TO

MUM AND DAD

A THESIS

submitted for the Degree of

DOCTOR OF PHILOSOPHY

in the

Australian National University

by

PACKIN SANTHEEPAN,

STUDIES ON A BIFUNCTIONAL ENZYME
FROM *ESCHERICHIA COLI*

Purification, properties and kinetic characteristics of chorismate mutase-prephenate dehydrogenase

A THESIS submitted for the degree of DOCTOR OF PHILOSOPHY in the Australian National University by PADMINI SAMPATHKUMAR

September, 1978
This thesis embodies the results of research carried out in the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, from October, 1974, to September, 1978 during the tenure of a Rotary Graduate Fellowship and an Australian National University Research scholarship.
STATEMENT

In accordance with the regulations of the Australian National University, I wish to state that, with the exception of the ultracentrifugal analysis and the preparation of peptide maps, the work described in this thesis was carried out by me under the supervision of Dr. J. F. Morrison.
ACKNOWLEDGEMENT

It is a pleasure to thank my supervisor, Dr. J. F. Morrison for his helpful guidance and encouragement throughout the course of this work.

My sincere thanks go to Dr. R.G. Duggleby for his constructive criticisms of the drafts of this thesis and for his helpful suggestions on many aspects of this work. In addition, I would like to express my gratitude to the following members of the department for their assistance in various ways: to Dr. K. J. Ellis, Dr. E. Heyde, Dr. D. Magrath, Dr. J. T. Powell, Dr. I. G. Young, Miss P. Dudziński, Mr. D. Fayle and Mr. A. Laird for helpful discussions, to Dr. J. Rood and Dr. J. Williams for proof-reading manuscripts, to Mrs. M. Sneddon for her cheerful and skilful technical assistance, to Mr. D. Abigail for his help in preparing cell extracts, and to Mr. L. James for amino acid analysis.

I am also grateful to Dr. H.R.P. Miller and Dr. K. J. Lafferty for their advice and comments on the immunological studies reported in this thesis and to Dr. S. Searjentson, Dr. D.O. Willenborg and Miss A. Boots for advice on immunological techniques. Further, I would like to express my appreciation to Professor L. W. Nichol for performing the ultracentrifugation experiments, to Dr. D.C. Shaw for the preparation of peptide maps and to Dr. D. Magrath for the preparation of the ligands used for affinity chromatography.

The excellent service provided by Miss. J. Fields and her staff from the 'Microanalysis' section and by Mr. R. Westen and his
staff from the 'Photography' section are gratefully acknowledged.

Thanks are also due to Mrs. M. May for her skill and patience in typing this manuscript and to Victor Solo for his assistance with preparation of diagrams and for his good humour and friendship.

Finally, I wish to thank my parents whose love, encouragement and many self-sacrifices have made these studies possible.
PREFACE

Throughout the thesis, Figures and Tables are presented on separate pages and in most instances follow the page of text on which the first reference to them has been made.

The following abbreviations have been used:

- **AMP**: Adenosine 5'-monophosphate
- **ADP**: Adenosine diphosphate
- **DEAE**: Diethylamino ethyl
- **EDTA**: Ethylene diamine tetra-acetic acid
- **Hg**: Mercury
- **NAD**: Nicotinamide-adenine dinucleotide
- **NADH**: Reduced nicotinamide-adenine dinucleotide
- **P_i**: Inorganic orthophosphate
- **Tris**: Tris (hydroxymethyl) aminomethane
The bifunctional enzyme chorismate mutase-prephenate dehydrogenase catalyzes the conversion of chorismate to prephenate which is subsequently oxidized to hydroxyphenylpyruvate in the presence of NAD. A pure, stable preparation of this enzyme has been obtained in good yield from a regulatory mutant of *E.coli*. The enzyme was purified from extracts of the organism by treatment with streptomycin sulphate, fractionation with ammonium sulphate, followed by chromatography on Sepharose-AMP, DEAE-cellulose and hydroxylapatite columns. The native enzyme has a molecular weight of 88,000 and is made up of two identical subunits as indicated by the results of amino acid composition, peptide mapping and electrophoresis in sodium dodecyl sulphate. The enzyme has a sedimentation coefficient of 4.8 S as determined in the ultracentrifuge and an isoelectric point of 5.3.

Kinetic studies have been carried out on both the mutase and dehydrogenase reactions. The results of steady state velocity studies in the presence of substrates only as well as product and dead-end inhibition studies indicate that the reaction conforms to a rapid equilibrium, random mechanism involving the formation of two dead-end complexes *viz* enzyme-NADH-prephenate and enzyme-NAD-hydroxyphenylpyruvate. The random nature of the reaction mechanism is confirmed by the results of binding studies which show that both NAD and prephenate combine with the free form of enzyme and that the values of the dissociation constants obtained from binding studies are in reasonable agreement with those obtained kinetically. A comparison of the magnitudes of kinetic parameters as determined from studies on the mutase and dehydrogenase reactions suggest that different active sites are responsible for the two catalytic
activities of the enzyme. This conclusion is further supported by the observation that the number of moles of prephenate bound to the free enzyme exceeds the number of moles of bound NAD, by a factor of two. Kinetic data for the overall reaction catalyzed by the enzyme suggest that there is some interaction between the two catalytic sites on the enzyme and that some of the prephenate produced at the mutase site may be channelled to the dehydrogenase site without being released into the medium.

Differential inhibition of the two activities of the enzyme is observed on titration of the enzyme with antiserum raised to a pure preparation of the enzyme. Prephenate and NAD lower the inhibition of both enzymic activities by the antiserum while NADH is effective only in protecting against loss of dehydrogenase activity. These results indicate that the inhibition of the two individual activities of the enzyme by the antiserum involves interaction with different antigenic determinants on the enzyme molecule. Such a conclusion is consistent with the results of kinetic and binding studies which suggest that the mutase and dehydrogenase reactions are catalyzed by the enzyme at different active sites.

Studies with antiserum to \textit{E.coli} chorismate mutase-prephenate dehydrogenase showed that cross reactions occurred with chorismate mutase-prephenate dehydrogenase from \textit{A.aerogenes} and chorismate mutase-prephenate dehydratase from \textit{E.coli} but not with either the aspartate or aromatic amino acid aminotransferase from \textit{E.coli}. These findings show that the \textit{E.coli} chorismate mutase-prephenate dehydrogenase exhibits a high degree of homology with the same enzyme from \textit{A.aerogenes} and a lesser degree of homology with the chorismate mutase-prephenate dehydratase from \textit{E.coli}. It appears therefore, that the three bifunctional enzymes may have similar evolutionary origins.
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CHAPTER I

GENERAL INTRODUCTION

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3. MULTIMERENZYMES AND MULTIFUNCTIONAL ENZYMES

4. PROPERTIES OF THE BIFUNCTIONAL ENZYMES CHOROSPIRE NITRASE-PHOSPHATE HYDROGENASE

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GENERAL INTRODUCTION
CHAPTER I

GENERAL INTRODUCTION

The enzyme chorismate mutase-prephenate dehydrogenase catalyzes two sequential reactions along the tyrosine biosynthetic pathway. It is an essential enzyme since the two activities reside on a single polypeptide chain. The mutase activity of the enzyme is responsible for the subsequent dehydrogenase activity of the enzyme as responsible for the subsequent oxidation state. The enzyme occurs at the beginning of the branch pathway leading to tyrosine and has an important role in the branch pathway leading to tyrosine and has an important role in the production of aromatic amino acids.

1. INTRODUCTION

2. THE PATHWAY OF BIOSYNTHESIS OF AROMATIC AMINO ACIDS

3. MULTIENZYMES COMPLEXES AND MULTIFUNCTIONAL ENZYMES

4. PROPERTIES OF THE BIFUNCTIONAL ENZYME CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE

5. AIMS OF THIS INVESTIGATION
1. **INTRODUCTION**

The enzyme chorismate mutase-prephenate dehydrogenase catalyzes two sequential reactions along the tyrosine biosynthetic pathway and is a bifunctional enzyme since the two activities reside on a single polypeptide chain. The mutase activity of the enzyme is responsible for the conversion of chorismate to prephenate and the dehydrogenase activity of the enzyme is responsible for the subsequent oxidative decarboxylation of prephenate to hydroxyphenylpyruvate in the presence of NAD (Fig.1.1). The enzyme occurs at the beginning of the branch pathway leading to tyrosine and has an important role in the regulation of tyrosine biosynthesis.

The work presented in this thesis is concerned with the elucidation of the properties of chorismate mutase-prephenate dehydrogenase from *Escherichia coli* with special reference to its bifunctional nature. The aim of this introduction is, therefore, to present the properties of chorismate mutase-prephenate dehydrogenase that were known at the time this work began and to compare these with some of the general features of other multifunctional enzymes and multienzyme complexes.

