L	T	A	**N**	I	T	A	G	**H**	L	L
Bovine	I	I	E	T	I	S	L	F	I	Q	P	M	A	**L**	A	V	R	L	T	A	**N**	I	T	A	G	**H**	L	L
Rat[a]	I	I	E	T	I	S	L	F	I	Q	P	M	A	**L**	A	V	R	L	T	A	**N**	I	T	A	G	**H**	L	L
X. laevis	I	I	E	T	I	S	L	F	I	R	P	L	A	**L**	G	V	R	L	T	A	**N**	L	T	A	G	**H**	L	L
D. melanogaster	C	I	E	T	I	S	N	I	I	R	P	G	T	**L**	A	V	R	L	T	A	**N**	M	I	A	G	**H**	L	L
T. glabrata	V	I	E	L	L	S	Y	V	A	R	A	F	S	**L**	G	L	R	L	S	A	**N**	I	F	S	G	**H**	L	L
S. cerevisiae	I	I	E	T	L	S	Y	I	A	R	A	I	S	**L**	G	L	R	L	G	S	**N**	I	L	A	G	**H**	L	L
A. nidulans	L	I	E	F	I	S	Y	L	S	R	N	V	S	**L**	G	L	R	L	A	A	**N**	I	L	S	G	**H**	M	L
N. crassa	L	I	E	F	I	S	Y	L	A	R	N	I	S	**L**	G	L	R	L	A	A	**N**	I	L	S	G	**H**	M	L
Synechococcus.6301	P	F	K	I	L	E	D	F	T	K	P	L	S	**L**	S	F	R	L	F	G	**N**	I	L	A	D	**E**	L	V
Chloroplast[b]	P	I	N	I	L	E	D	F	T	K	P	L	S	**L**	S	F	R	L	F	G	**N**	I	L	A	D	**E**	L	I
Esch. coli	I	L	E	G	V	S	L	L	S	K	P	V	S	**L**	G	L	R	L	F	G	**N**	M	Y	A	G	**E**	L	I
														207[c]							214					219		

a sequence is identical to subunit-6 sequence of chinese hamster and mouse.
b Spinach chloroplast CF_0IV
c Numbers correspond to the amino acid sequence for *Esch. coli*

presented in bold print. Conserved residues are underlined. The working hypothesis for proton translocation detailed in Fig 6.6, positions the subunit-*a* residue Asn-214 in the middle of the membrane, adjacent to the ring of subunit-*c* Asp-61 residues. The Asn-214 residue of subunit-*a* was originally postulated to be a component of the transmembranous proton wire (*q.v.* Chapter 1, section 1.5.(i)), acting via the hydrogen-bonding facility of the amide group. Positioning Asn-214 of subunit-*a* adjacent to the *c*-subunit Asp-61 residues demands that the asparagine is not functionally involved in translocating protons. If the asparagine residue is essential at position 214, the working hypothesis cannot be correct, as the postulated proton wire then bypasses the Asp-61 subunit-*c* residue, effectively enabling subunit-*a* helix IV to function independently as a protonophore, dissipating any membrane potential. This theory was challenged by substituting residue Asn-214 for the hydrophobic residue, glycine.

The subunit-*a* residue Leu-207 is positioned, in the working hypothesis, near the membrane-cytoplasmic interface (Fig 6.6). The substitution of a smaller hydrophobic residue for Leu-207 may affect the close packing arrangement of the helices in the quaternary structure of the complex, perhaps causing some displacement of the side chains responsible for proton translocation in a manner similar to that proposed for the L31F substitution in subunit-*c* (Cox *et al.*, 1983). An attempt was therefore made to substitute the subunit-*a* residue Leu-207 by the small, hydrophobic residue, alanine.

7.2 Production of the subunit-*a* substitutions N214G,L207A, H245L and E219Q

7.2.(i) Mutagenesis

Mutants were prepared using the standard double priming technique detailed in chapter 3, section 3.3, with single-stranded template prepared from cells transformed by the phage replicative form pAN461, and screened using the differential-temperature hybridisation method. The relevant [γ-^{32}P] labeled oligonucleotide was used to probe the filters, which were then washed until potential mutants were clearly visible (45^{o}C for 1.5 min). Phage carrying the possible base substitution(s) were isolated, and the nucleotide sequence around the mutation site determined. After confirmation of the correct base substitution(s), the oligonucleotide used to introduce the substitution(s) was utilised as a sequencing primer, and phage exhibiting a single sequencing pattern were retained. However, the 21 base oligonucleotide containing the base substitution coding for E219Q, competently annealed to the complementary

TABLE 7.2

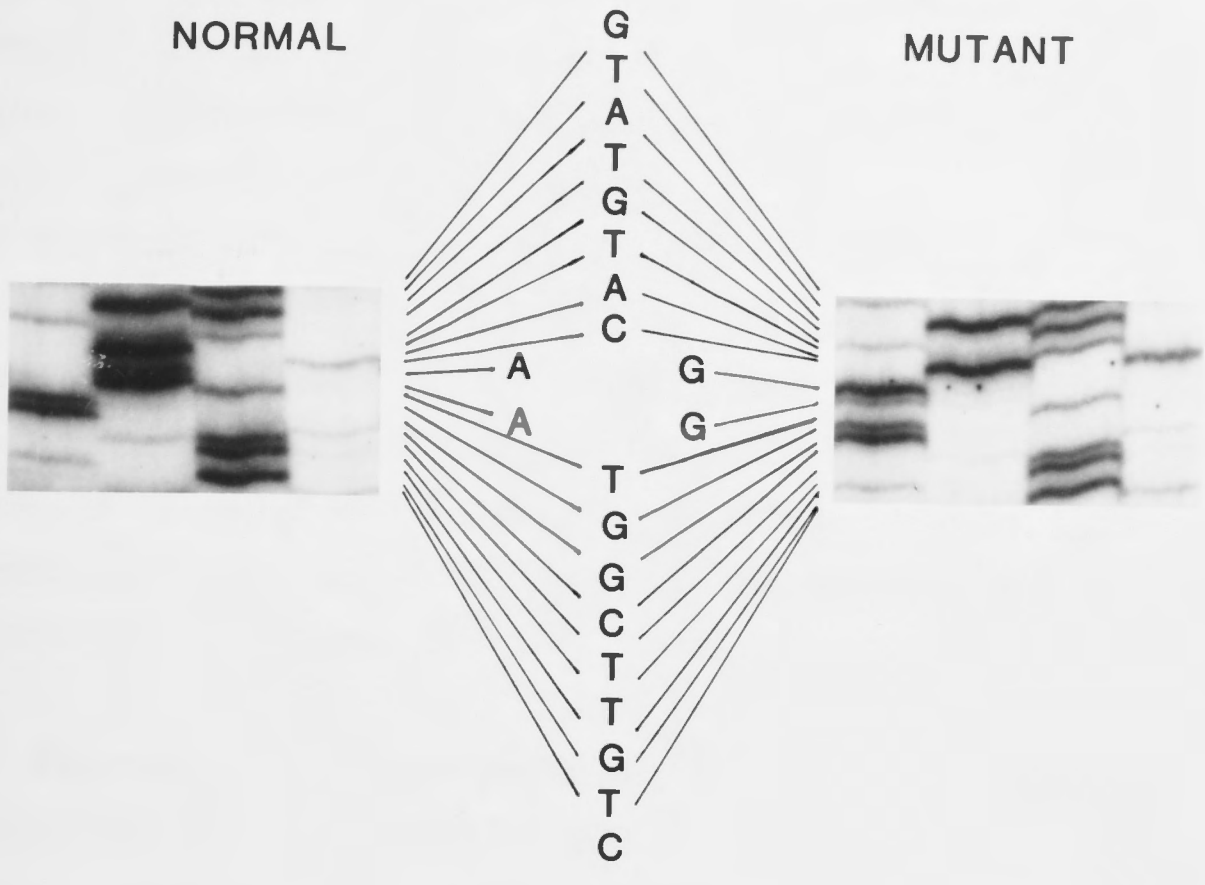
SYNTHETIC OLIGONUCLEOTIDES USED IN THE PREPARATION AND SEQUENCE CONFIRMATION OF THE SUBUNIT-*A* SUBSTITUTIONS N214G, L207A, H245L AND E219Q.

Preparation of the oligonucleotides is detailed in Chapter 2, section 2.7.(i). The base which is underlined signifies a base substitution from the normal
sequence.

Synthetic oligonucleotide	Relevant amino acid substitution	Notes
5'-CTGTTCGGTGGCATGTATGC-3'	N214G	
5'-CCAGTTTCAGCCGGTTTGCG-3'	L207A	
5'-CCATTTTCCTCATCCTGAT-3'	H245L	
5'-CATGTATGCCGGTCAGCTGATTTTCATTCTG-3'	E219Q	
5'-ATGGCACTGGGCGTATTTA-3'		Sequencing primer used for sequencing from nucleotide 475 in the *uncB* gene.
5'-GTAAAACGACGGCCAGT-3'		Phage M13 universal primer which anneals onto the viral strand approximately 50 bases 5' from the *Hin*d III restriction site in the mp9 polylinker.

Fig. 7.1. Sections of DNA sequencing gels showing the *uncB568*(N214G) and *uncB582*(L207A) nucleotide substitutions. Samples of DNA from both normal and mutant were sequenced and the sequencing reactions electrophoresed through 6% (w/v) polyacrylamide as described in Chapter 2, section 2.6. Sequencing gels may be read in the order G, A, T, C from left to right. The indented nucleotides indicate the nucleotides which have been substituted.

DNA SEQUENCE CHANGE IN UNCB568

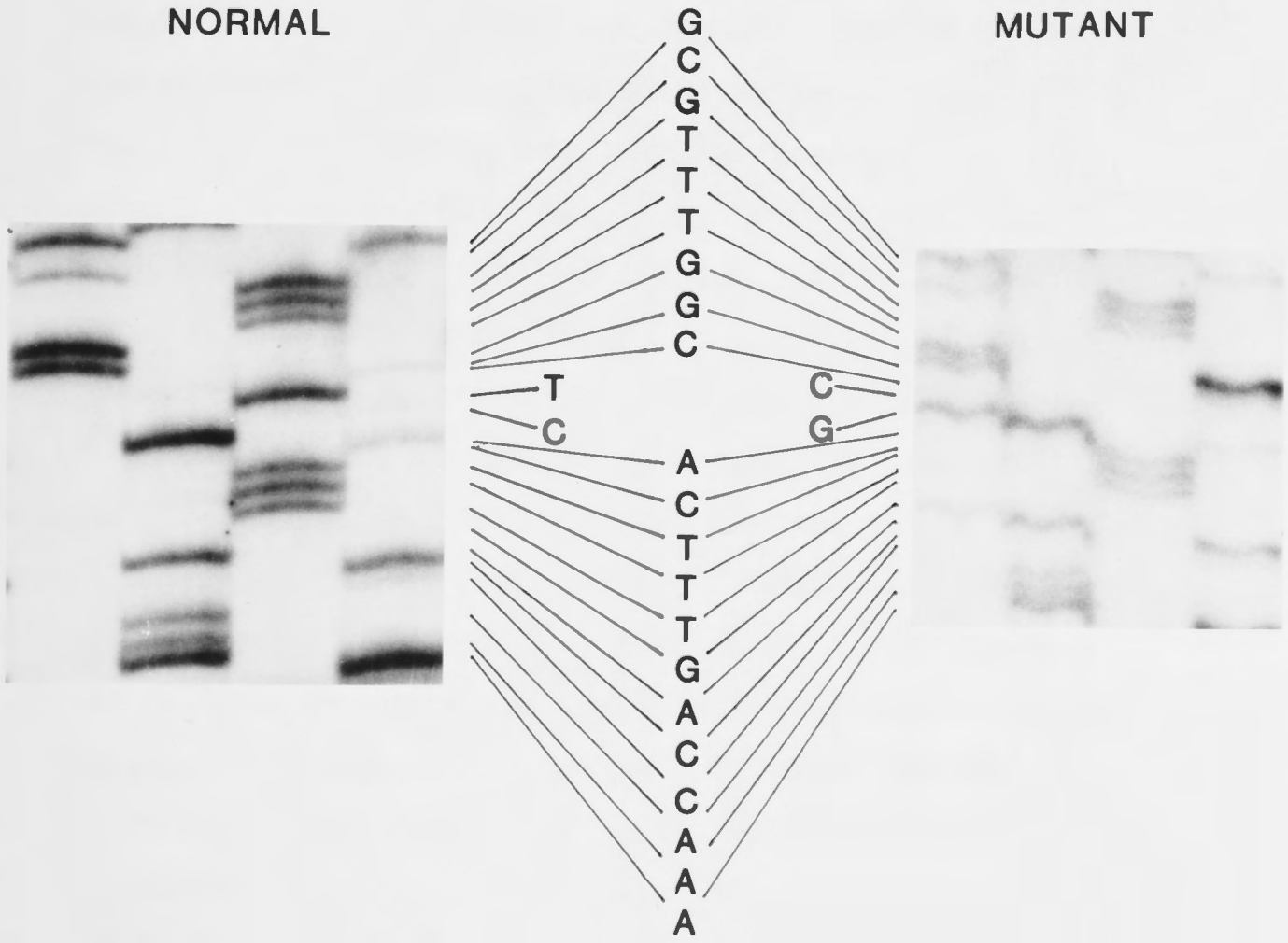


TRIPLET CHANGE

AAC ⟶ GGC

N214G

DNA SEQUENCE CHANGE IN UNCB582



TRIPLET CHANGE

CTC ⟶ GCC

L207A

sequence in the single stranded template, but unfortunately proved to be 'promiscuous', annealing to at least one other site in the template. Computer search programs failed to indicate any region within the template which had higher than 60% homology to the synthetic oligomer.

This problem was successfully circumvented by synthesising a longer oligonucleotide, maintaining the same base substitution. Sequencing gels showing the base substitutions for each of the four mutagenesis experiments are shown in Figs 7.1, 7.2 and 7.3. Oligonucleotides used to produce the substitutions are shown in Table 7.2 with the substituted bases underlined. Each base replacement is highlighted in Fig 7.4 with the altered bases underlined and the substituted amino acids in bold type.

7.2.(ii) Production of plasmids pAN416(N214G), pAN456(L207A), pAN451(H245L) and pAN467(E219Q)

The mutant derivatives of pAN461 carrying the N214G, L207A and H245L substitutions were each mixed with pAN36 and vector pAN174. These mixtures were then cleaved with nuclease *Hin*d III and ligated with T4 DNA ligase at 20^{o}C for 12 hours. Strain AN1273(*uncG428*) was again used as the recipient strain for the transformation experiments as only those plasmids which carried the complete *unc* operon would be able to transform the strain to growth on succinate (*q.v.* section 3.3.(iv)). After transformation with each of the ligation mixes, twelve colonies which were able to utilise succinate as their sole carbon source in the presence of chloramphenicol were purified from each transformation mix and their plasmids isolated. After analysis of the plasmid preparations, a plasmid similar in size to pAN45 carrying the deleted vector was retained carrying one of each of the three subunit-*a* substitutions.