2. **THE PATHWAY OF BIOSYNTHESIS OF AROMATIC AMINO ACIDS**

The pathway of biosynthesis of aromatic amino acids is now well established (Umbarger and Davis, 1962; Gibson and Pittard, 1968) and is shown in Fig.1.2. The first reaction in this pathway involves the condensation of erythrose-4-phosphate and phosphoenolpyruvate, both compounds being derived from glucose, to form 3-deoxy D-arabino
FIG.I.1. THE REACTIONS CATALYZED BY THE BIFUNCTIONAL ENZYME
CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE.
FIG. 1.2. PATHWAYS FOR THE BIOSYNTHESIS OF AROMATIC AMINO ACIDS

The biosynthesis of tryptophan proceeds through a series of reactions that have been studied in prokaryotes and eukaryotes so far studied. The first reaction in the sequence is catalyzed by the enzyme anthranilate synthase and involves an amidopyruvyl elimination accompanied by a glutamine amide transfer. The second reaction is the addition of the phosphoribosyl moiety of 5-phosphoribosyl-1-pyrophosphate (PRPP) to anthranilate catalyzed by phosphoribosylanthranilate isomerase. An amidopyruvyl elimination and ring closure catalyzed by indoleglycerol phosphate synthase. The final step is the removal of the glycerol phosphate side-chain from indoleglycerol phosphate and its replacement by the alanin moiety of L-serine, catalyzed by the enzyme, tryptophan synthase. The pathway is effectively regulated by tryptophan, which acts as a feed back inhibitor of anthranilate synthase, the enzyme catalyzing the first tryptophan-specific reaction in the sequence.
heptulosonic acid-7-phosphate (DAHP), which is a straight chain, 7-carbon compound. This compound is then cyclized and converted in a number of steps to chorismate. Chorismate, which is the end product of what is referred to as the 'common pathway', serves as a branch point intermediate in the synthesis of a variety of aromatic compounds including phenylalanine, tyrosine, tryptophan, Vitamin K, menaquinone, ubiquinone, folic acid and enterochelin (Gibson and Pittard, 1968).

The biosynthesis of tryptophan from chorismate proceeds through a series of reactions that has not varied in any of the prokaryotes and eukaryotes so far studied. The first reaction in the sequence is catalyzed by the enzyme anthranilate synthase and involves an enolpyruvyl elimination accompanied by a glutamine amido transfer. The second reaction is the addition of the phosphoribosyl moiety of 5-phosphoribosyl-1-pyrophosphate to the 3-position of anthranilate, catalyzed by anthranilate phosphoribosyltransferase. An Amadori rearrangement of phosphoribosylanthranilate catalyzed by phosphoribosylanthranilate isomerase is followed by a step involving decarboxylation and ring closure catalyzed by indoleglycerol phosphate synthase. The final step is the removal of the glycerol phosphate side-chain from indoleglycerol phosphate and its replacement by the alanyl moiety of L-serine, catalyzed by the enzyme, tryptophan synthase. The pathway is effectively regulated by tryptophan, which acts as a feed back inhibitor of anthranilate synthase, the enzyme catalyzing the first tryptophan-specific reaction in the sequence.

One of the remarkable features of the tryptophan biosynthetic pathway is that a number of enzymes catalyzing individual reactions in
this pathway occur as multifunctional enzymes or multienzyme complexes in prokaryotes as well as eukaryotes. Thus, in some enteric bacteria, the enzyme anthranilate synthase is associated with the enzyme anthranilate phosphoribosyltransferase, forming a multienzyme complex and in all enteric bacteria studied to date, the enzymes phosphoribosylanthranilate isomerase and indoleglycerol phosphate synthase are linked forming a multifunctional enzyme. The synthesis of tryptophan from indoleglycerol phosphate and serine is also catalyzed by a multienzyme complex in enteric bacteria and by a multifunctional enzyme in *Neurospora crassa*.

In contrast to the tryptophan biosynthetic pathway, the biosynthesis of phenylalanine and tyrosine diverge from prephenate, which is an intermediate derived from chorismate by a Claisen rearrangement. In the pathway leading to phenylalanine, prephenate is converted to phenylpyruvate which undergoes a transamination reaction to form phenylalanine. In the branch pathway leading to tyrosine, the oxidative decarboxylation of prephenate yields hydroxyphenylpyruvate reaction, which then undergoes a transamination reaction forming tyrosine.

The intermediates between chorismate, phenylalanine and tyrosine namely prephenate, phenylpyruvate and 4-hydroxyphenylpyruvate appear to be common to all organisms synthesizing phenylalanine and tyrosine from chorismate. However, several patterns of control mechanisms and enzyme organization have been described for the two pathways in prokaryotic as well as eukaryotic systems.

In enteric bacteria including *E.coli, Aerobacter aerogenes* and *Salmonella typhimurium*, two distinct chorismate mutases are involved
one of which is linked with prephenate dehydratase while the other
forms a bifunctional enzyme with prephenate dehydrogenase (Cotton and
Gibson, 1965; Davidson et al, 1972; Schmit and Zalkin, 1969).
Phenylalanine is an end product inhibitor of the enzyme chorismate
mutase-prephenate dehydratase and tyrosine is a strong inhibitor of
the dehydrogenase activity of the enzyme chorismate mutase-prephenate
dehydrogenase (Fig.I.3a).

Multiple molecular forms of chorismate mutase have been
separated from extracts of *Bacillus subtilis* (Lorence and Nester, 1967).
Another strain of the same organism has a single chorismate mutase that
is linked with DAHP synthase, the first enzyme of the aromatic amino
acid pathway, to form a bifunctional protein (Huang et al, 1974a, b).
No associations of chorismate mutase with succeeding enzymes have been
found in *N.crassa* (Baker, 1968), *Saccharomyces cerevisiae* (Sprossler
et al, 1970), *Streptomyces aureofaciens* (Görisch et al, 1974), and

In the hydrogen bacterium *Alcaligenes eutrophus*, chorismate
mutase and prephenate dehydratase activities occur as a bifunctional
enzyme, while the enzyme prephenate dehydrogenase is not linked to any
other protein of the pathway (Fig.I.3b). Both activities of the
bifunctional enzyme are inhibited by phenylalanine while tyrosine
activates only the dehydratase reaction catalyzed by the enzyme and
causes the inhibition of the monofunctional prephenate dehydrogenase
(Friedrich et al, 1976a,b).

In blue-green bacteria, the enzymic reactions leading from
prephenate to tyrosine occur in a different sequence to that described
FIG. 1.3. ORGANIZATION OF ENZYMES INVOLVED IN PHENYLALANINE AND TYROSINE BIOSYNTHESIS IN (a) ENTERIC BACTERIA (b) ALCALIGINES EUTROPHUS (c) NEUROSPORA CRASSA.

ENZYMES: (1) CHORISMATE MUTASE-PREPHENATE DEHYDRATASE, (2) PHENYLPYRUVATE AMINOTRANSFERASE, (3) CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE, (4) 4-HYDROXYPHENYLPYRUVATE AMINOTRANSFERASE, (5) PREPHENATE DEHYDROGENASE, (6) CHORISMATE MUTASE AND (7) PREPHENATE DEHYDRATASE (BRACKETS DENOTE BIFUNCTIONAL ENZYMES).
above (Fig.I.4a). This sequence involves an enzyme prephenate transaminase which catalyzes the transamination of prephenate to pretyrosine. The second reaction in this pathway is the dehydrogenation of pretyrosine, catalyzed by the enzyme pretyrosine dehydrogenase. The enzymes for the biosynthesis of phenylalanine appear to be identical to those observed in other microorganisms and include a single prephenate dehydratase and a phenylalanine transaminase (Stenmark et al., 1974). The regulation of both the phenylalanine and tyrosine biosynthetic pathways appears to be mediated through the enzyme prephenate dehydratase whose activity is modulated by the end products phenylalanine and tyrosine. Phenylalanine is a strong allosteric inhibitor of the enzyme while tyrosine is a powerful activator.

The enzymes involved in the biosynthesis of phenylalanine and tyrosine as well as their regulation are even more complex in P. aeruginosa (Fig.I.4b) than in the organisms mentioned above. Pretyrosine appears to be an intermediate in the biosynthesis of both phenylalanine and tyrosine in this organism (Jensen and Stenmark, 1975; Patel et al., 1977). In addition, this organism also retains the phenylpyruvate and hydroxyphenylpyruvate pathway to the biosynthesis of phenylalanine and tyrosine. The organism also possesses two proteins with chorismate mutase activity, one of which is bifunctional and possesses both chorismate mutase and prephenate dehydratase activities. The other chorismate mutase, known as chorismate mutase II, is an enzyme of lower molecular weight and may be involved in the tyrosine biosynthetic pathway. The phenylpyruvate produced by the bifunctional enzyme undergoes transamination to phenylalanine by phenylalanine transaminase. The flux along this pathway is regulated by phenylalanine which is an end product inhibitor of the bifunctional enzyme. Prephenate
FIG. 1.4. REACTIONS OF ENZYMES INVOLVED IN PHENYLALANINE AND TYROSINE BIOSYNTHESIS IN (A) BLUE-GREEN BACTERIA AND (B) PSEUDOMONAS AERUGINOSA.

(1) CHORISMATE MUTASE (2) PREPHENATE DEHYDRATASE (3) PHENYLPYRUVATE AMINOTRANSFERASE (4) PREPHENATE AMINOTRANSFERASE (5) PRETYROSINE DEHYDROGENASE (6) PRETYROSINE DEHYDRATASE (7) PREPHENATE DEHYDROGENASE (8) 4-HYDROXYPHENYLPYRUVATE AMINOTRANSFERASE.

In PSEUDOMONAS AERUGINOSA, THERE ARE TWO ENZYMES CATALYZING REACTION (1), ONE OF WHICH IS LINKED TO THE ENZYME CATALYZING REACTION (2) TO FORM A BIFUNCTIONAL ENZYME (DENOTED BY THE BRACKET). REACTIONS (5) AND (7) MAY BE CATALYZED BY A SINGLE DEHYDROGENASE AND REACTIONS (3), (4) AND (8) BY A SINGLE AMINOTRANSFERASE.
formed by chorismate mutase II is converted to pretyrosine by the enzyme prephenate transaminase or to hydroxyphenylpyruvate by prephenate dehydrogenase. When pretyrosine is produced in the above reaction, it is converted either to phenylalanine by the action of pretyrosine dehydratase or to tyrosine by the action of pretyrosine dehydrogenase. Both prephenate dehydrogenase and pretyrosine dehydrogenase activities are feedback inhibited by tyrosine (Patel et al., 1977).

The existence of both the pretyrosine and the 4-hydroxyphenylpyruvate pathway in *P. aeruginosa* may be related to the broad substrate specificity exhibited by some of the enzymes in the pathway. Thus it appears that a single dehydrogenase may catalyze the oxidation of both prephenate and pretyrosine in this pathway. Similarly a single transaminase may be responsible for the transamination reactions involving prephenate, phenylpyruvate or 4-hydroxyphenylpyruvate as substrates (Patel et al., 1977).

Jensen and Pierson (1975) have discussed the evolutionary implications of the presence of such diverse organizations of the tyrosine biosynthetic enzymes in different bacteria. They have suggested that the 4-hydroxyphenylpyruvate pathway of tyrosine biosynthesis of modern enteric bacteria may have originated as a pretyrosine sequence. Thus the pretyrosine pathway in blue-green bacteria may represent an ancestral pathway while the dual pathway operating in *P. aeruginosa* may represent a stage of pathway evolution intermediate between that in blue-green bacteria and *E. coli*.

3. **MULTIENZYME COMPLEXES AND MULTIFUNCTIONAL ENZYMES**

In contrast to multifunctional enzymes in which multiple
reactions are catalyzed by a single polypeptide chain, the various enzyme activities in a multienzyme complex are contributed by different subunits held together by noncovalent interactions (Ginsberg and Stadtman, 1970). Thus it is not possible to separate a multifunctional protein into non identical subunits, each of which carries out one of the multiple reactions catalyzed by the intact protein (Kirschner and Bisswanger, 1976).

Most of the known multifunctional enzymes and multienzyme complexes appear to occur in bacteria or fungi and are predominantly found in pathways of amino acid biosynthesis. However, notable examples have also been discovered in mammals. These include the fatty acid synthases from chicken and rat liver (Stoops et al, 1975; Qureshi et al, 1974) and carbamyl phosphate synthetase-aspartate transcarbamylase isolated from hamster cells (Coleman et al, 1977). However, it must be noted that the organization of enzymes into multienzyme complexes and multifunctional enzymes does not occur uniformly in all organisms. Thus enzymes along metabolic pathways may occur in different organisms as individual proteins, multienzyme complexes or multifunctional enzymes. This is true for the multienzyme complex anthranilate synthase-anthranilate phosphoribosyl transferase which occurs as a multienzyme complex only in some enteric bacteria (Crawford, 1975) and for the multifunctional enzymes phosphoribosylantranilate isomerase-indoleglycerol phosphate synthase (Crawford, 1975), chorismate mutase-prephenate dehydratase and chorismate mutase-prephenate dehydrogenase (Fig.1.3), which occur as multifunctional enzymes in enteric bacteria and as two single proteins in other bacteria and fungi. These observations have led to the suggestion that the evolution of multifunctional enzymes from small, individual proteins may have occurred via multienzyme complexes (Smith, 1970; Bonner et al, 1965; Yourno
et al, 1970, 1972). The evolution of a multifunctional enzyme from a multienzyme complex may have involved the translocation and subsequent fusion of genes causing the polypeptide product of one gene to be linked through peptide bond formation to the polypeptide product of adjacent genes. The fused polypeptide chains could have retained the ability to fold independently into catalytically active domains connected by a flexible region. Although such ideas on the evolution of multifunctional enzymes remain hypothetical, the conservation of primary structure observed between domains of multifunctional enzymes and the corresponding monofunctional enzymes in different organisms is consistent with the proposed pattern of evolution (Li et al, 1974a,b). These suggestions also imply that multifunctional enzymes possess a number of independent catalytic sites at which the individual reactions are catalyzed. Many multifunctional enzymes catalyzing nonsequential reactions have been shown to possess such independent active sites, based on evidences from selective chemical inactivation, partial proteolytic digestion or the production of mutants with one or the other activity (Kirschner and Bisswanger, 1976). Among multifunctional enzymes catalyzing sequential reactions, biochemical and genetic studies on the enzyme chorismate mutase-prephenate dehydratase from E.coli clearly indicate that the enzyme possesses two distinct catalytic sites (Gething, 1973; Baldwin, 1974; Duggleby et al, 1978). The immunochemical evidence presented by Kaplan et al (1964) suggests that the bifunctional enzyme tryptophan synthase from N.crassa may also possess two independent polypeptide "domains" associated with the two activities of the enzyme.

One of the possible advantages of organizing enzymes into multienzyme complexes or multifunctional enzymes is the possible
enhancement of the catalytic properties of the individual enzymes comprising the aggregates (Reed and Cox, 1966; Welch, 1977). This may be illustrated with tryptophan synthase, a well-known multienzyme complex, which is composed of two types of subunits (α and β₂ subunits), formerly designated the A and B proteins (Crawford and Yanofsky, 1958). The subunits are completely different proteins with quite different physical and catalytic properties (Yanofsky, 1960; Creighton and Yanofsky, 1970). Separately, the α and β₂ units have distinct, but only partial activities on the enzymic half reactions:

\[
\begin{align*}
\text{Indole glycerol -P} & \xrightarrow{\alpha} \text{indole + glyceraldehyde-3-P} \quad (I-1) \\
\text{Indole} + \text{L-serine} & \xrightarrow{\beta_2:pyridoxal-P} \text{L-tryptophan} \quad (I-2) \\
\text{Indole glycerol -P} + \text{L-serine} & \xrightarrow{\alpha_2\beta_2:pyridoxal-P} \text{L-tryptophan + glyceraldehyde-3-P} \quad (I-3)
\end{align*}
\]

With reconstituted α₂β₂ complex, the overall reaction (I-3) is catalyzed with a turnover number 30-to 100-fold greater than that of the individual subunits for the half reactions. The coupling of reactions I-1 and I-2 to yield the overall functionally significant reaction I-3 occurs only when the α and β₂ subunits are physically associated. Spectral studies (Miles et al, 1968; Goldberg et al, 1968) have suggested that conformational changes are induced in both types of subunits as a result of the interactions between them and that these changes lead to an increased efficiency of their inherent catalytic functions.

Similar enhancement of the rate of the overall reaction has been observed for the arom enzyme conjugate from N.crassa (Gaertner et al, 1970) which is a multifunctional protein with five distinct sequential enzymic activities residing on a dimer of a single
polypeptide chain (Lumsden and Coggins, 1977; Gaertner and Cole, 1977). Welch (1977) has suggested that such enzyme aggregates may possess some form of an energy transfer scheme where the activation energy for each step may be derived by direct excitation within the aggregate, leading to an enhancement of the observed overall rate. The transfer of intermediates from site-to-site in optimal steric positions may also facilitate catalysis.

Another advantage attributable to multienzyme complexes or multifunctional proteins catalyzing sequential reactions is their potential for channelling or compartmentalizing the intermediates of a pathway. Several lines of evidence suggest that channelling may play an important role in the operation of diverse pathways that produce common intermediates and use separate isozymes. In *N. crassa*, there exist two isofunctional carbamyl phosphate synthetases, one of which is complexed with aspartate transcarbamylase and is under the genetic control of genes for pyrimidine biosynthesis while the other is under the control of genes for arginine biosynthesis (Davis, 1963; Reissig, 1963). Deficiencies of either enzyme causes the organism to become auxotrophic for the end product of the corresponding pathway; thus the carbamyl phosphate formed by either carbamyl phosphate synthetase is not free to equilibrate in a common intracellular pool, but is compartmented or segregated into separate pathways (Davis et al, 1967). Complete channelling of intermediates has also been observed for the bifunctional enzyme tryptophan synthase from *N. crassa* (Gaertner et al, 1970) and for the fatty acid synthase complex from yeast and pigeon liver although in the latter case, the intermediates formed are covalently bound to sulphydryl binding sites on the enzyme (Lynen, 1970; Phillips et al, 1970). Thus intracellular compartmentation of
intermediates common to more than one pathway may be a function of multienzyme aggregates in which enzymes which catalyze consecutive steps in a sequence are complexed, thereby protecting intermediates from degradation or diversion into other pathways.

For multifunctional enzymes catalyzing nonsequential reactions, it is possible that the advantages are related to the regulatory properties of the component enzymes. The arrangement of multiple catalytic activities on a single protein molecule would permit coordinate inhibition or activation of enzyme activities by a single effector ligand. The bifunctional enzyme Aspartokinase I-homoserine dehydrogenase I from *E.coli* is an example of a multifunctional enzyme catalyzing non sequential reactions (Patte *et al*, 1966) which constitute the first and the third steps in the pathway of biosynthesis of amino acids derived from aspartate (Fig.I.5). Both activities of the enzyme are inhibited by the end product threonine. Aspartokinase II-homoserine dehydrogenase II is also a bifunctional enzyme in the same pathway, both activities of which are feedback inhibited by methionine (Patte *et al*, 1967; Falcoz-Kelly *et al*, 1969).

The catalytic and regulatory advantages described above are not apparent for some multifunctional enzymes. Chorismate mutase-prephenate dehydratase from *E.coli* is a bifunctional enzyme whose catalytic and regulatory properties have been studied in detail (Gething, 1973; Baldwin, 1974; Duggleby *et al*, 1978). The enzyme has been shown to possess two separate and distinct active sites. Despite the fact that the enzyme catalyzes two sequential reactions along the phenylalanine biosynthetic pathway, no interaction between the sites or any channelling of the intermediate, prephenate, have been observed.
FIG. 1.5. END PRODUCT-REGULATION OF THE BIOSYNTHETIC PATHWAY OF AMINO ACIDS OF THE ASPARTATE FAMILY.