As the plasmid carrying the subunit-*a* substitution was to be used to transform strain AN727(*uncB402*), it was not necessary to form a plasmid carrying the *unc* operon *in toto*. Thus, the phage derivative carrying the E219Q substitution was mixed with vector pAN174, cleaved with nuclease *Hin*d III, and ligated with T4 DNA ligase. The ligation mix was used to transform strain AN2015(*uncH243*) to chloramphenicol resistance, and several colonies which were able to grow on succinate were purified and their plasmids isolated. A plasmid identical in size and nuclease *Hin*d III-generated restriction pattern to plasmid pAN51 was retained.

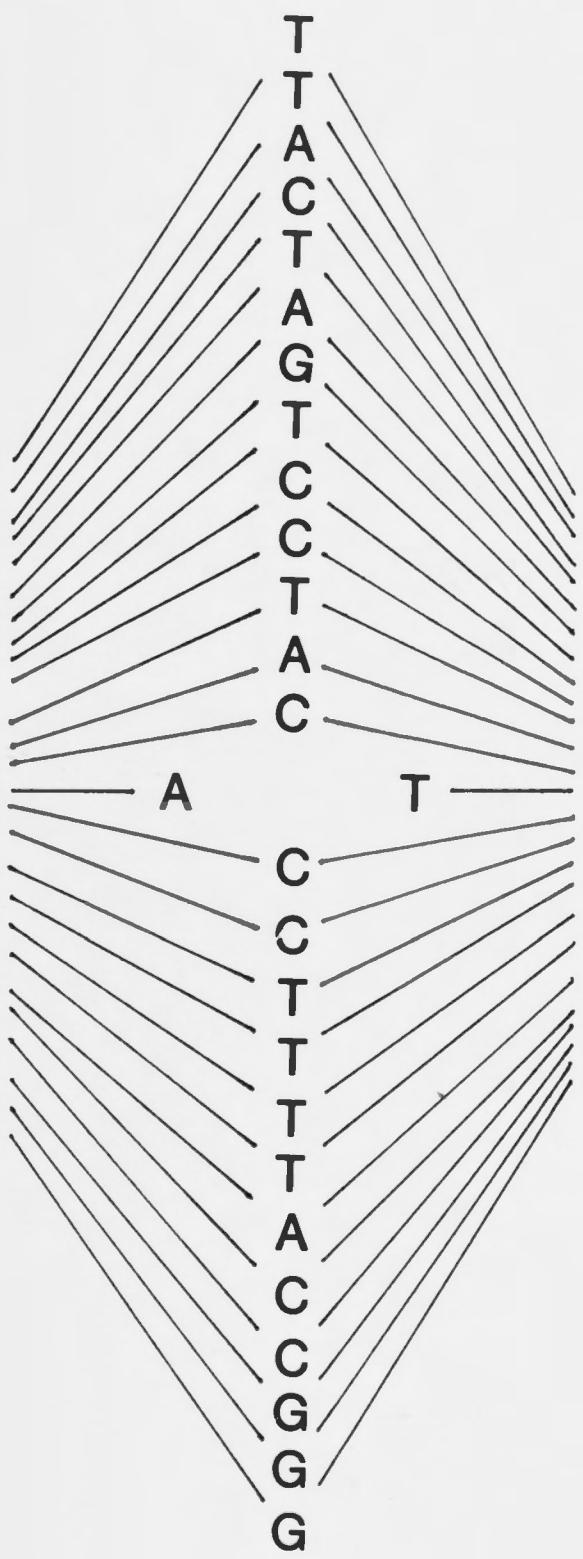
Plasmids carrying one of each of the four subunit-*a* amino acid substitutions were used to transform strain AN727(*uncB402*) to chloramphenicol resistance. One colony from each of the transformation mixes was purified and

Fig. 7.2. Sections of DNA sequencing gels showing the *uncB577*(H245L) nucleotide substitution. Samples of DNA from both normal and mutant were sequenced and the sequencing reactions electrophoresed through 6% (w/v) polyacrylamide as described in Chapter 2, section 2.6. Sequencing gels may be read in the order G, A, T, C from left to right. The indented nucleotides indicate the nucleotide which have been substituted.

DNA SEQUENCE CHANGE IN UNCB577

NORMAL

MUTANT



TRIPLET CHANGE

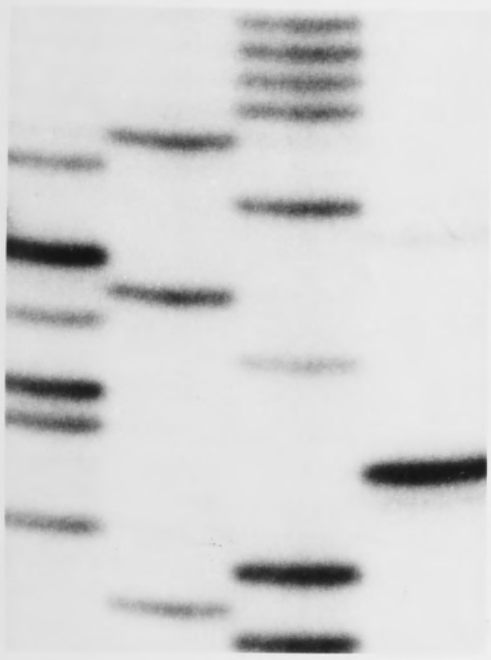
CAC ⟶ CTC

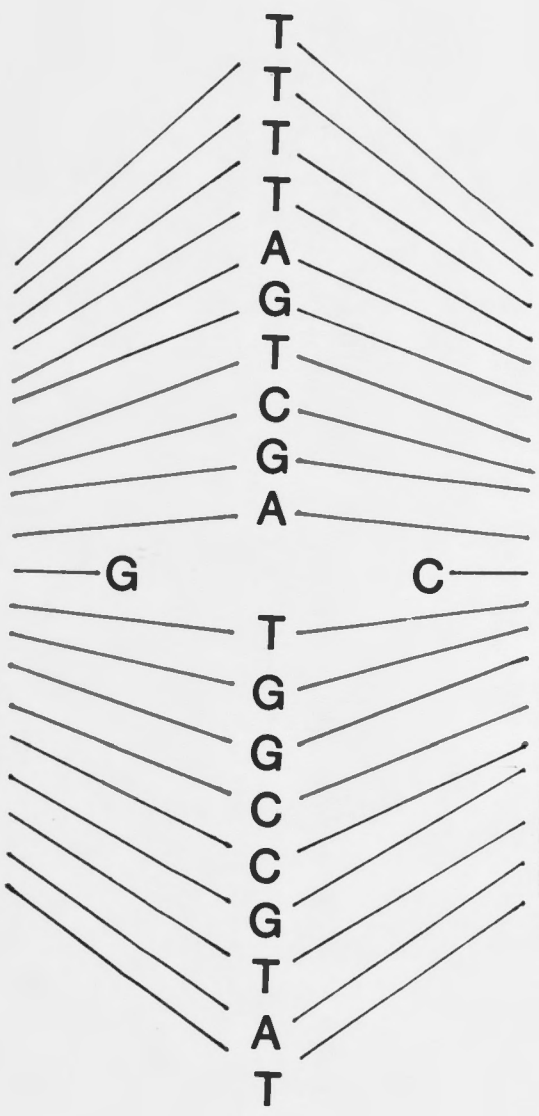
H245L

Fig. 7.3. Sections of DNA sequencing gels showing the *uncB587*(E219) nucleotide substitution. Samples of DNA from both normal and mutant were sequenced and the sequencing reactions electrophoresed through 6% (w/v) polyacrylamide as described in Chapter 2, section 2.6. Sequencing gels may be read in the order G, A, T, C from left to right. The indented nucleotides indicate the nucleotide which have been substituted.

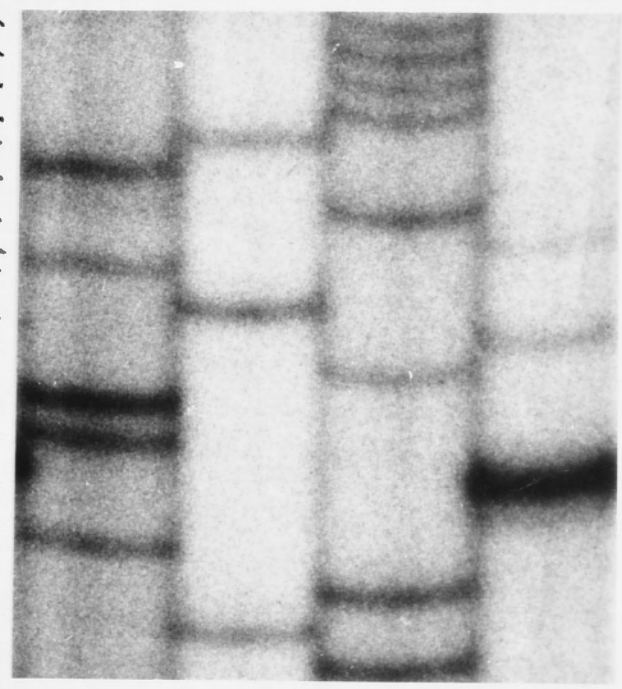
DNA SEQUENCE CHANGE IN UNCB587

NORMAL





MUTANT



TRIPLET CHANGE GAG⟶ CAG

E219Q

Fig 7.4 Nucleotide sequence of the *uncB* gene coding for the *E. coli* F_oF_1-ATPase subunit-*a*, together with the amino acid sequence. The sequence shown incorporates all the base substitutions in *uncB568*(N214G), *uncB582*(L207A), *uncB77(*H245L) and *uncB587*(E219Q) with the replaced nucleotides underlined at positions 619,620, 640,641, 656 and 734 respectively and the substituted amino acids printed in bold type.

```
 M  A  S  E  N  M  T  P  Q  D  Y  I  G  H  H  L  N  N  L
ATGGCTTCAGAAAATATGACGCCGCAGGATTACATAGGACACCACCTGAATAACCTT
         10         20         30         40         50
 Q  L  D  L  RL  T  F  S  L  V  D  P  Q  N  P  P  A  T  F
CAGCTGGACCTGCGTACATTCTCGCTGGTGGATCCACAAAACCCCCCAGCCACCTTC
 60         70         80         90        100        110
 W  T  I  N  I  D  S  M  F  F  S  N  N  L  G  L  L  F  L
TGGACAATCAATATTGACTCCATGTTCTTCTCGGTGGTGCTGGGTCTGTTGTTCCTG
   120        130        140        150        160        170
 V  L  F  R  S  V  A  K  K  A  T  S  G  V  P  G  K  F  Q
GTTTTATTCCGTAGCGTAGCCAAAAAGGCGACCAGCGGTGTGCCAGGTAAGTTTCAG
      180        190        200        210        220        2
 T  A  I  F  I  V  I  G  F  V  N  G  S  V  K  D  M  Y  H
ACCGCGATTGAGCTGGTGATCGGCTTTGTTAATGGTAGCGTGAAAGACATGTACCAT
30         240        250        260        270        280
 G  K  S  K  L  I  A  P  L  A  L  T  I  F  V  W  V  F  L
GGCAAAAGCAAGCTGATTGCTCCGCTGGCCCTGACGATCTTCGTCTGGGTATTCCTG
  290        300        310        320        330        340
 M  N  L  M  D  I  L  P  I  D  L  L  P  Y  I  A  E  G  V
ATGAACCTGATGGATTTACTGCCTATCGACCTGCTGCCGTACATTGCTGAACATGTA
     350        360        370        380        390        40
 L  G  L  P  A  L  R  N  N  P  S  A  D  V  N  V  T  L  S
CTGGGTCTGCCTGCACTGCGTGTGGTTCCGTCTGCGGACGTGAACGTAACGCTGTCT
0        410        420        430        440        450
 M  A  L  G  V  F  I  L  I  L  F  Y  S  I  Q  M  Q  G  I
ATGGCACTGGGCGTATTTATCCTGATTCTGTTCTACAGCATCAAAATGAAAGGCATC
 460        470        480        490        500        510
 G  G  F  T  K  E  L  T  L  Q  P  F  N  H  W  A  F  I  P
GGCGGCTTCACGAAAGAGTTGACGCTGCAGCCGTTCAATCACTGGGCGTTCATTCCT
    520        530        540        550        560        570
 V  N  L  I  L  A  G  V  S  L  L  S  K  P  V  S  A  G  L
GTCAACTTAATCCTTGAAGGGGTAAGCCTGCTGTCCAAACCAGTTTCAGCCGGTTTG
       580        590        600        610        620
 R  L  F  G  G  M  Y  A  G  Q  L  I  F  I  L  I  A  G  L
CGACTGTTCGGTGGCATGTATGCCGGTGTGCTGATTTTCATTCTGATTGCTGGTCTG
630        640        650        660        670        680
 L  P  W  W  S  Q  W  I  L  N  V  P  W  A  I  F  L  I  L
TTGCCGTGGTGGTCACAGTGGATCCTGAATGTGCCGTGGGCCATTTTCCTCATCCTG
   690        700        710        720        730        740
 I  I  T  L  Q  A  F  I  F  M  V  L  T  I  V  Y  L  S  M
ATCATTACGCTGCAAGCCTTCATCTTCATGGTTCTGACGATCGTCTATCTGTCGATG
      750        760        770        780        790        8
 A  S  E  E  H
GCGTCTGAAGAACAT
00       810
```

retained. Strains were designated as follows; AN2769(pAN416, carrying *uncB568*); AN2766(pAN456, carrying *uncB582*); AN2757(pAN451, carrying *uncB577)*; AN2792(pAN467, carrying *uncB587*). For ease of reference, these strains will be referred to as strains AN2769(N214G), AN2766(L207A), AN2757(H245L) and AN2792(E219Q).

7.3. Growth characteristics and membrane properties of strains AN2769(N214G), AN2766(L207A), AN2757(H245L) and AN2792(E219Q)

7.3.(i) Growth characteristics

Mutant strains were assayed along with their respective coupled and uncoupled control strains for their ability to utilise succinate minimal medium for growth, and for growth yields on limiting (5 mM) glucose (Table 7.3). Strains AN2769(N214G) and AN2766(L207A) were able to grow well on solid succinate minimal media, whilst strains AN2757(H245L) and AN2792(E219Q) showed no apparent growth after 36 hours. Strains AN2769(N214G) and AN2766(L207A) also exhibited similar growth yields on limiting glucose to the coupled control strain AN2709(pAN436/*uncB402*). However, both strain AN2757(H245L) and strain AN2792(E219Q) gave growth yields similar to the uncoupled control strain AN2736 (pAN174/*uncB402*). Mutant strains AN2769(N214G) and AN2766(L202A) exhibited similar growth yields to the coupled control strain AN2709 on limiting glucose when all strains were grown at 42^{o}C.

7.3.(ii) ATPase activities

Subcellular fractions were prepared from the mutant strains together with relevant control strains, AN2709(pAN436/*uncB402*), AN2402(pAN51/*uncB402*) and uncoupled control strain AN2736(pAN174/*uncB402*). The ATPase activities of the cytoplasmic fraction from the four mutant strains were negligible (Table 7.3). The ATPase activities of the membrane fractions from mutant strains AN2769(N214G) and AN2766(L207A) were identical to the activities in membranes from the coupled control strain AN2709(pAN436/*uncB402*). However, the ATPase activities from the membrane fraction from mutant strains AN2757(H245L) and AN2792(E219Q) were only approximately 55% and 45% of the membrane-bound activities found from the coupled control strains AN2709(pAN436/*uncB402*) and AN2402(pAN51/*uncB402*) respectively.

TABLE 7.3

GROWTH CHARACTERISTICS AND ATPase ACTIVITIES OF SUBCELLULAR FRACTIONS FROM MUTANT STRAINS AN2769(N214G), AN2766(L207A), AN2757(H245L) AND AN2792(E219Q).

The subcellular preparations from the mutant and respective control strains were isolated simulataneously as described in Chapter 2. Removal of the F_1-ATPase from the membranes was performed by dialysing the membranes against low ionic strength buffer in the absence of *p*-aminobenzamidine. Further details of strains are given in the text to Chapter 7 or in Table 2.1 and 2.2.

Bacterial strain (Plasmid)	Plasmid encoded amino acid substitution	Growth on succinate	Growth yield on 5mM glucose (Klett units)	ATPase activities (µmol Pi/min/mg protein) Cytoplasmic	Membrane Pre-dialysis	Membrane Post dialysis
AN2769(pAN416)	N214G	+	230	<0.1	0.7	0.7
AN2766(pAN456)	L207A	+	221	0.1	0.6	0.7
AN2757(pAN451)	H245L	-	143	0.2	0.4	0.6
AN2709(pAN436)[a]	+	+	231	0.1	0.7	0.7
AN2792(pAN467)	E219Q	-	148	<0.1	0.5	0.9
AN2402(pAN51)[b]	+	+	234	0.1	1.3	1.2
AN2736(pAN174)[c]	-	-	144	0.2	0.2	0.2

a Coupled control strain for strains AN2769, AN2766 and AN2757
b Coupled control strain for strain AN2792
c Uncoupled control strain

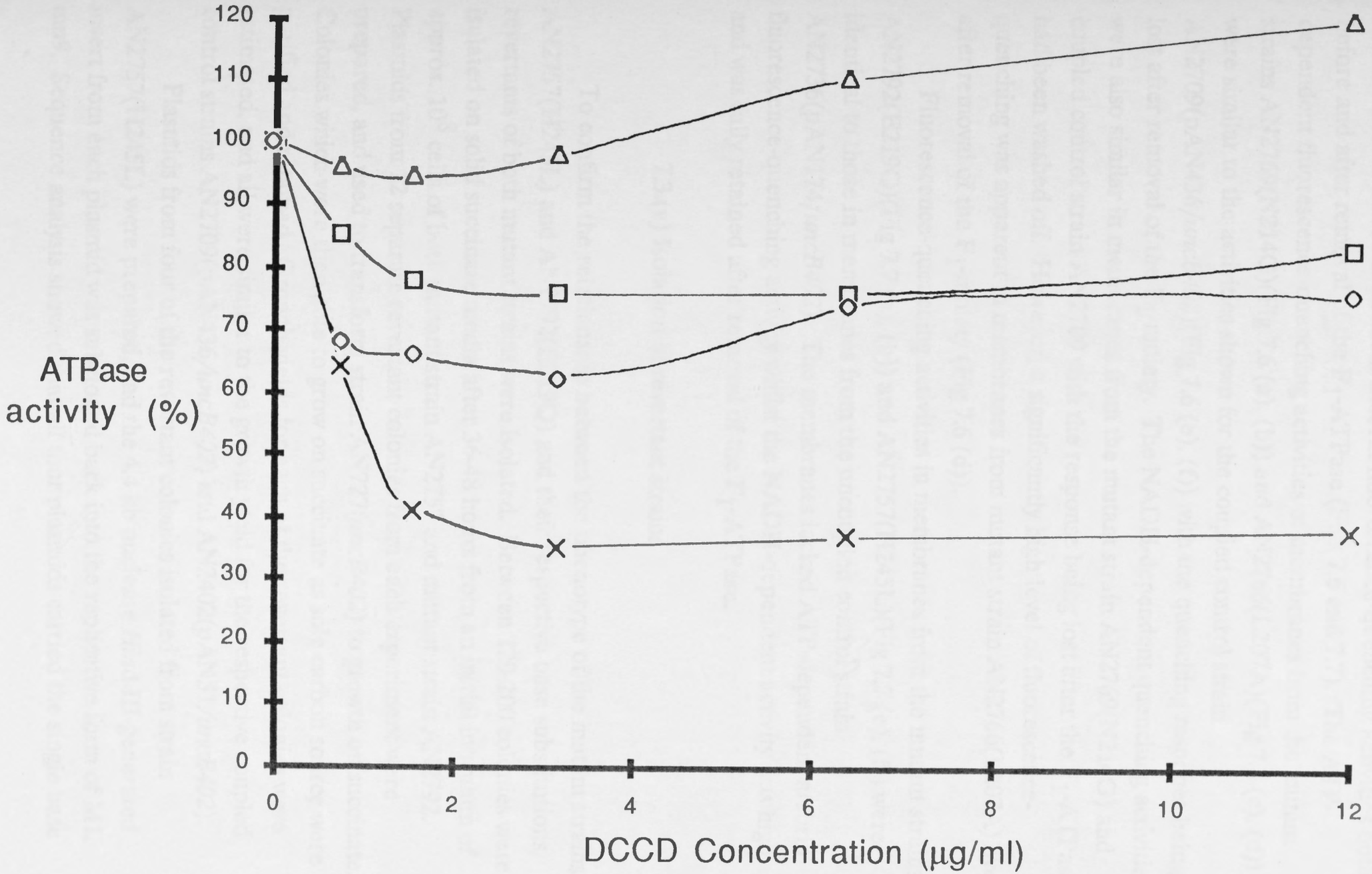
If the membrane fractions from the two mutant strains AN2757(H245L) and AN2792(E219Q) were dialysed against low ionic strength buffer in the absence of *p*-aminobenzamidine and reassayed, the ATPase activity increased to 85% of the comparable coupled control value for the membrane fraction from strain AN2757, and 75% of the comparable coupled control value for the membrane fraction from strain AN2792. The ATPase activities of the other two mutant strains AN2769(N214G) and AN2766(L207A), and the coupled and uncoupled control strains were unaffected by this treatment (Table 7.3). Confirmation that the dialysis treatment had released the F_1-ATPase from the membranes of the two mutant strains AN2757(H245L) and AN2792(E219Q) was achieved by showing the loss of ATPase activity on assaying the membranes after centrifugation of the dialysed preparation.

7.3.(iii) Sensitivities to the inhibitor DCCD

The sensitivities to the inhibitor DCCD of the ATPase activities in the membrane preparations from all strains were measured (Fig 7.5). Membrane-bound ATPase activities from the coupled control strains AN2709(pAN436/*uncB402*) and AN2402(pAN51/*uncB402*) were maximally inhibited by approximately 65%, similar to the levels of inhibition noted for the mutant strain AN2769(N214G). However, membrane-bound ATPase activities from mutant strains AN2757(H245L) and AN2792(E219Q) were relatively insensitive to the inhibitor, with activities from strain AN2792 being maximally inhibited by 25% and the activities from strain AN2757 inhibited by less than 10% The ATPase activity in membranes from mutant strain AN2757(H245L) was also stimulated at higher concentrations of DCCD (>4 $\mu g.ml^{-1}$ DCCD per 300 μg membrane protein), similar to the affect of DCCD treatment on ATPase activities from strain AN2735(R210Q)(*q.v.*section 4.2.(iv)). This stimulation was not noted in membrane-bound ATPase activities from mutant strain AN2792(E219Q).

Membrane-bound ATPase activities from mutant strain AN2766(L207A) were maximally inhibited by approximately 45% and appeared significantly less sensitive to the inhibitor than the ATPase from the coupled control strain AN2709(pAN436/*uncB402*).

Fig 7.5 Inhibition of ATPase activity by DCCD. Membranes (0.3 mg of protein) were incubated at 30°C in 5 ml of the assay mixture together with the indicated levels of DCCD. The mixture was sampled at intervals, the rate determined for each DCCD concentration and the percent inhibition was calculated (*q.v.* Chapter 2, section 2.8.(iii)). Symbols: ×, membranes from coupled control strain AN2709(pAN436/*uncB402*); ◇, membranes from mutant strain AN2766(L207A); □, membranes from mutant strain AN2792(E219Q); △, membranes from mutant strain AN2757(H245L). Inhibition of ATPase activity in membranes from mutant strain AN2769(N214G) was similar to the inhibition in membranes from the coupled control strain AN2709(*unc*$^{+}$).


ATPase activity (%)
120
110
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
DCCD Concentration (μg/ml)

7.3.(iv) Atebrin fluorescence-quenching activities

Membrane preparations from the mutant and control strains were assayed for ATP- and NADH-dependent atebrin fluorescence-quenching activities both before and after removal of the F_1-ATPase (Figs 7.6 and 7.7). The ATP-dependent fluorescence-quenching activities of membranes from the mutant strains AN2769(N214G)(Fig 7.6 (a), (b)) and AN2766(L207A)(Fig 7.6 (c), (d)) were similar to the activities shown for the coupled control strain AN2709(pAN436/*uncB402*)(Fig 7.6 (e), (f)) with the quenching respones being lost after removal of the F_1-moiety. The NADH-dependent quenching activities were also similar in membranes from the mutant strain AN2769(N214G) and coupled control strain AN2709 with the response being lost after the F_1-ATPase had been washed off. However, a significantly high level of fluorescence-quenching was apparent in membranes from mutant strain AN2766(L207A) even after removal of the F_1-moiety (Fig 7.6 (d)).

Fluorescence-quenching activities in membranes from the mutant strains AN2792(E219Q)(Fig 7.7 (a), (b)) and AN2757(H245L)(Fig 7.7 (e), (f)) were identical to those in membranes from the uncoupled control strain AN2736(pAN174/*uncB402*). The membranes lacked ATP-dependent atebrin fluorescence-quenching activity whilst the NADH-dependent activity was high and was fully retained after removal of the F_1-ATPase.

7.3.(v) Isolation of revertant strains

To confirm the relationship between the phenotype of the mutant strains AN2757(H245L) and AN2792(E219Q) and their respective base substitutions, revertants of both mutant strains were isolated. Between 120-200 colonies were isolated on solid succinate media after 36-48 hours from an initial inoculum of approx. 10^8 cells of both mutant strain AN2757 and mutant strain AN2792. Plasmids from 12 separate revertant colonies from each experiment were prepared, and used to transform strain AN727(*uncB402*) to growth on succinate. Colonies which were then able to grow on succinate as sole carbon source were purified and retained. Growth yields for each of the revertant colonies were estimated, and all were similar to the growth yield for the respective coupled control strains AN2709(pAN436/*uncB402*) and AN2402(pAN51/*uncB402*).

Plasmids from four of the revertant colonies isolated from strain AN2757(H245L) were prepared, and the 4.4 kb nuclease *Hin*d III-generated insert from each plasmid was subcloned back into the replicative form of M13 mp9. Sequence analysis showed that all four plasmids carried the single base

Fig. 7.6. Atebrin fluorescence-quenching in membranes prepared from strains of *E.coli*. Atebrin fluorescence-quenching activities were assayed as detailed in Chapter 2, section 2.8.(ii). Atebrin was added to give a final concentration of 4 μM, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to 2 μM. (a) membranes from strain AN2769(N214G); (b) stripped membranes from strain AN2769(N214G); (c) membranes from strain AN2766(L207A); (d) stripped membranes from strain AN2766(L207A); (e) membranes from coupled control strain AN2709(pAN436/*uncB402*) (f) stripped membranes from coupled control strain AN2709(pAN436/*uncB402*).