--- REPRESENTS INHIBITION.
(Duggleby et al, 1978). Thus the behaviour of the enzyme appears to be virtually identical to that expected for an equimolar mixture of two monofunctional enzymes. Duggleby et al (1978) have suggested that the bifunctional enzyme may have evolved as a result of accidental fusion of the chorismate mutase and prephenate dehydratase genes and may be retained by some organisms simply because it is not detrimental. In this connection, it must be noted that mutations resulting in the fusion of adjacent genes have been observed in *S. typhimurium* (Yourn et al, 1970). The kinetic properties of the resulting bifunctional enzyme imidazoleacetol phosphate aminotransferase-histidinol dehydrogenase have been shown to differ little from those of the corresponding monofunctional enzymes, indicating that the correct folding of individual domains is retained and that the individual catalytic activities are not enhanced when they are connected by a stretch of polypeptide chain (Rechler and Bruni, 1971).

Gene fusion appears to be a plausible mechanism for the evolution of multifunctional enzymes. Whether this fusion is a recent event or whether single polypeptides were derived from fused ancestors is not known. A knowledge of the structure of some multifunctional and the corresponding monofunctional enzymes might allow conclusions to be reached about the order of these events.

4. PROPERTIES OF THE BIFUNCTIONAL ENZYME CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE

The bifunctional nature of chorismate mutase-prephenate dehydrogenase was demonstrated by Cotton and Gibson (1965) who showed that cell free extracts of *E.coli* and *A.aerogenes* contained two chorismate
mutases that were easily separable by DEAE-cellulose chromatography. One of the peaks of mutase activity coincided with that of prephenate dehydratase activity and the other with that of prephenate dehydrogenase activity. During the purification of the enzyme from *A. aerogenes* (Cotton and Gibson, 1967), no separation of the mutase and dehydrogenase activities was observed. The ratio of the two activities also appeared to remain virtually constant throughout the purification procedure, confirming the bifunctional nature of the protein. Subsequently, Koch *et al* (1970a, 1971a) reported the isolation of chorismate mutase-prephenate dehydrogenase in pure form from both *E. coli* and *A. aerogenes*. In a study of the comparative properties of the two enzymes, they observed that the enzymes had similar molecular weights of around 80,000 and were composed of subunits of molecular weight 40,000. The two enzymes also appeared to share a considerable similarity in amino acid composition. In a preliminary study of the kinetic properties of the enzymes, Koch *et al* (1970a, 1971a) reported hyperbolic saturation curves for all substrates using the enzyme from *A. aerogenes*. On the other hand for the enzyme from *E. coli*, the substrate saturation curves were reported as being sigmoidal with both chorismate and prephenate, while hyperbolic with NAD. In the presence of tyrosine and with prephenate as the variable substrate, both enzymes were observed to exhibit clearly sigmoidal kinetics.

A detailed study of the kinetic properties of chorismate mutase-prephenate dehydrogenase from *A. aerogenes* has been recently reported by Heyde and Morrison (1978) who established the kinetic mechanism for the dehydrogenase reaction catalyzed by the enzyme as being rapid equilibrium random with the formation of E-NADH-prephenate and E-NAD-hydroxyphenylpyruvate dead-end complexes. These authors
have also compared the values for the dissociation constant of the common intermediate prephenate, as obtained in the dehydrogenase reaction and in the mutase reaction. Similarities in the observed value have led them to suggest that the enzyme possibly possesses a single catalytic site at which both the mutase and the dehydrogenase reactions may be catalyzed or two identical or closely similar sites at which the individual reactions take place. No such kinetic evidence regarding the number of catalytic sites is available for the E.coli enzyme. But parallel inactivation of both the mutase and the dehydrogenase activity of the enzyme from E.coli, has been observed with a number of modifying reagents (Koch et al, 1972). Prephenate were observed to protect both activities, to the same extent, against inactivation. This observation has led to the suggestion that the two active sites of the enzyme from E.coli may be close together or contiguous.

5. AIMS OF THIS INVESTIGATION

The elucidation of the kinetic and thermodynamic properties of the bifunctional enzyme chorismate mutase-prephenate dehydrogenase from E.coli was the prime objective of the studies presented in this thesis. Pure and stable preparations of chorismate mutase-prephenate dehydrogenase were required for this purpose and hence a simple purification procedure that gave good yields of pure and stable enzyme was developed (Chapter II). Since the dehydrogenase reaction involves two substrates, it was necessary, as a first step towards understanding the overall reaction, to determine the order of addition of the substrates in this reaction. Steady state kinetic techniques have been used to determine the kinetic mechanism of the dehydrogenase reaction
(Chapter IV). As the enzyme catalyzes two sequential reactions, it was of interest to determine whether the two reactions are catalyzed at a single active site or at two independent sites on the enzyme. Towards this end, the relationship between prephenate binding sites in the mutase and in the dehydrogenase reaction has been determined in kinetic and thermodynamic studies (Chapter V). The relationship between mutase and dehydrogenase sites has been investigated further in immunological studies (Chapter VI) which have also been used to detect any evolutionary relationship that may exist between the two bifunctional enzymes chorismate mutase-prephenate dehydratase and chorismate mutase-prephenate dehydrogenase from *E.coli*. 
CHAPTER II

PURIFICATION OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE

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6. SUMMARY

Isolation of chloromethyl dianese-propanoate dehydrogenase in pure form has been reported previously (Koch et al., 1971a). However, attempts to reproduce their procedure in this laboratory have been unsuccessful. The enzyme obtained by following the reported procedure had a specific activity of only 5 units/mg as compared to a specific activity of 12 units/mg expected for the pure enzyme on the basis of results reported by Koch et al. (1971a). The preparation was heterogeneous as judged by polyacrylamide gel electrophoresis and could lose as much as 30% of its activity in 24 hr under recommended conditions for storage. It was therefore necessary to develop a new purification procedure that would enable the production of large quantities of pure enzyme that could be stored for long periods without loss of activity.

The purification procedure used by Koch et al. (1971a) consisted of a combination of conventional techniques such as ammonium sulphate fractionation, ion exchange chromatography and gel filtration. Since the above procedures are laborious when employed in large scale fractionations, it was decided to attempt the development of an alternative purification procedure based on affinity chromatographic principles. Affinity chromatography has been used for the rapid, large scale purification of a number of enzymes and other proteins (Cuatrrocasa et al., 1968; Cuatrrocasa, 1970a and b). The technique is based on the specific affinity of the protein for a ligand that is bound covalently to a supporting matrix such as Sepharose. Removal of the bound protein may be accomplished by the use of a solution of the ligand, by altering the pH or the ionic strength of buffer solutions, or by a combination of these procedures.
1. INTRODUCTION

The isolation of chorismate mutase-prephenate dehydrogenase in pure form has been reported previously (Koch et al, 1971a). However, attempts to reproduce their procedure in this laboratory have been unsuccessful. The enzyme obtained by following the reported procedure had a specific activity of only 3 units/mg as compared to a specific activity of 12 units/mg expected for the pure enzyme on the basis of results reported by Koch et al (1971a). The preparation was heterogeneous as judged by polyacrylamide gel electrophoresis and could lose as much as 30% of its activity in 24 hr under recommended conditions for storage. It was therefore necessary to develop a new purification procedure that would enable the production of large quantities of pure enzyme that could be stored for long periods without loss of activity.

The purification procedure used by Koch et al (1971a) consisted of a combination of conventional techniques such as ammonium sulphate fractionation, ion exchange chromatography and gel filtration. Since the above procedures are laborious when employed in large scale fractionations, it was decided to attempt the development of an alternative purification procedure based on affinity chromatographic principles. Affinity chromatography has been used for the rapid, large scale purification of a number of enzymes and other proteins (Cuatracasas et al, 1968, Cuatracasas, 1970a and b). The technique is based on the specific affinity of the protein for a ligand that is bound covalently to a supporting matrix such as Sepharose. Removal of the bound protein may be accomplished by the use of a solution of the ligand, by altering the pH or the ionic strength of buffer solutions or by a combination of these procedures.
Two possible ligands were considered for the purification of chorismate mutase-prephenate dehydrogenase by affinity chromatography. One was tyrosine, which is an allosteric effector of the enzyme (Cotton and Gibson, 1965), and the other was AMP, an analogue of NAD which functions as a substrate for the dehydrogenase reaction. This chapter presents the results obtained with the use of the latter and reports the development of a new purification procedure based on this technique. Preliminary details of the procedure have been reported elsewhere (Sampathkumar and Morrison, 1977).

2. MATERIALS

Cyanogen bromide-activated Sepharose was obtained from Pharmacia, DEAE cellulose (DE 52) from Whatman Biochemicals Ltd., and hydroxylapatite (Bio-gel HTP) from Bio-Rad Laboratories. Agarose-hexane-NAD Type 3 was supplied by P-L Biochemicals. p-Phenylnethylsulfonyl fluoride and dithiothreitol were obtained from Calbiochem and streptomycin sulphate and N-ethylmorpholine were from the Sigma Chemical Co. N-ethylmorpholine was generally redistilled before use at 35°C under a pressure of 15 mm/Hg. L-tyrosine was a product of Merck, while AMP, NAD and NADH were supplied by P-L Biochemicals. Crystallized and lyophilized bovine serum albumin was a product of Sigma and Polyox WSR 301, a product of Union Carbide.

3. METHODS

(a) Preparation of chorismate and prephenate

*E. aerogenes* 62-1 was used to produce chorismic acid which was isolated and recrystallized by the method of Gibson (1968).
Sodium prephenate was prepared from chorismate by the enzymic action of chorismate mutase-prephenate dehydrogenase (Dudziński and Morrison, 1976).