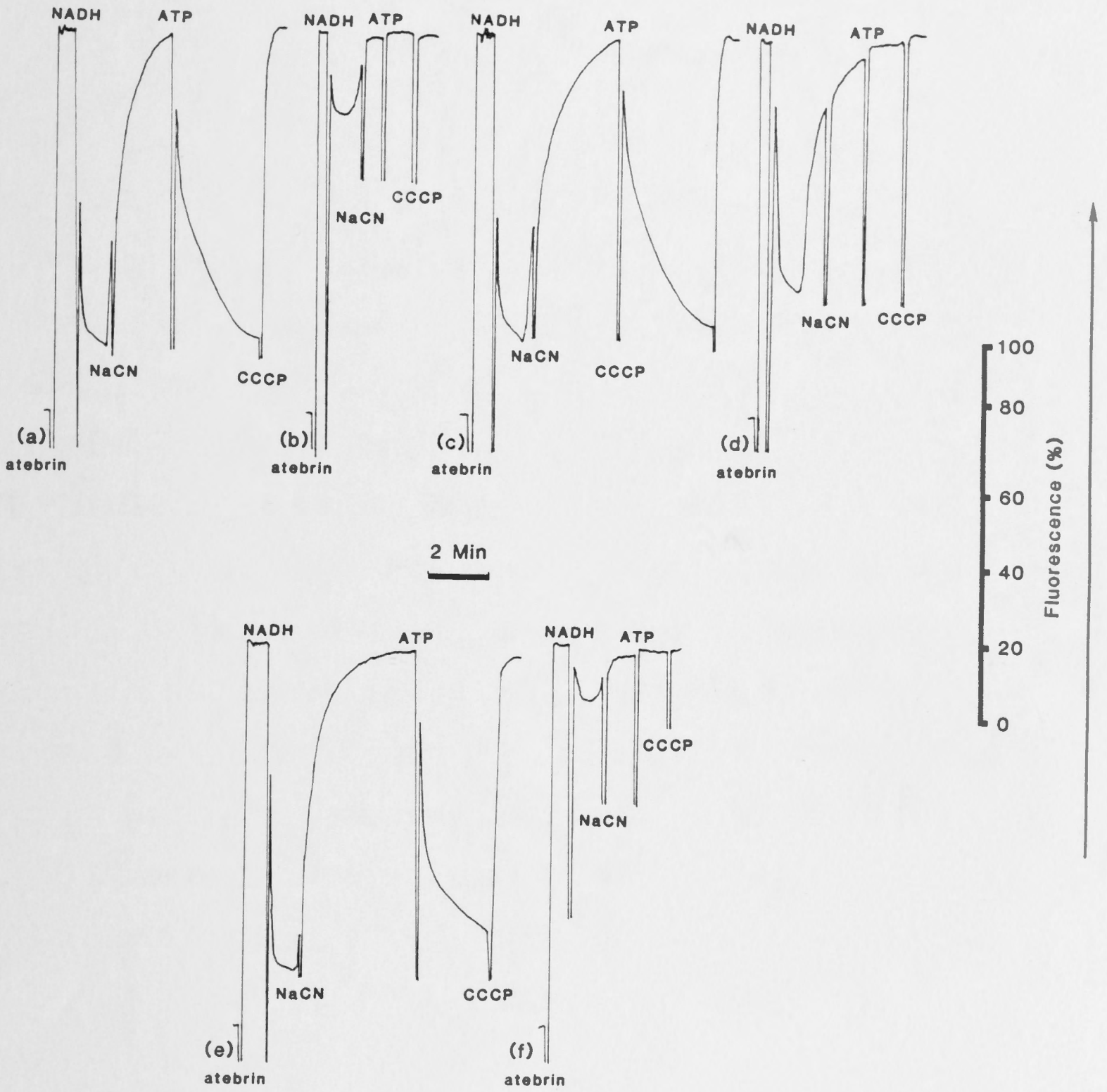
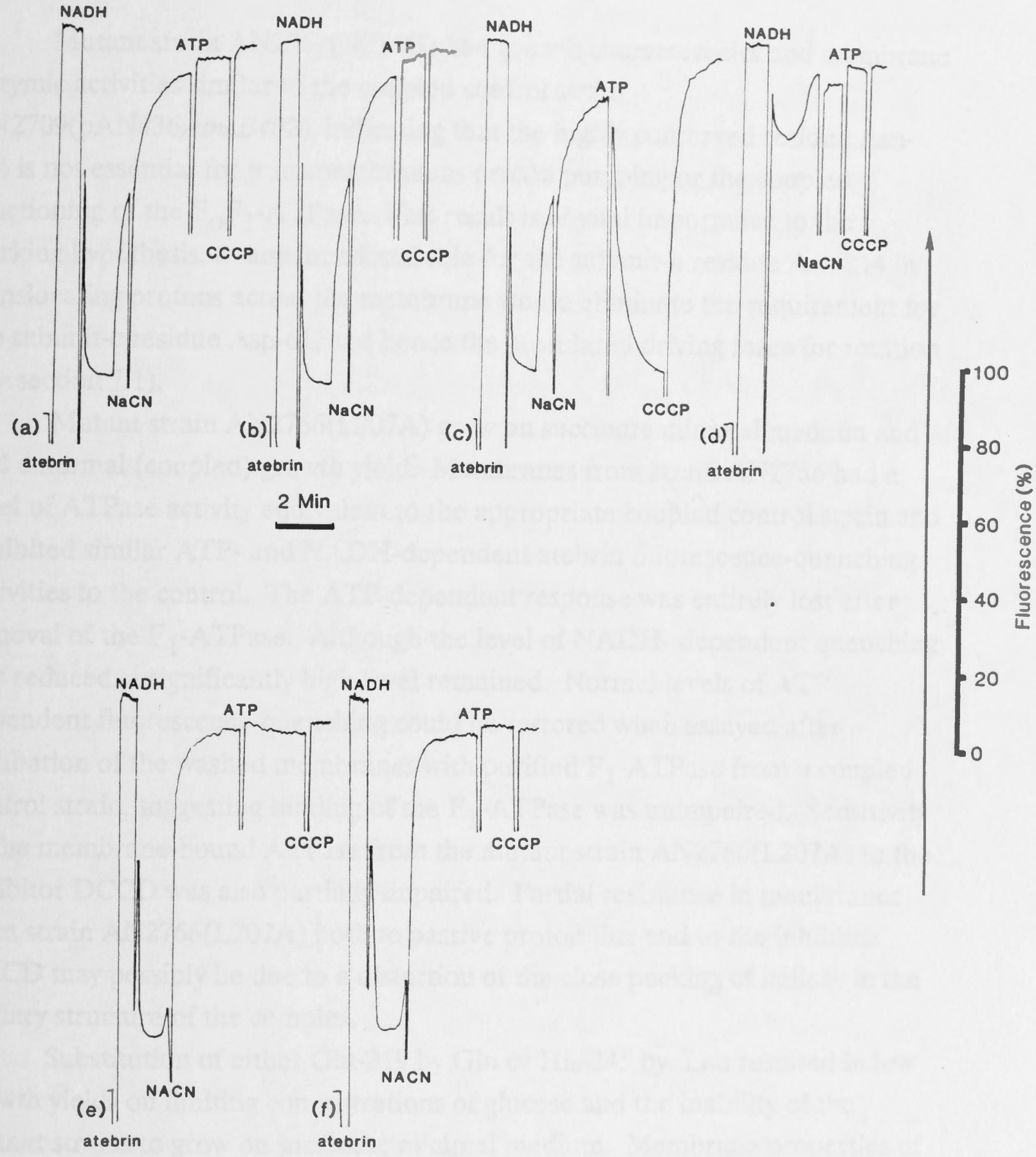

NADH
ATP
NaCN
CCCP
(a)
atebrin
(b)
(c)
(d)
2 Min
(e)
(f)
100
80
60
40
20
0
Fluorescence (%)

Fig. 7.7. Atebrin fluorescence-quenching in membranes prepared from strains of *E.coli*. Atebrin fluorescence-quenching activities were assayed as detailed in Chapter 2, section 2.8.(ii). Atebrin was added to give a final concentration of 4 μM, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to 2 μM. (a) membranes from strain AN2792(E219Q); (b) stripped membranes from strain AN2792(E219Q); (c) membranes from coupled control strain AN2402(pAN51/*uncB402*); (d) stripped membranes from strain AN2402(pAN51/*uncB402*); (e) membranes from strain AN2757(H245L); (f) stripped membranes from strain AN2757(H245L). Fluorescence quenching activities of membranes from the uncoupled control strains were similar to traces (a) and (e) and stripped membranes were similar to traces (b) and (f).


NADH
ATP
CCCP
NaCN
(a)
atebrin
(b)
(c)
(d)
2 Min
(e)
(f)
NACN
100
80
60
40
20
0
Fluorescence (%)

reversion to the normal sequence. Although plasmid-borne reversion was noted for strain AN2792(E219Q) (see above), none of the revertant-bearing plamids were subjected to sequence analysis.

7.4 Discussion

Mutant strain AN2769(N214G) had growth characteristics and membrane enzymic activities similar to the coupled control strain AN2709(pAN436/*uncB402*), indicating that the highly conserved residue Asn-214 is not essential for transmembranous proton pumping or the coupled functioning of the F_0F_1-ATPase. This result is of vital importance to the working hypothesis, as any functional role for the subunit-*a* residue Asn-214 in translocating protons across the membrane would eliminate the requirement for the subunit-*c* residue Asp-61, and hence the postulated driving force for rotation (*q.v.*section 7.1).

Mutant strain AN2766(L207A) grew on succinate minimal medium and had a normal (coupled) growth yield. Membranes from strain AN2766 had a level of ATPase activity equivalent to the appropriate coupled control strain and exhibited similar ATP- and NADH-dependent atebrin fluorescence-quenching activities to the control. The ATP-dependent response was entirely lost after removal of the F_1-ATPase. Although the level of NADH- dependent quenching was reduced, a significantly high level remained. Normal levels of ATP-dependent fluorescence-quenching could be restored when assayed after incubation of the washed membranes with purified F_1-ATPase from a coupled control strain, suggesting binding of the F_1-ATPase was unimpaired. Sensitivity of the membrane-bound ATPase from the mutant strain AN2766(L207A) to the inhibitor DCCD was also partially impaired. Partial resistance in membranes from strain AN2766(L207A) both to passive proton flux and to the inhibitor DCCD may possibly be due to a distortion of the close packing of helices in the tertiary structure of the complex.

Substitution of either Glu-219 by Gln or His-245 by Leu resulted in low growth yields on limiting concentrations of glucose and the inability of the mutant strains to grow on succinate minimal medium. Membrane properties of the two mutant strains AN2757(H245L) and AN2792(E219Q) were similar, lacking ATP-dependent fluoresence-quenching activities and remaining proton impermeable after removal of the F_1-moiety. Both sets of membrane preparations exhibited significant stimulation of ATPase activity on release of the F_1-ATPase from the F_0-moiety, and both were relatively insensitive to the inhibitor DCCD. However, the membrane-bound ATPase from strain

AN2792(E219Q) failed to show any stimulation of activity at higher levels of DCCD as noted in membranes from strains AN2735(R21Q)(*q.v.* section 4.2.(iii)) and AN2757(H245L). These properties are consistent with mutant strains in which phosphorylation has been uncoupled from electron transport. The observation that the substitution of Glu-219 by Gln in subunit-*a* uncoupled oxidative phosphorylation, provides support for the procedure used in the previous three chapters, where the structure of subunit-*a* was altered to position ionisable amino-acids, which were shown not to be functionally involved in coupling oxidative phosphorylation, outside the membrane.

Cain and Simoni (1986), isolated a subunit-*a* mutant carrying a S206L substitution, which partially affected transmembranous proton translocation. Membranes from this strain had a significant level of DCCD-sensitive ATP-dependent ACMA fluorescence-quenching activity showing that the mutated ATPase complex is still partially capable of pumping protons across the membrane, and the authors express some doubt concerning the involvement of Ser-206 in the proton pore. Thus, it remains a possibility that one of the functions of the serine residue may be to stabilise an intramembranous ionisable residue which is functionally important and has yet to be identified. The subunit-*a* residue Arg-61 is positioned just outside the membrane in the structure illustrated in Fig.7.8. Repositioning of residue Arg-61 to an intramembranous position similar to that postulated for Ser-206, may enable the Arg-61 residue to function as a member of the transmembranous proton pore, and would also position a carboxylate near the phospholipid head-group region in the subunit-*a* transmembrane helices I, II and III which may function to 'anchor' the helices on the periplasmic face of the membrane. The location of the carboxylates may be stabilised by interactions with hydronium ions, other cations, or the dipole moments of water molecules which are believed to form an ordered layer along the surface of the membrane, , similar to the Stern-Graham, Gouy-Chapman layers around a charged electrode surface (Kell, 1979; see Bockris and Reddy, 1970).

The amino-acid substitutions detailed in this, and the previous three chapters, gave experimental evidence for a molecular mechanism by which protons may pass through a protein embedded within a membrane. Accepting the doubts concerning the involvement of residue Ser-206, it is postulated that protons pass across the membrane via the side-chains of discrete amino acid residues. The proton pore through the F_O-moiety can be defined as a chain involving the side-chains of residues Ser-206, Arg-210, Glu-219 and His-245 from subunit-*a*, and residue Asp-61 from subunit-*c*. Residues Ser-206, Arg-210 and Glu-219 are located in helix IV of subunit-*a*, His-245 in helix V of subunit-*a*, and

Asp-61 in helix II of subunit-*c*. The residues are located in the helices at approximately 0.6 nm intervals across the membrane, at positions which facilitate transfer from one functionally essential amino acid side-chain to the next. The integration of residues from both subunit-*a* and subunit-*c* in the functional proton pore suggests that, as there are at least six copies of subunit-*c* per F_oF_1-ATPase complex, and only one copy of subunit-*a* (*q.v.*section 1.2.(ii)), and that it is likely that all copies of subunit-*c* are involved in tranmembranous proton pumping (*q.v.*section 1.5.(i)), it appears that subunit-*a* must move with respect to the multimer of *c*-subunits (see Chapter 9).

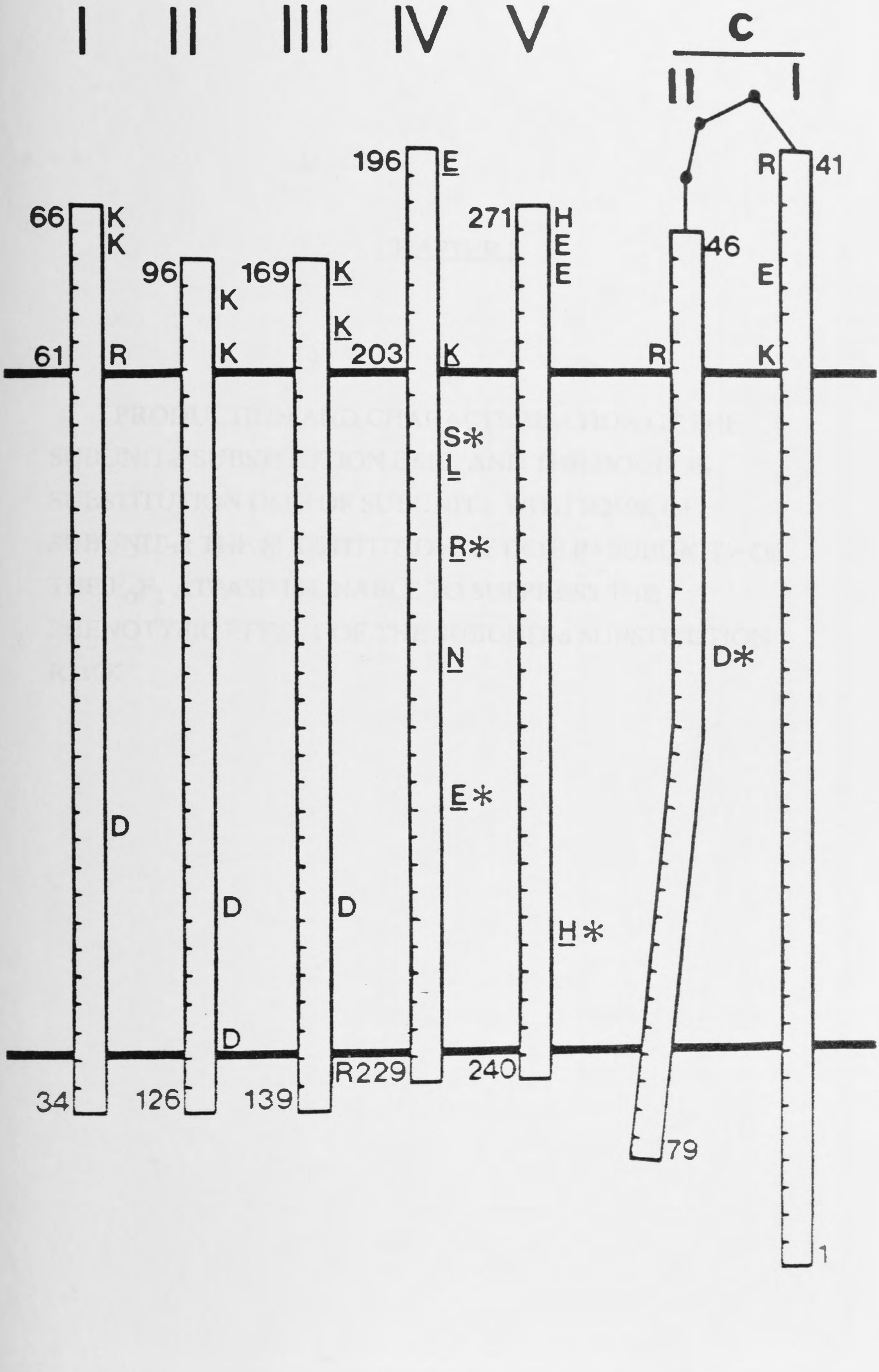
There were two characteristics of the membranes from strain AN2792(E219Q) and strain AN2735(R210Q), also evident in membranes from strain AN2757(H245L), which differ from the His-245 - Tyr mutant described by Cain and Simoni (1986). These were the approximately 50% inhibition of ATPase activity when the F_1-ATPase was bound to the mutant F_o-moiety, and the sensitivity to the inhibitor DCCD. The exact mechanism by which DCCD exerts its influence on the F_1-ATPase after binding to the F_o-moiety is unknown. However, many mutations in the intramembranous F_o subunits -*a*, -*b* and -*c* have now been characterised at the molecular level and the H245Y substitution reported by Cain and Simoni (1986) is the first F_o mutation that boasts impermeable membranes whilst retaining a DCCD-sensitive membrane-bound F_1-ATPase activity.