(b) Preparation of Sepharose-tyrosine

Carboxybenzoyl 6-amino-hexanoic acid was condensed with tyrosine methyl ester in the presence of N-N'dicyclohexyl carbodiimide. After removal of the protecting groups, the resulting product, 6-amino hexanoyl-L-tyrosine, was reacted with cyanogen bromide-activated Sepharose 4B essentially as described by Cuatrecasas et al (1968). The concentration of the ligand used was 0.2 M and the reaction was carried out overnight at 4°C in 0.1 M Na₂CO₃ (pH 9.0). The mixture was filtered and the Sepharose was washed with two volumes of distilled water, then with two cycles of 0.1 M sodium acetate buffer (pH 4.0) containing 0.5 M NaCl and finally with two cycles of 0.1 M Tris-HCl buffer pH 8.0 containing 0.5 M NaCl. Amino acid analyses indicated that the concentration of ligand coupled to the gel was approximately 4 µmoles per ml of wet packed Sepharose.

(c) Preparation of Sepharose-AMP

N⁶-(6-amino hexyl)-AMP was prepared by Dr. D. Magrath of the Department of Biochemistry, Australian National University, according to the procedure described by Trayer et al (1974). Inosine was converted in three steps (Brederick and Martini, 1947; Brown and Welicky, 1953; Gerster et al, 1963) into 6-chloro-9-β-D-ribofuranosyl purine which was then selectively phosphorylated at the 5' position according to Guilford et al, (1972). The phosphorylated compound was converted directly into N⁶-(6-amino hexyl)-AMP by reaction with 1,6-diaminohexane and the subsequent purification of the product on Dowex 1. The purified product, at a concentration of 40 mM, was coupled to cyanogen bromide-activated Sepharose 4B by leaving the mixture in 0.1 M Na₂CO₃ (pH 9.0) overnight.
at 4°C. Blank gels were prepared as above except for the omission of \( \text{N}^6-(6\text{-amino hexyl})-\text{AMP} \). The columns were washed as described above for Sepharose-tyrosine and the gels stored at 4°C in 0.1 M N-ethylmorpholine-HCl buffer (pH 7.5) containing 0.01% sodium azide.

The concentration of ligand coupled to the gel was estimated both by phosphate analysis and by measuring the absorbance of the adenine moiety. Phosphate analysis was routinely carried out according to the method of Ma and McKinley Jr. (1953) but the micro method of Lueck and Boltz (1956) was employed when the phosphorus content of the samples was in the low range of 5-20 µg. The absorbance of the adenine moiety was measured both before and after hydrolysis of Sepharose-AMP. UV-spectra of the matrix-bound nucleotide were run with the unhydrolyzed gels kept uniformly suspended in a solution of Polyox WSR 301 (Larrson and Mosbach, 1971). Blank gels were used as controls. Known amounts of the free ligand were used as standards and the amount of bound AMP was calculated from spectrophotometric measurements. The samples of Sepharose-AMP were hydrolyzed in 5 ml of 0.5 M HCl at 100°C for 15 min (Larrson and Mosbach, 1971) and after dilution with water to a total volume of 10 ml, the amount of nucleotide material was calculated from the absorption obtained at 263 nm using as standards, hydrolysates of the blank gel together with known amounts of the free ligand.

The value for bound AMP as determined by phosphate analysis and by measurement of the adenine moiety in either hydrolyzed or unhydrolyzed samples of Sepharose-AMP were normally in good agreement. The amount of bound ligand was usually in the range of 7-8 µmoles per ml of wet packed Sepharose.
(d) Preparation of Sepharose-ADP

\[ p^1-(6\text{-aminohex-1-yl})p^2-(5'\text{-adenosyl}) \text{ pyrophosphate} \]

was also prepared by Dr. D. Magrath of the Department of Biochemistry, Australian National University, according to the method of Trayer et al. (1974).

N-trifluoroacetyl 6-amino hexan-1-ol phosphoryl imidazolide prepared as described by Barker et al. (1972, 1974) was condensed with the anhydrous tributyl ammonium salt of AMP and the resulting product purified to yield trifluoroacetyl amino hexanol-ADP which was then isolated as dilithium salt. The trifluoroacetyl group was removed by hydrolysis at pH 11 for 3 hr at room temperature and the free amine was reacted with cyanogen bromide-activated Sepharose. The concentration of ligand used, conditions of coupling and methods of estimation of bound ligand were similar to those used for Sepharose-AMP. Under these conditions, 8-10 µmoles of the ligand were usually bound per ml of wet packed Sepharose.

(e) Measurement of enzyme activity

The chorismate mutase activity of the various enzyme fractions obtained in the course of enzyme purification was determined at 30°C by a stopped time assay (Koch et al, 1970a). Reaction mixtures (0.4 ml) contained 2.5 mM chorismate, 0.5 mM EDTA, 0.5 mM dithiothreitol and 50 mM Tris-HCl buffer (pH 7.5). Reactions were stopped with 0.4 ml of 1 M HCl and incubated for 10 min to allow the complete conversion of prephenate to phenylpyruvate. The absorbance of phenylpyruvate was then measured at 320 nm under alkaline conditions. The molar extinction coefficient of phenylpyruvate was taken to be 17,600. Blanks to which enzyme was added after the addition of HCl were included to overcome any nonspecific contribution to absorbance at 320 nm by substances present in the enzyme fraction.
Prephenate dehydrogenase activity was measured in reaction mixtures (1.0 ml) containing 50 mM Tris-HCl buffer (pH 7.5), 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM prephenate, and 1.0 mM NAD. The formation of NADH from NAD at 30°C was followed spectrophotometrically by the increase in absorbance at 340 nm. Since 4-hydroxyphenylpyruvate contributes to absorbance at 340 nm, reaction velocities were calculated using a corrected extinction coefficient of 6500 for an equimolar mixture of the two products. A Cary 118 spectrophotometer fitted with masks was routinely used for these measurements. (The masks narrowed the width of the light beam and reduced artifacts resulting from light passing through the translucent side walls of the cuvette).

A unit of enzyme is defined as the quantity of enzyme required to produce one µmole of product per min at 30°C. Specific activity of the enzyme is reported as units of enzyme activity per mg of protein.

(f) Estimation of protein

Protein concentrations in crude extracts and in fractions obtained in early stages of purification were measured by the Biuret method using bovine serum albumin as a standard. Effluent fractions from chromatographic columns were routinely monitored by measuring their absorbance at 280 nm with a Gilford 240 spectrophotometer. The amount of purified enzyme protein was also determined in the spectrophotometer using 0.82 as the $E_{280}^{1%}$ value (see Chapter III).

(g) Polyacrylamide gel electrophoresis

Disc gel electrophoresis in 7% (w/v) acrylamide was run as
described by Davis (1964) without sample or spacer gels. Samples of 10 to 50 µl containing bromophenol blue and 40% sucrose were layered under buffer on the upper cathodic gel surface. The gels were run at 4°C using a current of 2.5 mA per tube.

Protein bands were detected by staining with Coomassie Blue. Prephenate dehydrogenase activity was located in the gels by soaking them at room temperature in the dark in a solution containing 50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM prephenate, 1 mM NAD, 0.3 mg/ml of tetrazolium nitroblue, and 50 µg/ml of phenazine methosulphate. Prephenate dehydrogenase activity was detected by the appearance of purple bands after 30 min. For some experiments prephenate was replaced with 2 mM chorismate.

(h) Organism and growth of cells

_E.coli_ JP2312 (_aroF, aroG, tyrR, trps_) carrying a regulator mutation causing constitutive synthesis of chorismate mutase-prephenate dehydrogenase was kindly provided by Professor J. Pittard. This strain has 5-fold higher levels of chorismate mutase-prephenate dehydrogenase as compared to the strain JP232 previously used for the purification of this enzyme (Koch _et al_, 1971a).

(i) Growth in minimal medium

During preliminary investigations of growth conditions for optimum enzyme production, the cells were grown in a glucose-mineral salts medium supplemented with histidine, valine, isoleucine, and very low quantities (1 x 10^-5 M) of tryptophan. Under these conditions, high levels of DAHP synthase, the first enzyme in the biosynthetic pathway of tyrosine, were known to be produced (Camakaris, H. and Pittard J.,
1976). It was speculated that elevated levels of chorismate mutase-prephenate dehydrogenase may also be produced under these conditions. Cells were therefore grown in batches of 10 l in 10 x 1 l flasks in the above medium at 37°C. The inoculum for each batch consisted of growth washed with the glucose-mineral salts medium, from five plates of nutrient agar incubated overnight at 30°C. The culture was agitated at 37°C in a New Brunswick shaker and the cells were harvested at various stages of growth by centrifugation. Crude cell extracts were prepared using 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol, 1 mM tyrosine (Koch et al., 1971a) and 0.1 mM phenylmethylsulfonyl fluoride, and the specific activity of the enzyme in each extract was recorded.

(ii) Growth in Luria broth

Cells were grown in batches of 10 l in 10 x 1 l flasks in Luria broth (Luria, 1957) at 37°C. The inoculum for each batch consisted of the growth, washed with Luria broth, from five plates of nutrient agar incubated overnight at 30°C. The culture was agitated at 37°C, and the cells harvested and extracted as described above, for cells grown in minimal medium. The cell yields and the specific activity of the enzyme in each extract were also recorded.