Strains carrying a missense mutation characterised in an F_o-subunit which does not appear to affect complex assembly but results in proton impermeable membranes normally exhibit inhibition of membrane-bound ATPase activity which can be alleviated on release of the F_1-ATPase from the membrane. ATPase complexes from these strains are also normally resistant to DCCD. Until the characterisation of the H245Y substitution, the only exception with respect to proton permeability and ATPase inhibition was the mutant carrying the D61G substitution isolated by Hoppe and Sebald, (1979). Why the substitution of H245Y should produce different membrane properties from the H245L substitution and from other missense mutations involved in proton translocation does not immediately appear obvious.

A histidine residue is postulated as being present at a similar position to the *E. coli* subunit-*a* Glu-219 in the mitochondrial ATPase subunit-*6* from many diverse species, whilst a glutamate is conserved at a similar position to the *E. coli* subunit-*a* His-245 residue in the mitochondrial analogues (*q.v.* section 7.1). However, no similar pairing is apparent for the chloroplast subunit-*a* analogue CF_oIV (Hennig and Herrmann, 1986), or the analogue from the cyanobacterium *Synechococcus 6301* (Cozens and Walker, 1987). As the molecular mechanism

for coupling both oxidative and photophosphorylation are believed to be similar it is important to try and suggest alternatives for the lack of similar residues at similar positions in the subunit-*a* analogues from both *Synechococcus* and spinach chloroplasts. A carboxylate residue is postulated as being at a comparable position to the *E. coli* subunit-*a* residue Glu-219 in both subunit-*a* analogues from spinach chloroplasts and *Synechococcus*, but there is no histidine residue in a comparable position to the *E. coli* subunit-*a* residue His-245. Both the spinach and *Synechococcus* subunit-*a* analogues contain five histidine residues, with two being present within the C-terminal final five residues in both cases. A possible candidate for involvement in the transmembranous proton pore is His-33 in the *Synechococcus* subunit-*a* analogue, and His-35 in the spinach chloroplast subunit-*a* analogue. These two residues are both postulated to be just within the membrane, in helix I. It is possible that the requirement for positioning either the histidine or carboxylate residue, both within the relay system involved in proton translocation, and within the intramembranous helices of the subunit-*a* analogues, is not strictly conserved. This possibility is supported by the apparent alternation of the positions of the two residues between *E. coli* and mitochondria from diverse species..

Fig. 7.8. Diagrammatic representation of the five predicted intramembranous helices of subunit-*a* and the two intramembranous helices of subunit-*c*. The number of the residue at both N- and C-terminals of each putative helix are given. The thick horizontal line towards the top of the page represents the membrane/cytoplasm border, whilst the lower thick line represents the membrane/periplasm border. Every ionisable residue predicted to be within the membrane is marked and all residues which have been substituted are underlined. An asterisk next to a residue denotes that a substitution of that residue had been shown to markedly affect proton permeability of the membrane.


I
II
III
IV
V
C
II
I
66
K
K
61
R
96
K
K
169
K
K
196
E
203
K
271
H
E
E
R
46
R
41
E
K
S*
L
R*
N
D*
E*
H*
D
D
D
D
34
126
139
R229
240
79
1

CHAPTER 8

PRODUCTION AND CHARACTERISATION OF THE SUBUNIT-*c* SUBSTITUTION D61E, AND THE DOUBLE-SUBSTITUTION D61E OF SUBUNIT-*c* WITH R210K OF SUBUNIT-*a*: THE SUBSTITUTION OF D61E IN SUBUNIT-*c* OF THE F_oF_1 ATPASE IS UNABLE TO SUPPRESS THE PHENOTYPIC EFFECT OF THE SUBUNIT-*a* SUBSTITUTION R210K

8.1.Introduction

The specific requirement of Arg-210 in subunit-*a* for maintaining oxidative phosphorylation has been shown in Chapters 4 and 5. Assuming the subunit-*a* residue Arg-210 and Asp-61 of subunit-*c* are in close proximity, it may be possible to partially compensate for the introduction of lysine at position 210 in subunit-*a* by replacing aspartate by glutamate at position 61 of subunit-*c*. If only one of the amine groups of the arginine residue is involved in proton translocation, by substituting lysine for arginine, the ε-amino group of the lysine may enable the lysine residue to function as a member of the putative proton pore, but is too far removed from the aspartate carboxyl group to allow efficient proton translocation. This distance may be optimised by substituting the subunit-*c* residue Asp-61 by a glutamate, whereby the extra methylene group in the side-chain of the glutamate residue may compensate for the shorter side-chain of the lysine residue at position 210 of subunit-*a*, bringing the ε-amino group of the lysine and carboxyl group of the glutamate close enough to establish an interaction in a similar manner to that envisaged for the normal aspartate-arginine pair. To investigate this possibility, an attempt was made to produce the double substitution of R210K,D61E in subunits -*a* and -*c* respectively. The production of the individual subunit-*c* substitution D61E was also attempted as any phenotypic effect produced by the double substitution must be shown to be caused by the Lys-210 and Glu-61 in tandem rather than as an effect produced by the subunit-*c* substitution, D61E, or subunit-*a* substitution R210K, alone.

8.2.Production of the double substitution R210K in subunit-*a*,D61E in subunit-*c* and the single subunit-*c* substitution D61E

8.2.(i) Mutagenesis

Mutants were prepared using the standard double-priming procedure detailed in Chapter 3, section 3.3 with single-stranded template prepared from cells transformed either by phage replicative form pAN461 or with phage carrying the base substitutions encoding the R210K mutation. Phage potentially carrying the required base replacements were selected using the differential-temperature hydridisation method, with the [γ-^{32}P] labeled oligonucleotide carrying the single base substitution encoding the D61E replacement used as the probe. The oligonucleotide encoding the D61E substitution annealed at more than one place on both templates as evidenced by a dual sequencing ladder obtained on sequence analysis. Filters were washed until plaques of possibly

TABLE 8.1

SYNTHETIC OLIGONUCLEOTIDES USED IN THE PREPARATION AND SEQUENCE CONFIRMATION OF THE SUBUNIT-*C* SUBSTITUTION D61E AND THE DOUBLE SUBSTITUTION D61E IN SUBUNIT-*C* WITH R210K IN SUBUNIT-*A*.

Preparation of the oligonucleotides is detailed in Chapter 2, section 2.7.(i). The underlined base signifies a base substitution from the normal sequence.

Synthetic oligonucleotide	Relevant amino acid substitution	Notes
5'-GTCTGGTGGAGGCTATCCCGAT-3'	D61E	The D61E substitution is within subunit-*c*
5'-AACACTACTACGTTTTAACT-3'		Sequencing primer used for sequencing the entire uncE gene. The primer anneals in the intergenic region, 20 bases 5' from the *uncE* start codon.
5'-GTAAAACGACGGCCAGT-3'		Phage universal primer which anneals onto the viral strand approximately 50 bases 5' from the *Hin*d III restriction site in the mp9 polylinker.

mutated phage could be clearly defined. Phage from the plaques which exhibited strong signals were isolated and the area around the mutation site sequenced using the primer shown in Table 8.1. Several phage contained small regions of nucleotide inserts, consistent with an irregular annealing of the oligonucleotide. However, one phage preparation containing only a single base substitution encoding the D61E mutation was isolated from each of the two mutagenesis experiments (Fig 8.1). The two base-substituted phage populations were purified to homogeneity. Single-stranded phage DNA from each of the two purified populations was isolated, and the complete nucleotide sequence of the altered *uncE* gene deduced. Phage DNA from each of the populations carried only single base substitutions, at nucleotide 183, and were retained.

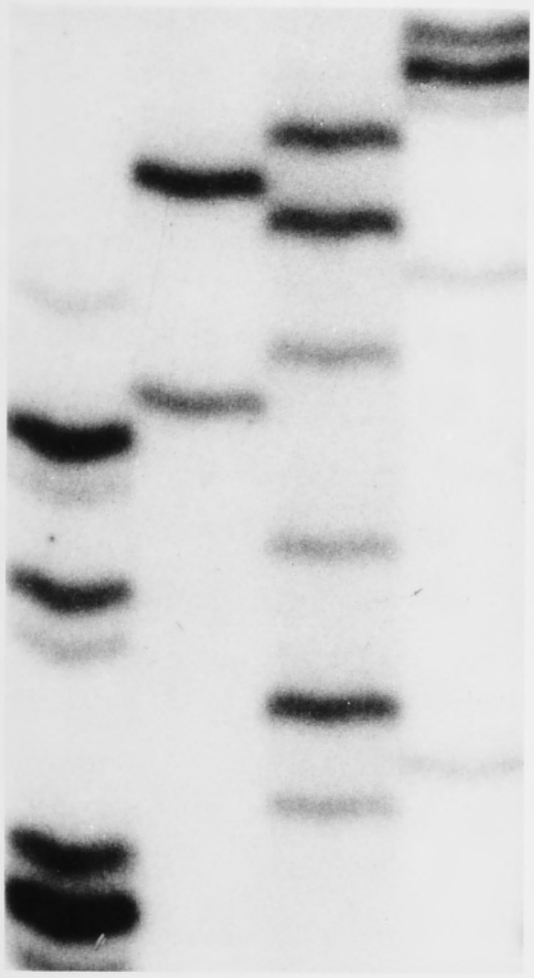
8.2.(ii) Production of plasmids pAN482(D61E) and pAN455(R210K,D61E)

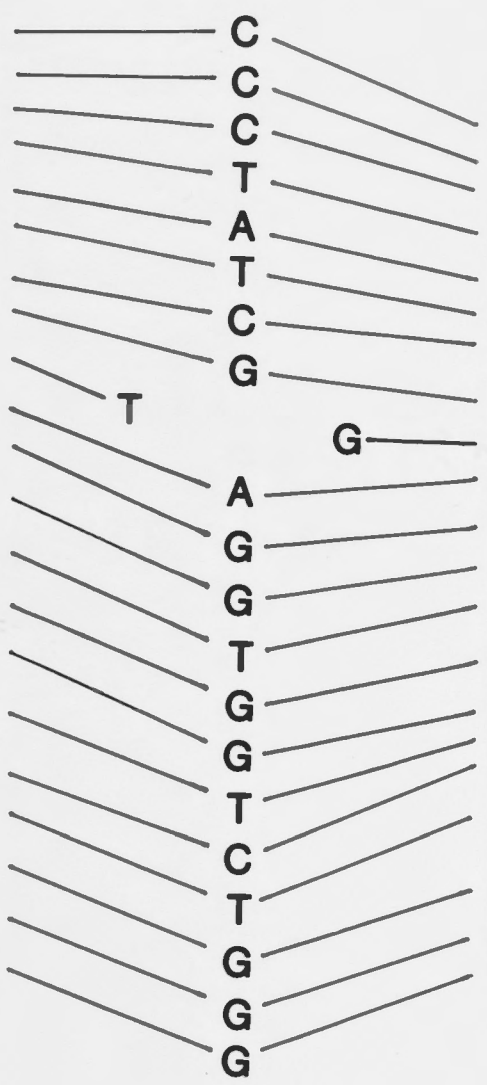
The mutant replicative form encoding the double mutation D61E,R210K was mixed with pAN36 and pAN174, the mixtures digested with nuclease *Hin*d III and ligated with T4 DNA ligase. Competent cells of strain AN1273(*uncG428*) were transformed with the ligation mix and colonies containing plasmids potentially carrying the 4.4 kb *Hin*d III-generated fragment selected by plating the transformants onto solid succinate minimal media supplemented with chloramphenicol. Plasmids were isolated from several colonies and one which proved to be similar in size and *Hin*d III-digested restriction pattern to pAN45 was retained and designated pAN455. All plasmids isolated carried pAN174 as vector. The derivative carrying the single base substitution encoding D61E was mixed with pAN174, the mixture digested with *Hin*d III and ligated with T4 DNA ligase. Competent cells from strain AN2015(*uncH241*) were transformed by the ligation mix and plasmids which were capable of rescuing growth on succinate were isolated from several colonies. A plasmid which proved to be identical in size and *Hin*d III-generated restriction pattern to pAN51 was retained and designated pAN482. Plasmid pAN455 was used to transform strain AN727(*uncB402*) to chloramphenicol resistance, with a purified resultant colony designated AN2765(pAN455 carrying *uncB569*,*uncE581*). Plasmid pAN482 was used to transform strain AN943(*uncE429*) to chloramphenicol resistance, with the purified resultant colony designated AN2814(pAN482, carrying *uncE581*). For ease of reference these two strains will be referred to as strain AN2765(*a*R210K,*c*D61E) and strain AN2814(*c*D61E), with the prefix *a* designating a mutation in subunit-*a*, and *c* designating a mutation in subunit-*c*..

Fig 8.1. Sections of DNA sequencing gels showing the *uncE581*(D61E) nucleotide substitution. Samples of DNA from both normal and mutant were sequenced, and the sequencing reactions electrophoresed through 6% (w/v) polyacrylamide as described in Chapter 2, section 2.6. Sequencing gels may be read in the order G, A, T, C from left to right. The indented nucleotides indicate the nucleotide which has been substituted.