4. RESULTS

(a) Choice of growth conditions for E.coli JP2312

In order to determine suitable growth conditions and optimum time of harvest, cells of E.coli were grown in both minimal medium and in Luria broth. Cells were harvested at various stages of growth and the
extracts prepared as described under 'Methods'. When grown in minimal medium, maximum specific activity of the enzyme was observed in extracts of cells harvested in the late logarithmic phase of growth (Fig.II.1A). The cell yields were 1.5 g/l of the medium and the specific activity of the enzyme was around 0.6. The enzyme in these extracts was found to be unstable and lost almost all activity on standing for 48 hr at 4°C. When cells were grown in Luria broth, the highest specific activity of the enzyme was obtained in extracts of cells harvested in the stationary phase (Fig.II.1B) where the cell yields were 5 g/l of medium. The specific activity of the enzyme had a lower value around 0.3 but the enzyme was relatively stable and the crude extracts could be stored at 4°C for up to five days without any loss of activity.

Hence strain JP2312 was grown routinely in Luria broth in 20 x 1 l batches at 37°C. Cells were harvested 17 hr after the addition of inoculum at a Klett reading of around 400.

(b) Stabilization of the enzyme

Although crude extracts prepared as above were stable for up to five days at 4°C, any attempts to further purify the extract resulted in progressive loss of activity of the enzyme. Thus it was necessary to develop conditions for stabilizing the enzyme at all stages of its purification using both the crude and partially purified preparations of the enzyme.

The stability of the enzyme was studied under a variety of buffer conditions with and without the addition of substrates, modifier, inhibitors of proteolytic enzymes, univalent and bivalent cations, metal
FIG.II.1. VARIATION IN SPECIFIC ACTIVITY OF CHORISMA TE MUTASE OBSERVED IN CELL EXTRACTS OF E.COLI JP2312 HARVESTED AT VARIOUS STAGES OF GROWTH. CELLS WERE GROWN IN MINIMAL MEDIUM SUPPLEMENTED WITH APPROPRIATE AMINO ACIDS (A) OR IN LURIA BROTH (B).

(•—•) LOG KLETT.
(■—■) SPECIFIC ACTIVITY.
A

Log Klett

Specific activity of chorismate mutase

Time (hr)

B

Log Klett

Specific activity of chorismate mutase

Time (hr)
chelators, and various sulphydryl protecting reagents. Contrary to previous reports (Koch et al, 1971a) neither the modifier tyrosine nor the substrate prephenate was effective as a stabilizing agent. NAD was only partially effective. A significant increase in stability of the enzyme was observed when the Tris-HCl buffer was replaced by N-ethylmorpholine-HCl buffer (pH 7.5) containing 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol (w/v). It was subsequently found that in addition to the above components, a low concentration of trisodium citrate (1 mM) was very effective in stabilizing the enzyme at later stages of purification. It was not included in the buffer at all stages as it interfered with chromatography of the enzyme on some of the columns used in the purification procedure. It was added during prolonged dialysis of dilute fractions of the enzyme and during storage of the enzyme for long periods.

On the basis of the above results, the composition of the main buffer, termed Buffer A, used in the purification consisted of 0.1 M N-ethylmorpholine-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol (w/v). At the end of the purification, the enzyme was dialyzed against 0.1 M N-ethylmorpholine-citrate buffer (pH 7.0) containing 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol (w/v). This buffer, termed Buffer B, was found to be most effective in stabilizing the enzyme over prolonged periods of storage. In addition to the above, crude cell extracts contained phenylmethylsulfonyl fluoride at a concentration of 0.1 mM.

(c) **Development of the purification procedure**

(i) **Removal of nucleic acids**

The procedure adopted previously for the removal of nucleic
acids from crude cell extracts involved the use of protamine sulphate at optimum concentrations (Koch et al, 1970b). Experiments with protamine sulphate indicated that most of the enzyme activity was also precipitated under conditions used for the precipitation of nucleic acids. Similar observations were made by Smith et al (1977), who noticed that significant losses of enzyme occurred at this step in the purification. Since all the enzyme activity could be precipitated by using higher concentrations of protamine sulphate, attempts were made to further purify the enzyme from the protamine-nucleic acid-protein pellet. This procedure resulted in appreciable losses of the enzyme as it was difficult to solubilize the pellet and fractionation of the solubilized material with ammonium sulphate was unsatisfactory. The resulting enzyme preparation showed only a two-fold increase in specific activity over those of the cell extracts.

It was therefore decided to employ an alternative procedure for removing nucleic acids. Streptomycin sulphate could be used satisfactorily to precipitate most of the nucleic acids without any loss of enzyme activity from the supernatant solution and this procedure was adopted for the removal of nucleic acids from cell extracts of E.coli.

(ii) Ammonium sulphate fractionation

After removal of nucleic acids with streptomycin sulphate most of the enzyme activity in the supernatants could be precipitated between 0.40 - 0.53 saturation of ammonium sulphate, with considerable purification of chorismate mutase-prephenate dehydrogenase. The precipitate could be stored at -20°C for periods of up to a year without any loss in enzyme activity. Hence, several preparations were brought to this stage and accumulated before further purification was commenced.
(iii) Chromatography on Sepharose-tyrosine and Sepharose-AMP

Extracts of enzyme precipitated with ammonium sulphate were chromatographed on columns of Sepharose-tyrosine after dialysis against Buffer A. Chorismate mutase-prephenate dehydrogenase did not bind to the column under these conditions. Similar results were obtained when the dialysates were chromatographed in Buffer A in the presence of 1 mM NAD, 1 mM prephenate or 1 mM NADH.

Following the reports that the enzyme chorismate mutase-prephenate dehydratase binds to Sepharose-phenylalanine column only under conditions of high ionic strength (Gething and Davidson, 1973), dialyzed extracts of enzyme were loaded on the tyrosine column in the presence of 0.5 M NaCl at pH 7.0, 7.5 or 8.0 in 50 mM N-ethylmorpholine-HCl buffer containing 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol (w/v). However, no binding of the enzyme to the column was observed. Chromatography of the enzyme at pH values below 7.0 or above 8.0 was not possible due to the instability of the enzyme under these conditions.

Chromatography of the enzyme on Sepharose-AMP columns was more successful. On passage of dialyzed extracts of the enzyme in Buffer A through columns of Sepharose 4B and Sepharose-AMP, it was observed that the enzyme was completely bound to Sepharose-AMP whereas no binding could be observed to the unsubstituted Sepharose. Extensive washing with Buffer A resulted in the removal of about 80% of the inert protein contained in the applied enzyme fraction. The bound chorismate mutase-prephenate dehydrogenase could be eluted as a broad peak with a gradient of 0-20 mM NAD or a gradient of 0-30 mM AMP. By contrast, there was sharp elution of the enzyme in a gradient of 0-0.5 M KCl in the same buffer. However, pooled fractions of eluted enzyme possessed similar
specific activities irrespective of the means used for elution. Hence a gradient of 0-0.5 M KCl was routinely used to elute the enzyme from Sepharose-AMP columns. The bulk of mutase and dehydrogenase activities were usually eluted as a single peak at a KCl concentration of about 0.35 M. A detailed elution profile of a column developed under these conditions is shown in Fig.II.2.

Polyacrylamide gels of the materials eluted as above indicated that in addition to chorismate mutase-prephenate dehydrogenase, other proteins may also be present. These contaminating proteins may be other dehydrogenases or kinases which also bind to Sepharose-AMP or proteins that interact nonspecifically with Sepharose-AMP. An 18-fold purification of the enzyme was usually observed at this step with a yield of 60% of enzyme activity. The procedure has since been used successfully both on a small scale (50 mg of protein) and on a large scale (800 mg of protein) and has been incorporated in the detailed purification procedure.

In addition to Sepharose-AMP and Sepharose-tyrosine, Sepharose-ADP in which ADP was linked through its \( \beta \) phosphoryl group (Trayer et al., 1974) and Agarose-NAD in which NAD was linked through the C-8 of the adenine moiety (Lee et al., 1974) were both tried as affinity columns. Both columns retarded the enzyme but were not as effective as Sepharose-AMP in separating the bulk of contaminating proteins from chorismate mutase-prephenate dehydrogenase.

(iv) Chromatography on DEAE-cellulose

It was possible that nonspecific ionic interactions were responsible for the presence of some of the contaminating proteins in
FIG.II.2. CHROMATOGRAPHY OF PARTIALLY PURIFIED CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE ON SEPHAROSE-AMP.

PARTIALLY PURIFIED CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE (110 UNITS OF MUTASE ACTIVITY WITH A SPECIFIC ACTIVITY OF 0.92) WAS CHROMATOGRAPHED ON A COLUMN (0.9 X 5 CM) OF SEPHAROSE-AMP AS DESCRIBED UNDER 'RESULTS'. THE COLUMN WAS ELUTED WITH A LINEAR GRADIENT OF KC1 (0-0.5 M) IN A TOTAL VOLUME OF 50 ML. THE FLOW RATE WAS 30 ML/HR AND 5 ML FRACTIONS WERE COLLECTED.

(○) CHORISMATE MUTASE ACTIVITY.
(■) PREPHENATE DEHYDROGENASE ACTIVITY.
the eluates from the Sepharose-AMP column. A chromatographic step based on the ion exchange properties of eluted proteins was therefore included in the procedure for the purification of chorismate mutase-prephenate dehydrogenase.

The eluate from the Sepharose-AMP column was dialyzed against Buffer A containing 1 mM citrate and chromatographed on DEAE-cellulose equilibrated with the same buffer. The enzyme was eluted in a gradient from 0-0.5 M KCl in Buffer A. Both the mutase and dehydrogenase activities of the enzyme were eluted as a single sharp peak at a chloride concentration of 0.15 M. This step also resulted in approximately 2-fold purification of the enzyme with a recovery of 70% of enzyme activity.