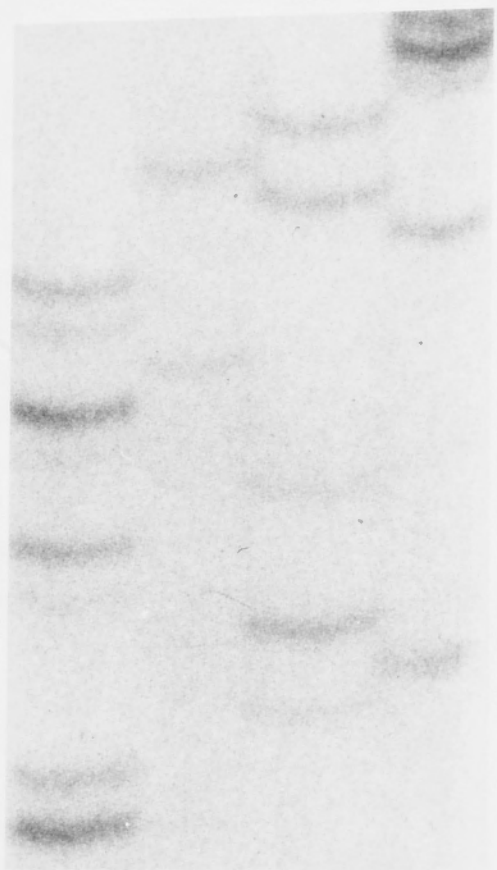
DNA SEQUENCE CHANGE IN UNCE589

NORMAL





MUTANT



TRIPLET CHANGE

GAT ⟶ GAG

D61E

8.3 Growth characteristics and membrane properties of strains AN2765(*a*R210K,*c*D61E) and AN2814(*c*D61E).

8.3.(i) Growth characteristics.

Table 8.2 shows the comparison between mutant strains AN2765(*a*R210K,*c*D61E) and AN2814(*c*D61E) and the relevant coupled and uncoupled control strains with their abilities to grow on succinate minimal media and their growth yields on limiting glucose. The growth yield of strain AN2765(*a*R210K,*c*D61E) was similar to the uncoupled control strain AN2736(*uncB402*/pAN174), whilst strain AN2814(*c*D61E) exhibited an intermediate growth yield between coupled and uncoupled control strains. Complementation analysis with plasmids pAN455 and pAN482 are also listed in Table 8.2. Plasmid pAN482(*uncE581*) was able to transform all reference strains, except strain AN943(*uncE429*), to growth on succinate. However, plasmid pAN455(*uncB569*,*uncE581*) rescued growth on succinate in strains AN1440(*uncF469*), AN1273(*uncG428*) and AN2015(*uncH241*) only. Growth yields were low when the plasmid pAN455(*uncB569*,*uncE581*) was transferred into all reference strains, with no strains obtaining growth yields similar to the coupled control strain.

8.3.(ii) ATPase activities

Subcellular fractions were prepared from the two mutant strains AN2765(*a*R210K,*c*D61E), AN2814(*c*D61E) and the relevant coupled and uncoupled control strains (Table 8.3). Cytoplasmic ATPase activities were negligible in both mutant strains AN2765 and AN2814, in comparison to the two uncoupled control strains AN2586(pAN174/*uncE429*) and AN2736(pAN174/*uncB402*). The membrane-bound ATPase activities exhibited by mutant strain AN2765(*a*R210K,*c*D61E) and by coupled control strain AN2709(pAN436/*uncB402*) were similar, and unaffected when assayed after removal of the F_1-ATPase from the F_o-moiety. However, the membrane-bound ATPase activity of mutant strain AN2814(*c*D61E) was only approximately 40% of the membrane-bound ATPase activity noted in the coupled control strain AN1873(pAN51/*uncE429*). After liberation of the F_1-sector by dialysis against a low ionic strength buffer in the absence of *p*-aminobenzamidine the ATPase activity was increased from 0.4 μM Pi.min^{-1}.mg membrane protein^{-1} to 0.9 μM

TABLE 8.2

COMPLEMENTATION ANALYSIS WITH pAN455(*a*R210K,*c*D61E) AND pAN482(*c*D61E).

Growth yields were measured in media containing 5mM glucose supplemented with 5% v/v Luria and 90 $\mu g.ml^{-1}$ chloramphenicol. The value shown is the average of at least three attempts. For further details of plasmids and relevant strains see either section 8.2 (ii) or see Tables 2.1. and 2.2.

BACTERIAL STRAIN	pAN455(*a*R210K,*c*D61E)		pAN482(*c*D61E)		pAN436(*unc*$^+$)	
	GY[a]	Succ[b]	GY	Succ	GY	Succ
AN727(*uncB402*)	121	-	226	+	232	+
AN943(*uncE429*)	130	-	165[c]	-	229	+
AN1440(*uncF476*)	144	+	210	+	220	+
AN2015(*uncH241*)	141	+	232	+	231	+
AN730(*uncA401*)	134	-	181	+	189	+
AN1273(*uncG428*)	129	+	201	+	199	+
AN818(*uncD409*)	133	-	201	+	219	+
AN802(*uncC424*)	121	-	189	+	205	+

a GY: Growth yield in 5 mM glucose measured in Klett units.
b Succ: The ability of cells to utilise succinate as sole carbon source.
c The uncoupled control strain AN1873(pAN174/*uncE429*) gave a growth yield of 139.

TABLE 8.3

ATPase ACTIVITIES OF SUBCELLULAR FRACTIONS PREPARED FROM THE TWO MUTANT STRAINS AN2765(*a*R210K,*c*D61E) AND AN2814(*c*D61E), COUPLED CONTROL STRAINS AN2709(pAN436/*uncB402*) AND AN1873(pAN51/*uncE429*) AND UNCOUPLED CONTROL STRAIN AN2736(*unc*$^-$)

The four subcellular preparations were isolated simulataneously as described in Chapter 2, section 2.8.(i), and ATPase activities assayed as described in Chapter 2, section 2.8.(iii). Removal of the F_1-ATPase from the membranes was performed by dialysing the membranes against low ionic strength buffer in the absence of *p*-aminobenzamidine. Further details of strains are given in the text to Chapter 8, or in Table 2.1 and 2.2.

BACTERIAL STRAIN (Plasmid)	ATPase ACTIVITY (μmol.min^{-1}.mg protein^{-1})		
	CYTOPLASM	MEMBRANE	
		Pre-dialysis	Post-dialysis
AN2765(*a*R210K,*c*D61E) (pAN455)	0.1	0.7	0.7
AN2709(*unc*$^+$) (pAN436)	<0.1	0.7	0.7
AN2814(*c*D61E) (pAN482)	<0.1	0.4	0.9
AN1873(*unc*$^+$) (pAN51)	<0.1	1.1	1.1
AN2736(*unc*$^-$)[a] (pAN174)	0.2	0.2	0.2

[a] Similar levels of ATPase activities were notices in membranes and cytoplasm from uncoupled control strain AN2586(pAN174/*uncE429*)

$Pi.min^{-1}.mg$ membrane $protein^{-1}$, a level almost comparable to the level attained after similar dialysis with membranes from the coupled control strain.

8.3.(iii) Sensitivities to the inhibitor DCCD

The sensitivities of the membrane-bound ATPase activities from both mutant strains were measured, and are shown, along with the sensitivities of the coupled control strain AN2709(pAN436/*uncB402*) in Fig 8.2. ATPase activities from the mutant strain AN2814(*c*D61E) were relatively insensitive to the inhibitor, being maximally inhibited by 20%, and were stimulated in higher concentrations of DCCD (>4 $\mu g.ml^{-1}$DCCD per 300 μg membrane protein), similar to the affect of DCCD treatment on ATPase activities from mutant strains AN2735(R210Q, *q.v.*Chapter 4, section 4.2.(iv)), and AN2757(H245L, *q.v.*Chapter 7, section 7.3.(iii)). Membrane-bound ATPase activities from mutant strain AN2765(*a*R210K,*c*D61E), interestingly, exhibited intermediate levels of inhibition to DCCD, being maximally inhibited by 38% (Fig 8.2).

8.3.(iv) Atebrin fluorescence-quenching activities

Membrane preparations from the mutant and control strains were assayed for ATP- and NADH-dependent atebrin fluorescence-quenching activities both before and after removal of the F_1-ATPase (Fig 8.3). The NADH-dependent fluorescence-quenching activities of membranes from the mutant strain AN2814(*c*D61E) were similar to the NADH-dependent activities from the coupled control strain AN1873(pAN51/*uncE429*)(Fig 8.3 (a) and (f)). On removal of the F_1-ATPase, the NADH-dependent quenching activity of membranes from the mutant strain AN2814(*c*D61E) was decreased by 10% (Fig 8.3 (b)), in comparison to similarly treated membranes from the coupled control strain which were decreased by 76% (Fig 8.3 (g)). An ATP-dependent fluorescence-quenching activity in membranes from mutant strain AN2814(*c*D61E) of 16% was apparent, and was lost after removal of the F_1-ATPase (Fig 8.3 (a) and (b)). On addition of DCCD to the membranes from mutant strain AN2814(*c*D61E), the ATP-dependent quenching activity was lost, and addition of the inhibitor to membranes from the same strain after removal of the F_1-ATPase, resulted in an increased NADH-dependent quenching activity (Fig 8.3 (c)).

Membranes from mutant strain AN2765(*a*R210K,*c*D61E) exhibited a low (54%) NADH-dependent fluorescence-quenching activity (Fig 8.3 (d)). This activity increased to 66% after removal of the F_1-moiety (Fig 8.3 (e)). No ATP-

Fig 8.2 Inhibition of ATPase activity by DCCD. Membranes (0.3 mg of protein) were incubated at 30°C in 5 ml of the assay mixture together with the indicated levels of DCCD. The mixture was sampled at intervals, the rate determined for each DCCD concentration and the percent inhibition was calculated (*q.v.* Chapter 2, section 2.8.(iii)). Symbols: ×, membranes from coupled control strain AN2709(*unc*$^+$); □, membranes from mutant strain AN2765(*a*R210K,*c*D61E); △, membranes from mutant strain AN2814(*c*D61E). Inhibition of ATPase activity in membranes from coupled control strain AN1873(pAN51/*uncE429*) was similar to the inhibition noted in membranes from strain AN2709(pAN436/*uncB402*).

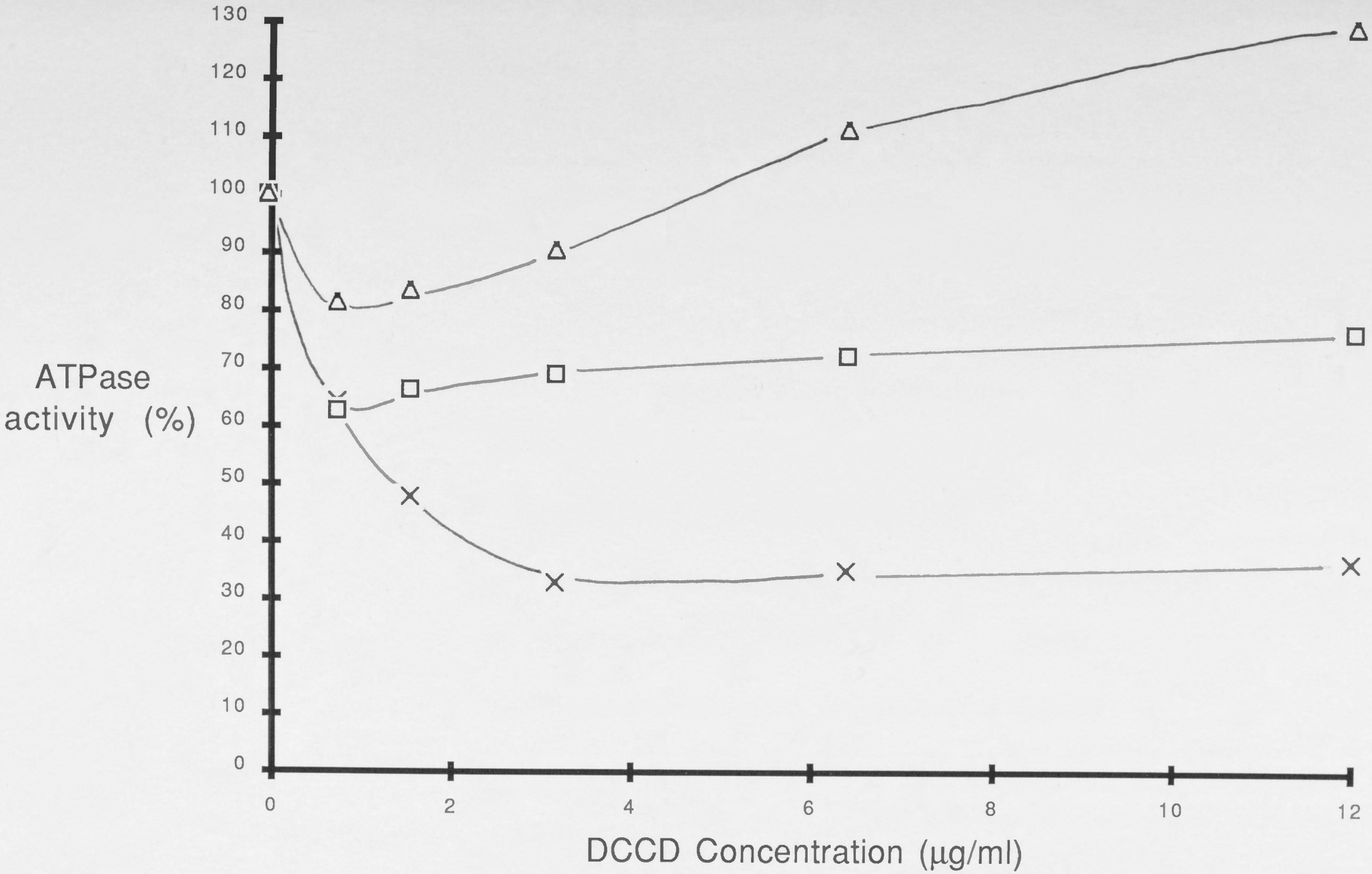
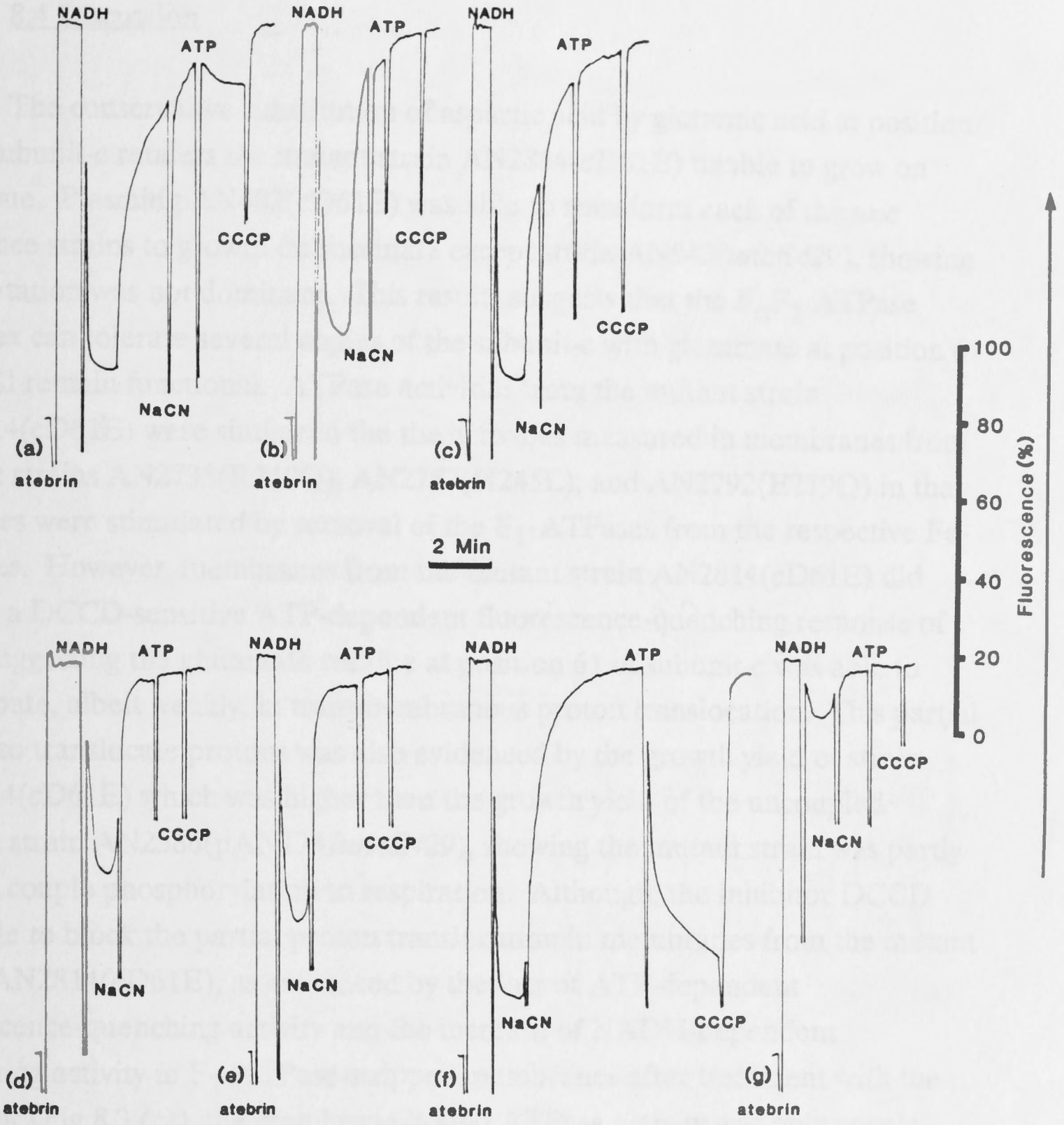

ATPase activity (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
DCCD Concentration (µg/ml)

Fig 8.3 Atebrin fluorescence-quenching in membranes prepared from strains of *E. coli*. Atebrin fluorescence-quenching activities were assayed as detailed in Chapter 2, section 2.8.(ii). Atebrin was added to give a final concentration of 4 μM, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to 2 μM. (a) membranes from mutant strain AN2814(*c*D61E); (b) stripped membranes from mutant strain AN2814(*c*D61E); (c) stripped membranes from mutant strain AN2814(*c*D61E) assayed after a 10 min preincubation in 50 μM (Final) DCCD; (d) membranes from mutant strain AN2765(*a*R210K,*c*D61E); (e) stripped membranes from mutant strain AN2765(*a*R210K,*c*D61E); (f) membranes from coupled control strain AN1873(*unc*$^+$); (g) stripped membranes from coupled control strain AN1873(*unc*$^+$).


NADH
ATP
CCCP
NaCN
atebrin
(a)
(b)
(c)
(d)
(e)
(f)
(g)
2 Min
100
80
60
40
20
0
Fluorescence (%)

dependent fluorescence-quenching activity was noted in membranes from the mutant strain AN2765(*a*R210K,*c*D61E) either before, or after removal of the F_1-ATPase.

8.4 Discussion

The conservative substitution of aspartic acid by glutamic acid at position 61 in subunit-*c* renders the mutant strain AN2814(*c*D61E) unable to grow on succinate. Plasmid pAN482(*c*D61E) was able to transform each of the *unc* reference strains to growth on succinate except strain AN943(*uncE429*), showing the mutation was not dominant. This results suggests that the F_oF_1 ATPase complex can tolerate several copies of the subunit-*c* with glutamate at position 61 and still remain functional. ATPase activities from the mutant strain AN2814(*c*D61E) were similar to the the activities measured in membranes from mutant strains AN2735(R210Q), AN2757(H245L), and AN2792(E219Q) in that activities were stimulated by removal of the F_1-ATPases from the respective Fo-moieties. However, membranes from the mutant strain AN2814(*c*D61E) did exhibit a DCCD-sensitive ATP-dependent fluorescence-quenching response of 16%, suggesting the glutamate residue at position 61 of subunit-*c* was able to participate, albeit weakly, in transmembranous proton translocation. This partial ability to translocate protons was also evidenced by the growth yield of strain AN2814(*c*D61E) which was higher than the growth yield of the uncoupled control strain AN2586(pAN174/*uncE429*), showing the mutant strain was partly able to couple phosphorylation to respiration. Although, the inhibitor DCCD was able to block the partial proton translocation in membranes from the mutant strain AN2814(*c*D61E), as evidenced by the loss of ATP-dependent fluorescence-quenching activity and the increase of NADH-dependent quenching activity in F_1-ATPase-stripped membranes after treatment with the inhibitor (Fig 8.3 (c)), the membrane-bound ATPase activity was only poorly inhibited (Fig 8.2).

Interpretation of the data concerning the double substitution of R210Q in subunit-*a* and D61E in subunit-*c* was complicated by using strain AN727(*uncB402*) as the recipient strain for the plasmid pAN455(*a*R210K,*c*D61E). Growth, and membrane-enzymic properties of strain AN2765(*a*R210K,*c*D61E) were similar to those documented for the strain AN2690(R210K)(*q.v.* Chapter 5), with a lower growth yield than the uncoupled control strain AN2736(pAN174/*uncB402*), and a membrane NADH-dependent fluorescence-quenching activity of 54%. However, membranes from strains AN2690(R210K) and AN2765(*a*R210K,*c*D61E) differed in their NADH-

dependent fluorescence-quenching activities after removal of the F_1-ATPase, and in the sensitivities of their ATPase activities to the inhibitor DCCD. Membranes from the double mutant strain AN2765(*a*R210K,*c*D61E) showed an increase in NADH-dependent fluorescence-quenching activity after removal of the F_1-ATPase, and the ATPase activity in membranes from this strain showed a maximal inhibition by DCCD of 38%. The membrane-bound ATPase activity from both strain AN2690(R210K)(*q.v.* Chapter 5, section 4.2.(iii)) and strain AN2814(*c*D61E) was uninhibited by DCCD. The observation that the membrane-bound ATPase activity from the double mutant strain AN2765(*a*R210K,*c*D61E) was inhibited by DCCD, whilst both individual mutations were uninhibited suggests there may be an interaction between the residues. Further experiments in this laboratory are being conducted to ascertain whether the binding of DCCD to the substituted subunit-*c* residue Glu-61 is promoted by a concomittant substitution of R210K in subunit-*a*.

The original hypothesis of being able to substitute both Arg-210 in subunit-*a*, and Asp-61 in subunit-*c* by lysine and glutamate respectively, without affecting oxidative phosphorylation was incorrect. However, this may not be due to the inability of the two substituted residues to participate in proton exchange, it may be due to the inability of the subunit-*c* residue Glu-61 to accept a proton from the subunit-*a* residue Glu-219, or conversely, to the inability of the Lys-210 residue to donate a proton to the next member of the proton pore. An interesting observation is that the *E. coli* subunit-*a* contains a glutamate residue at position 219 and a histidine residue at position 245, with an aspartate at residue 61 in subunit-*c*, whilst subunit-*6* from the mitochondria of all species sequenced include a histidine at a comparable position to glutamate 219 and a glutamate at the histidine 245 equivalent, along with a glutamate in subunit-*9* at a comparable position to the *E. coli* *c*-subunit residue Asp-61. To operate efficiently in proton tranlocation, the functional residues may be forced to adopt a specific ordering, such that ASP-GLU-HIS may be tolerated, as may GLU-HIS-GLU, whilst GLU-GLU-HIS is non-functional in translocating protons across the membrane. Although purely hypothetical, this possibility may be explored by introducing substitutions in the *E. coli* F_oF_1-ATPase, whereby Asp-61 in subunit-*c* may be replaced by glutamate, Glu-219 of subunit-*a* by histidine, and His-245 of subunit-*a* by glutamate, effectively modelling half of the postulated mitochondrial F_oF_1-ATPase proton pore. This experiment gains more credibility on the observation that arginine is the integral residue of the postulated pore, from mitochondrial and bacterial F_oF_1-ATPases, which is predicted to accept a proton from either the subunit-*c* aspartate in *E. coli* or the subunit-9 glutamate in mitochondria.

CHAPTER 9

GENERAL DISCUSSION

9.1 The proton pore of the E. coli F_0F_1-ATPase

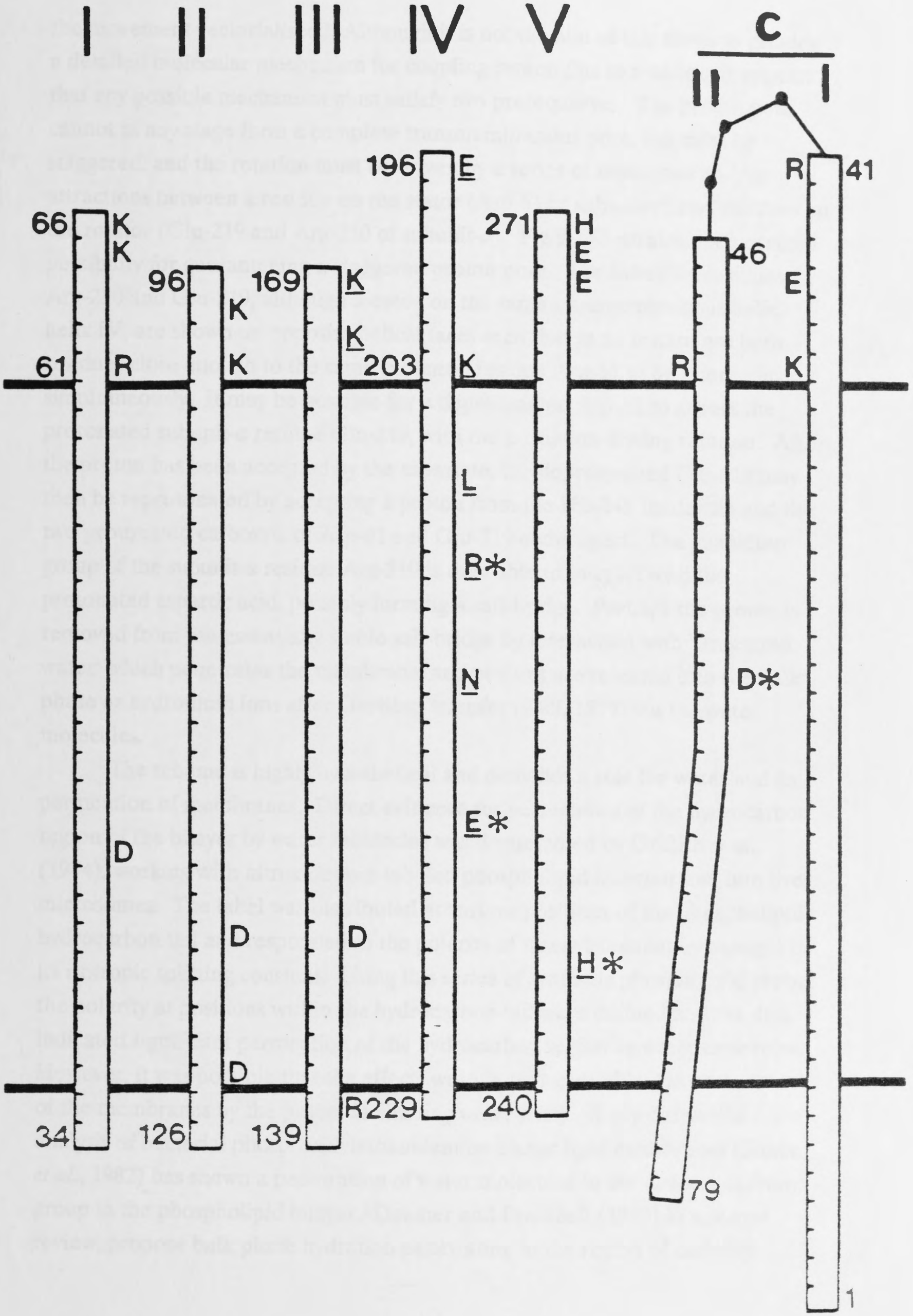
The data presented in the previous five chapters show unequivocally that substitution of residues Arg-210 and Glu-219 of subunit-*a* helix IV and His-245 of subunit-*a* helix V prevent translocation of protons through an assembled F_0F_1-ATPase complex. The involvement of subunit-*c* residue Asp-61 in proton translocation has been well documented (*q.v.* Chapters 1 and 8). However, data to support the direct involvement of the subunit-*a* residue Ser-206 as an essential member of the proton pore is not definitive (*q.v.* Chapter 7, section 7.4, see Cain and Simoni, 1986) and recent experiments from this laboratory (S.M. Howitt, personal communication) have shown that the substitution of Ser-206 by Ala results in a strain which is capable of growth on succinate and retains a growth yield comparable to a coupled control strain when both growth yields are measured at 37°C. Thus, it would appear that Ser-206 is not essential for proton translocation.

Fig 9.1 summarises the substitutions discussed in this study, with residues that have been shown to be involved in proton translocation denoted with an asterisk. The figure also represents the present secondary structural model for subunit-*a*, utilising the information gained after substitution of the highlighted residues. The combined data establishes experimentally, for the first time, a transmembranous proton pore delineated at the molecular level.

Having identified the integral residues of the transmembranous proton pore, the problem of discerning the exact molecular mechanism for coupling a membrane potential to phosphorylation has only partly been solved. The working model used and updated throughout this thesis postulates a conformational change in the F_1-ATPase to be produced by rotation of an inner intramembranous cluster of helices constructed out of two subunit-*b* helices and five subunit-*a* helices, rotating within an outer ring of *c*-subunit helices. The rotation is thought to be driven by the transmembranous proton flux. This rotation of the inner ring of intramembranous subunits produces a postulated cyclic conformational change at the three active sites on the F_1-ATPase mediated via the asymmetric arrangement of the three minor subunits -γ, -δ and -ε connected to the rotating cytoplasmic helices of the two *b*-subunits (*q.v.* Chapter 1, section 1.5).