Chromatography on Hydroxylapatite column

Polyacrylamide gel electrophoresis of the enzyme fraction obtained after chromatography on DEAE-cellulose revealed the presence of a single major band with traces of contaminating proteins. It was therefore decided to further purify the enzyme to homogeneity by adsorption chromatography on a hydroxylapatite column. Eluates of the enzyme from the DEAE-cellulose step were found to adsorb strongly to hydroxylapatite, but attempts to elute the enzyme with a gradient of phosphate resulted in huge losses of the enzyme activity. It has been suggested by Bernardi et al. (1972) that the adsorption of some proteins to hydroxylapatite crystals may be based on the binding of their acidic groups to calcium sites on the hydroxylapatite. Subsequent elution of such proteins by phosphate may involve their displacement from the calcium sites by phosphate. It was therefore conceivable that other anions capable of competing for the calcium sites may serve as alternative agents.
for eluting acidic proteins from hydroxylapatite columns.

Among the anions that were tested as eluting agents, sodium citrate was found to be most effective causing complete elution of the enzyme at a concentration of about 7 mM. The mutase and dehydrogenase activities were eluted as a single sharp peak with a constant specific activity in all the eluted enzyme fractions. A recovery of 95% was observed at this step and the resulting enzyme showed a single band of protein on polyacrylamide gel electrophoresis.

(d) The purification procedure for chorismate mutase-prephenate dehydrogenase from E. coli JP2312

The final procedure adopted for the purification of chorismate mutase-prephenate dehydrogenase is as follows:

Step 1 Preparation of crude extracts

Cells of E. coli strain JP2312 (45 g, stored at -20°C) were thawed and suspended in 270 ml of Buffer A containing 0.1 mM phenylmethylsulfonyl fluoride. The cells were disrupted in a Ribi cell fractionator at 20,000 psi and cell debris removed by centrifuging at 23,000 x g for 30 min. The centrifugation and all further steps were performed at -4°C.

Step 2 Removal of nucleic acid

A solution of streptomycin sulphate (40% w/v) in Buffer A was added dropwise with stirring to the cell extract until a final concentration of 6% streptomycin sulphate was reached. The mixture was stirred for a further 30 min and the precipitate removed by centrifuging for 30 min at 23,000 x g.

Step 3 Ammonium sulphate fractionation

Solid ammonium sulphate (22.6 g/100 ml) was added slowly with continual
stirring to the supernatant solution from the previous step. The mixture was stirred for a further 30 min and then centrifuged at 23,000 x g for 30 min. The precipitate was discarded and ammonium sulphate (7.66 g/100 ml) was added to the supernatant solution. The mixture was stirred for one hr and then centrifuged for 20 min at 23,000 x g. The precipitate could be stored at -20°C for several months without loss of activity.

**Step 4  Chromatography on Sepharose-AMP**

The precipitate obtained as described above was dissolved in a minimum volume of Buffer A to which 20 mM KH₂PO₄ had been added. Residual ammonium sulphate was removed by dialysis for 15 hr against two changes (2 l) of the same buffer. The dialyzed solution was clarified by centrifugation at 23,000 x g for 10 min and applied to a column (2.5 x 8 cm) of Sepharose-AMP equilibrated with the buffer described above.

The column was washed with 100 ml of this buffer and the enzyme eluted with a linear gradient of KCl (0-0.5 M) in a total volume of 400 ml of Buffer A. Elution of the enzyme occurs at a KCl concentration of about 0.35 M. The active fractions were pooled and dialyzed for 15 hr against 2 l of Buffer A containing 1 mM sodium citrate.

**Step 5  DEAE-cellulose chromatography**

The dialyzed enzyme was applied to a column (2.6 x 14 cm) of DEAE-cellulose (DE 52) which had been equilibrated with Buffer A containing 1 mM sodium citrate. The column was washed with 70 ml of the same buffer and eluted with a linear gradient of KCl (0-0.5 M) in a total volume of 400 ml of Buffer A. The enzyme was eluted at a KCl concentration of about 0.15 M.

**Step 6  Chromatography on hydroxylapatite**

The active fractions from the DEAE-cellulose column were pooled and applied to a hydroxylapatite column (1.6 x 14 cm) equilibrated with
Buffer A containing 0.3 M KCl. The column was washed with the same buffer and eluted with a linear gradient of citrate (0 to 20 mM) in Buffer A in a total volume of 150 ml. The enzyme is eluted at a citrate concentration of about 7 mM. The active fractions were pooled and dialyzed against 1 l of Buffer B.

Both mutase and dehydrogenase activities were measured and the ratio of the two activities calculated for the various fractions obtained during the course of purification. The yields and specific activities of the various fractions obtained during the course of the purification are listed in Table II.1. This table also records the ratio of the mutase and dehydrogenase activities of the enzyme in each of the various fractions. It can be seen from Table II.1 that this ratio remains constant throughout the purification procedure and that a 100-fold purification of the enzyme from crude extracts has been achieved with an overall yield of 35%.

(e) Storage of the purified enzyme

The purified enzyme in Buffer B was stable when stored at 4°C for up to four weeks. It could be stored for extended periods without loss of activity when snap frozen in liquid nitrogen and left at -20°C.

(f) Homogeneity of the purified enzyme

The symmetrical pattern of elution of protein and enzyme activity from hydroxylapatite indicated the homogeneity of the final enzyme preparation. This was confirmed by the constant specific activity
### TABLE II.1.  
YIELDS AND SPECIFIC ACTIVITIES OF FRACTIONS OBTAINED DURING COURSE OF PURIFICATION OF CHORISMA TE MUTASE-PREPHENATE DEHYDROGENASE

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total mutase activity (units)</th>
<th>Specific activity (units/mg protein)</th>
<th>Mutase Dehydrogenase ratio</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude cell extract</td>
<td>240</td>
<td>5820</td>
<td>2050</td>
<td>0.35</td>
<td>- b</td>
<td>100</td>
</tr>
<tr>
<td>2. Streptomycin sulphate treated extract</td>
<td>264</td>
<td>5658</td>
<td>1978</td>
<td>0.35</td>
<td>- b</td>
<td>96</td>
</tr>
<tr>
<td>3. Ammonium sulphate fractionation</td>
<td>42</td>
<td>1693</td>
<td>1558</td>
<td>0.92</td>
<td>1.31</td>
<td>76</td>
</tr>
<tr>
<td>4. Chromatography on Sepharose-AMP</td>
<td>105</td>
<td>68</td>
<td>1096</td>
<td>16</td>
<td>1.32</td>
<td>53</td>
</tr>
<tr>
<td>5. Chromatography on DEAE-cellulose</td>
<td>51</td>
<td>28</td>
<td>712</td>
<td>25</td>
<td>1.31</td>
<td>35</td>
</tr>
<tr>
<td>6. Chromatography on hydroxylapatite</td>
<td>21</td>
<td>19</td>
<td>656</td>
<td>35</td>
<td>1.31</td>
<td>32</td>
</tr>
</tbody>
</table>

a  Weight of cells was 45 g.

b  Dehydrogenase activity could not be measured accurately.
of the enzyme in these fractions. Polyacrylamide gel electrophoresis of the purified enzyme revealed only a single protein band (Fig.II.3). When the gels were stained for prephenate dehydrogenase activity, only one band was seen and this was also coincident with the protein band. On the basis of these observations, it was concluded that the purified enzyme was homogeneous.

(g) Kinetic properties of the enzyme

Preliminary studies of both the mutase and dehydrogenase reactions showed that the enzyme exhibits Michaelis-Menten kinetics with respect to each of its three substrates. Double reciprocal plots were linear with chorismate, prephenate or NAD as the varying substrate.

5. DISCUSSION

In this Chapter is described the development of a new purification procedure which yields homogeneous preparations of chorismate mutase-prephenate dehydrogenase from *E.coli* in good yield and with a three-fold higher specific activity than previously reported for this enzyme (Koch *et al*, 1971a). The purification of the enzyme was facilitated by the availability of a mutant which produces the enzyme in 5-fold higher quantities than the strain JP232 which was used previously. Chorismate mutase-prephenate dehydrogenase was purified to the extent that the specific activity of the purified enzyme was 100-fold higher than the specific activity of cell free extracts of *E.coli* JP2312.

The development of the purification procedure took considerable time and was hampered by the instability of the enzyme (cf. Koch *et al*,
FIG.II.3. POLYACRYLAMIDE GEL ELECTROPHORESIS OF PURIFIED CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE FROM E.COLI. THE GELS WERE STAINED FOR PROTEIN.

(a) 20 MICROGRAMS OF ENZYME.

(b) 80 MICROGRAMS OF ENZYME.
1971a). Efforts were made to overcome losses due to instability of the enzyme both during purification and during prolonged storage.

Significant improvement in stability was achieved by the replacement of Tris-HCl buffer by N-ethylmorpholine-HCl buffer and by the inclusion of 10% (w/v) glycerol. Addition of low concentrations of citrate in the storage buffer facilitated the storage of enzyme over prolonged periods without loss in activity.

In an endeavour to determine the mechanism by which citrate contributed to the stability of the enzyme, the effect of citrate has been tested on the mutase and dehydrogenase activities of the enzyme. No inhibition or activation of either enzymic activity was observed with chorismate mutase-prephenate dehydrogenase when tested at 30°C in 50 mM N-ethylmorpholine-morpholinoethane sulfonic acid buffer over the pH range from 7.0-8.5. It is therefore possible that the protecting effect of citrate observed at pH 7.0 is mediated through a conformational change which has no significant kinetic effect or which is manifested only at lower temperatures. In addition to stabilizing the enzyme, low concentrations of citrate were observed to interfere with the binding of chorismate mutase-prephenate dehydrogenase to Sepharose-AMP columns. Enzymes loaded in the presence of 1 mM citrate were retarded only slightly on passage through columns equilibrated with buffer containing 1 mM citrate. An attempt was made to take advantage of this behaviour to bring about specific elution of the enzyme from Sepharose-AMP columns. With a gradient of 0-50 mM citrate, the enzyme was eluted as a broad peak and although the specific activity of the eluate was slightly higher than that obtained after elution with a gradient of KCl, the recovery of enzyme was lower. The eluate after chromatography on DE 52 appeared pure and the use of any further step in purification was thus avoided.
In spite of its advantage as a shorter purification procedure, this method was not routinely used as the overall recovery of enzyme was low and the final preparation of the enzyme was so dilute, that it had to be concentrated before storage. The majority of investigations described with chorismate mutase-prephenate dehydrogenase in subsequent chapters were carried out using the enzyme purified as described under 'Results'.