There are two questions which may now be addressed given that the amino acids members of the proton pore have been identified. Firstly, if one accepts the possibility that rotation is the driving force behind coupling oxidative phosphorylation, how does the proton flux produce relative movement of the inner, rotator, helices with respect to the outer, stator, helices? Secondly, how is

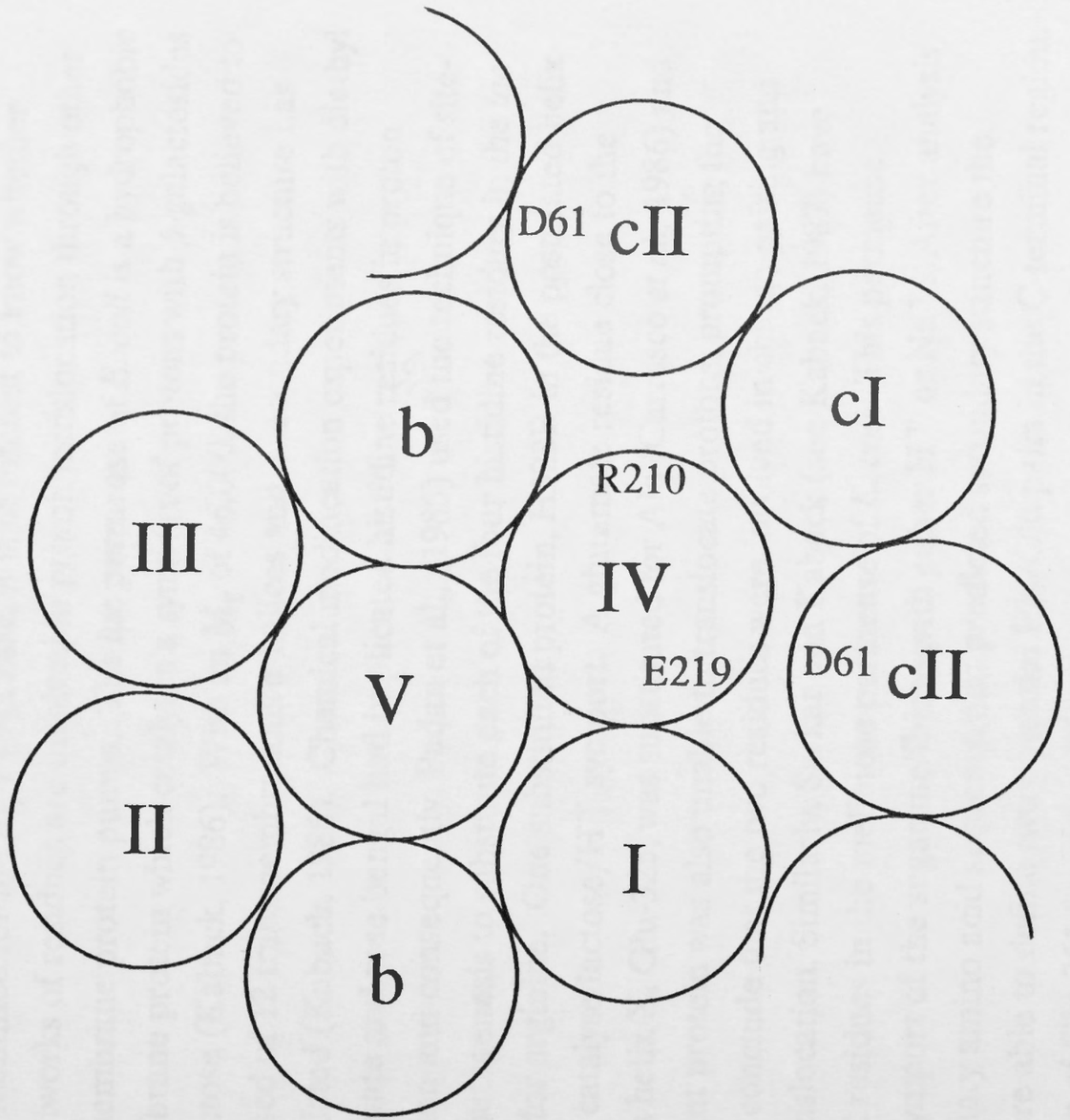
Fig. 9.1. Diagrammatic representation of the five predicted intramembranous helices of subunit-*a* and the two intramembranous helices of subunit-*c*. The number of the residue at both N- and C-terminals of each putative helix are given. The thick horizontal line towards the top of the page represents the membrane/cytoplasm border, whilst the lower thick line represents the membrane/periplasm border. Every ionisable residue predicted to be within the membrane is marked and all residues which have been substituted are underlined. An asterisk next to a residue denotes the residue is involved in proton translocation.


I
II
III
IV
V
C
II
I
66
K
K
61
R
D
96
K
K
D
169
K
K
D
D
196
E
203
K
L
R*
N
E*
D
D
R229
240
271
H
E
E
H*
R
46
D*
R
41
E
K
79
1
34
126
139

the movement vectorialised? Although it is not the aim of this thesis to provide a detailed molecular mechanism for coupling proton flux to rotation, it appears that any possible mechanism must satisfy two prerequisites. The proton pore cannot at any stage form a complete transmembranous pore, but must be staggered, and the rotation must be driven by a series of repulsions and/or attractions between a residue on the stator (Asp-61 of subunit-*c*) and residues on the rotator (Glu-219 and Arg-210 of subunit-*a*). Fig 9.2 illustrates one potential possibility for guaranteeing a staggered proton pore. The subunit-*a* residues Arg-210 and Glu-219, although located on the same intramembranous helix, helix IV, are shown on opposite helical faces such that at no instant are both residues close enough to the same subunit-*c* residue Asp-61 to be interacting simultaneously. It may be possible for a deprotonated Asp-61 to attract the protonated subunit-*a* residue Glu-219, with the attraction driving rotation. After the proton has been accepted by the aspartate, the deprotonated Glu-219 may then be reprotonated by accepting a proton from the His-245 imidazole and the two protonated carboxyls of Asp-61 and Glu-219 come apart. The guanidino group of the subunit-*a* residue Arg-210 is then able to interact with the protonated aspartic acid, possibly forming a salt-bridge. Perhaps the proton is removed from the essentially stable salt-bridge by interaction with 'structured water' which penetrates the membrane, and protons are released into the bulk phase as hydronium ions after Grotthus transfer (Kell, 1979) via the water molecules.

The scheme is highly hypothetical and demands a role for water and its permeation of membranes. Direct evidence for permeation of the hydrocarbon region of the bilayer by water molecules was documented by Griffith *et al.*, (1974), working with nitroxide spin-labeled phospholipid incorporated into liver microsomes. The label was distributed at various positions of the phospholipid hydrocarbon tail and responded to the polarity of its environment by changes in its isotropic splitting constant. Using this series of synthetic phospholipid probes the polarity at positions within the hydrocarbon tail were defined and the data indicated significant permeation of the hydrocarbon region by water molecules. However, it was possible that the effects were in part caused by the perturbation of the membranes by the probe, facilitating water entry. X-ray diffraction analysis of bacterial phosphatidylethanolamine planar lipid membranes (Simon *et al.*, 1982) has shown a penetration of water molecules to the deepest carbonyl group in the phospholipid bilayer. Deamer and Bramhall (1986) in a recent review, propose bulk phase hydration penetrating to the region of carbonyl

Fig. 9.2. Diagrammatic representation of the asymmetrical positioning of subunit-*a* residues Arg-210 and Glu-219 with respect to the subunit-*c* residues Asp-61. The five intramembranous helices of subunit-*a* are marked I to V and the two helices of the subunit-*b* dimer are marked b. Together, the seven helices constitute the membrane-internal 'rotator'. The outer ring, or stator, is comprised of a multimer of *c* subunits, with cI and cII referring to the two helices of each monomer. Residues Arg-210 and Glu-219 are shown on different helical faces of the amphipathic subunit-*a* helix IV. The subunit-*c* residue Asp-61 is shown on helix II.


D61 cII
b
cI
III
R210
IV
V
E219
D61 cII
II
I
b

oxygen of each monolayer, with a gradient of water monomers toward the centre of the hydrocarbon region. The proposed role for water molecules within the membrane is in accord with this data.

9.2. The application of site-directed mutagenesis in the definition of proton pores within other membrane-spanning proteins

As the residues involved in transmembranous proton translocation have now been identified for the F_o-F_1 ATPase, it is of interest to know whether similar networks of residues are involved in proton translocation through other integral membrane protein pumps. The *lac* permease of *E. coli* is a hydrophobic transmembrane protein which catalyses a symport of protons with β-galactosides such as lactose (Kaback, 1986). With an M_r of 46,000, the protein is believed to be composed of 12 transmembranous α-helices and a secondary structure has been predicted (Kaback, 1986). Chemical modification experiments with diethyl pyrocarbonate and rose bengal had implicated histidine residues in proton translocation and consequently, Padan et al., (1985) used the technique of site-directed mutagenesis to substitute each of the four histidine residues in the *lac* permease for arginine. One substituted protein, H322R in the postulated helix X, did not catalyse lactose/H^+ symport. A glutamate residue close to the arginine in helix X, Glu-325, was substituted for Ala (Carrasco *et al.*, 1986) and the resultant protein was also unable to translocate protons, prompting the authors to conclude that the two residues were involved in charge-pairing and proton translocation. Similarly, Sarkar and Kaback (see Kaback, 1987) have substituted residues in the melibiose permease of *E. coli*. This permease catalyses symport of the sugar melibiose with either H^+ or Na^+. After analysis of the primary amino acid sequence and predicted secondary structure the authors were able to define two potential His/Glu pairs in the C-terminal region. Substitution of Glu-361 for either Gly or Asp prevented proton/melibiose transport. The importance of a His-Glu pair located in the protein C-terminal one third has been shown conclusively in the work detailed in this thesis for proton translocation through the F_o-F_1 ATPase.

Computer modelling of helices IX and X of the *lac* permease revealed the side-chain of Ser-300 was in close proximity to the imidazole ring of His-322. Blow *et al.*,(1969), after crystallographic analysis of chymotrypsin, postulated a catalytic mechanism for the enzyme involving a charge-relay system consisting of an Asp-His-Ser chain. Thus, the potential triad in the *lac* permease of Glu-325,

His-322, Ser-300 was challenged by substituting Ser-300 for Ala. The resultant protein was unaffected in H^+/lactose symport (see Kaback, 1987). Although it may be premature on the basis of this data and on the data detailed in this thesis to assume that nonionisable polar residues are not functionally involved in proton translocation, only ionisable residues have so far been implicated in any transmembranous proton-pumping protein. Analysis of primary and predicted secondary structures of integral membrane proteins reveal that even hydrophobic regions postulated to be intramembranous often contain several of the polar residues asparagine, glutamine, serine, threonine or tyrosine. In the five postulated transmembranous helices for subunit-*a* of the *E. coli* F_oF_1-ATPase there are 25 polar residues. The subunit-*a* residue Asn-214 is potentially positioned optimally to interact with Arg-210, Glu-219 or both, but substitution of Asn-214 for Gly does not affect proton translocation (*q.v.* Chapter 7). Analysis of the X-ray crystallographic data obtained from the photosynthetic reaction centre of the purple bacterium *Rhodopseudomonas viridis* (Deisenhofer *et al.*, 1985) revealed eleven intramembranous helices. Of the 296 residues which comprise theses eleven helices, 49 were polar residues (Michel *et al.*, 1985, 1986). If these residues were able to form a type of hydrogen-bonded network similar to that proposed by Nagle and Morowitz (1979) it appears likely that several possible pathways for transmembranous proton translocation could be formed. It would also be highly likely that any integral membrane protein consisting of several transmembranous helices would be permeable to protons. As mentioned in Chapter 3, section 3.4, ionisable residues, buried in soluble proteins as charge-pairs, are stabilised by extensive hydrogen-bonding with neighbouring polar residues or protein backbone components. It is therefore proposed that the role of intramembranous polar residues is solely to provide stability to individual ionisable residues, or to the structure of the complex by intermolecular hydrogen-bonding.

Bacteriorhodopsin, an integral membrane protein of *Halobacterium halobium*, carries out light-dependent proton translocation from the inside to the outside of the cell. A recent series of papers by members of the Khorana group (Dunn *et al.*, 1987; Karnik *et al.*, 1987; Nassal *et al.*, 1987; Braiman *et al.*, 1987; Hackett *et al.*, 1987)) detail a system of expression and purification of bacteriorhodopsin in cells of *E.coli*. The gene encoding bacteriorhodopsin has been artificially synthesised and incorporates several uniformly spaced unique recognition sites for restriction endonucleases, facilitating site-directed mutagenesis. A secondary structural model with seven transmembrane helices has been predicted using electron and neutron diffraction data, proteolysis

studies and cross-linking experiments. The effects of specific amino acid substitutions can be calculated by purification of the altered protein, reconstitution of the protein into liposomes and measuring proton flux with a pH electrode. Four residues on the predicted helix F have been replaced, but these substitutions did not alter proton translocation. Analysis of the predicted secondary structure highlights several ionisable residues which appear to be well placed to facilitate proton translocation. The three residues Asp-85, Asp-96 and Arg-82 are all predicted to be members of Helix C, Asp-115 in helix D and two membrane-peripheral arginines, Arg-175 in helix F and Arg-225 on helix G are all candidates for involvement in the proton flux. However, the whole protein molecule does not contain a histidine residue, showing the His/Glu pairing may not be essential for transmembranous proton pumping.

9.3. The application of site-directed mutagenesis in the definition of intramembranous structure.

The assumption, that ionisable residues, if not involved in either proton translocation or structure stability are not located within the membrane, has been used in this thesis to rationalise the overall secondary structure of subunit-*a*. It is an important assumption as, if correct, it enables information to be gained about the structure of the protein. For example, one substitution documented in both the *lac* permease and bacteriorhodopsin involved ionisable residues being replaced by nonionisable amino acids with no effect on proton translocation. However, these two residues, Lys-319 of the *lac* permease and Glu-194 in bacteriorhodopsin, are represented as being integral membrane residues. These residues may therefore not be intramembranous and the predicted secondary structure should be corrected to compensate for this result. Hackett *et al.*,(1987) state their intentions to substitute all nine glutamate residues and all seven aspartate residues in bacteriorhodopsin for nonionisable residues in an attempt to define the residues involved in proton translocation. The results should assist in assessing whether or not the assumption used in this thesis is valid.

In conclusion, the mutant approach can be used *ab initio* to study the structure and function of any particular intramembranous enzyme by following a series of well defined stages. Firstly, the gene encoding the particular protein of interest must be cloned and the primary amino acid sequence determined after DNA sequence analysis. Mutants containing the altered protein are then produced by random mutagenesis with a chemical mutagen, and the specific

amino acid substitutions deduced again by DNA sequence analysis. Mutations which affect the assembly of the protein or the function of the protein can be defined, eventually enabling predictions of the effects of specific amino acid substitutions to be made and a structural model of the enzyme to be built. Then, by the method of site-directed mutagenesis, these predictions can be challenged. This approach has been successfully used in the study of the *E. coli* F_o-F_1 ATPase despite the absence of the X-ray crystallographic structure.

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