Chromatography on Sepharose-AMP was an important step in the purification procedure as it led to an 18-fold purification of the enzyme. It was observed that, with repeated use, there was a gradual reduction in the capacity of the column to bind enzyme. The loss of binding capacity occurred with a concomitant loss in the phosphorus content of the column while the adenine content of the inactive column appeared to be unchanged. No such loss of phosphate was observed in samples of Sepharose-AMP stored at 4°C for similar periods, and as yet unused in the purification procedure. It was therefore possible that prolonged usage of the Sepharose-AMP column resulted in a loss of phosphate either by removal of the orthophosphate due to hydrolysis of the phosphate ester bond or by removal of ribose-5-phosphate by hydrolysis of the carbon-nitrogen linkage in AMP. Using N^6-aminohexyl-AMP as substrate, dialyzed extracts of enzyme purified to the ammonium sulphate stage were tested for the presence of enzymes capable of catalyzing the above reactions. Both activities were found to be present in these fractions but the phosphatase activity was predominant and appeared in significant levels. Hence 20 mM P_i was included in all buffers used in the chromatography on Sepharose-AMP on the basis that P_i is a product inhibitor of phosphatases and a concentration of 20 mM would be high relative to the K_i value for P_i of most phosphatases. At this concentration, phosphate did not cause any loss in the activity of
chorismate mutase-prephenate dehydrogenase. The presence of phosphate in the buffer considerably prolonged the life of Sepharose-AMP columns which have now been continuously in use for the last two years with only a 10% loss in the phosphorus content of the bound ligand.

As described earlier, attempts to use Sepharose-tyrosine columns to purify the enzyme were unsuccessful. It was possible that the binding of the enzyme to Sepharose-tyrosine was weak in the absence of substrates but chromatography in the presence of NAD, prephenate or NADH did not result in the binding of enzyme to the column. It is therefore possible that the amino group of tyrosine is essential for its binding to the enzyme and hence no binding is observed to an affinity column in which the tyrosine is linked through the amino group to Sepharose. The results of preliminary inhibition studies with analogues of tyrosine support this idea and indicate that both the carboxyl and amino groups may play a role in the inhibition of the enzyme activity by tyrosine.

Because it was of importance to obtain high recoveries of stable enzyme, it was necessary to replace phosphate as an eluting agent for hydroxylapatite chromatography. The enzyme was unstable in concentrations of phosphate above 50 mM (cf. Koch et al., 1971a). Citrate was therefore used as an alternative eluting agent and found to be very effective. It had been assumed that citrate functions by displacing the acidic groups of proteins which bind to calcium sites on the hydroxylapatite complex (Bernardi et al., 1972), but it appears more likely that elution by citrate occurs as a result of its ability to destroy the crystal structure of hydroxylapatite and thereby cause a decrease
in its protein binding capacity. This was suggested by the observation that it was not possible to reuse hydroxylapatite columns from which the enzyme had been eluted with citrate. The columns had lost their capacity to bind proteins and had very poor flow rates. That this was due to the disruptive effect of citrate on the crystal structure was evident from the observation that equilibration of a fresh hydroxylapatite column with 1 mM citrate resulted in an increase in the quantity of fines in the supernatant which paralleled a decrease in flow rate and a decrease in its capacity to bind proteins.

The kinetic properties of the purified enzyme appear to differ from those reported by Koch et al (1971a). No nonlinearity was observed in any of the double reciprocal plots with chorismate, prephenate or NAD as the varied substrate and hence both the mutase and the dehydrogenase reactions catalyzed by the enzyme conform to Michaelis-Menten kinetics. It is therefore possible that the nonlinearity observed by Koch et al (1971a) may have been caused by the presence of small quantities of tyrosine in the enzyme fractions used in their kinetic studies. This remains a possibility in view of the fact that the enzyme used by these workers was normally stored in the presence of 1 mM tyrosine.

6. SUMMARY

A new procedure has been developed for the purification of chorismate mutase-prephenate dehydrogenase from crude extracts of *E. coli* strain JP2312. The overall recovery of the enzyme following this procedure was about 35%. The enzyme preparation was homogeneous as indicated by a single band of both protein and activity on polyacrylamide gel electrophoresis. The ratio of chorismate mutase to
prephenate dehydrogenase activity remained virtually constant through the purification procedure, confirming the bifunctional nature of the purified protein. Preliminary studies on the kinetic properties of the enzyme indicated that both the mutase and the dehydrogenase reactions catalyzed by the enzyme conform to Michaelis-Menten kinetics.
CHAPTER III

PHYSICAL AND CHEMICAL PROPERTIES OF
CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE

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   (c) Absorption spectra and dry weight determinations
   (d) Amino acid analysis
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   (d) Absorption spectra and extinction coefficients of the enzyme
   (e) Amino acid composition, minimum molecular weight and partial specific volume of the enzyme
   (f) Peptide maps of the enzyme
CHAPTER III

PHYSICAL AND CHEMICAL PROPERTIES OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE

1. INTRODUCTION

2. MATERIALS

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      (ii) In sodium dodecyl sulphate
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   (c) Absorption spectra and dry weight determinations
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   (e) Preparation of peptide maps
   (f) Analytical ultracentrifugation
   (g) Sucrose density centrifugation

4. RESULTS
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   (e) Amino acid composition, minimum molecular weight and partial specific volume of the enzyme
   (f) Peptide maps of the enzyme
5. DISCUSSION

5(g) Sedimentation coefficient for the enzyme

6. SUMMARY

(ii) In sucrose density gradient centrifugation

The primary aim of this chapter is the elucidation of some of the physical and chemical properties of chorismate mutase-prephenate dehydrogenase from peas. Some of the general properties of the enzyme including its molecular weight, amino acid composition and apparent kinetic constants for its various substrates have been reported by Tocher et al. (1973a). However, in the course of purification and stabilization of the enzyme and in preliminary kinetic studies, it was observed that the behaviour of chorismate mutase-prephenate dehydrogenase differed in many respects from that reported by those workers. In addition, there was some uncertainty about the results reported by Koch et al. (1973a) because of the non-specific activity of the enzyme used in their studies. It was therefore necessary to reinvestigate the general characteristics of the enzyme as a prerequisite to further kinetic and binding studies. The studies presented in this chapter provide information on the molecular size, subunit structure, isoelectric point and extinction coefficient of the enzyme.

2. MATERIALS

Gradiplak gradient gel (4x261 concave gradient of acrylamide) were supplied by Tomasono and Nerone Pty. Ltd. and carrier ampholytes pH 3 to 10 by LKB Products. The membranes used for molecular separation were a product of Millipore Corporation. Sodium dodecyl sulphate was obtained from Matheson, Coleman and Bell, and cross-linked ovalbumin prepared according to the method of Page et al. (1973), was a gift from Dr. D. T. Powell. Trypsin, pepsin, alcohol dehydrogenase (horse-liver) and ovalbumin were products of Worthington Biochemicals while glutamate...
1. **INTRODUCTION**

The primary aim of the studies described in this chapter is the elucidation of some of the physical and chemical properties of chorismate mutase-prephenate dehydrogenase from *E.coli*. Some of the general features of the enzyme including its molecular weight, amino acid composition and apparent Michaelis constants for its various substrates have been reported by Koch *et al* (1971a). However, in the course of purification and stabilization of the enzyme and in preliminary kinetic studies, it was observed that the behaviour of chorismate mutase-prephenate dehydrogenase differed in many respects from that reported by these workers. In addition, there was some uncertainty about the results reported by Koch *et al* (1971a) because of the low specific activity of the enzyme used in their studies. It was therefore necessary to reinvestigate the general characteristics of the enzyme as a prerequisite to further kinetic and binding studies. The studies presented in this chapter provide information on the molecular size, subunit structure, isoelectric point and extinction coefficient of the enzyme.

2. **MATERIALS**

Gradipore gradient gels (4-26% concave gradient of acrylamide) were supplied by Townson and Mercer Pty. Ltd. and carrier ampholytes pH 3 to 10 by LKB Products. The membranes used for molecular separation were a product of Millipore Corporation. Sodium dodecyl sulphate was obtained from Matheson, Coleman and Bell and cross-linked ovalbumin prepared according to the method of Payne *et al* (1973), was a gift from Dr. J. T. Powell. Trypsin, pepsin, alcohol dehydrogenase (horse liver) and ovalbumin were products of Worthington Biochemicals while glutamate...
dehydrogenase was a product of Calbiochem. Catalase (bovine liver) was obtained from Boehringer Mannheim and bovine serum albumin from Sigma. Creatine kinase was a gift from Dr. K. J. Ellis.

3. METHODS

(a) Estimation of the molecular weight by gel electrophoresis

(i) Native enzyme

The method of Margolis and Kenrick (1968) was used to estimate the molecular weight of the enzyme by pore-limit electrophoresis on a gradient of polyacrylamide gel. Gradient gels (4-26% concave gradient of acrylamide) were used with a gradipore electrophoresis apparatus (Townson and Mercer Pty. Ltd.). The electrophoresis buffer, pH 8.3 at 20°C, contained 10.75 g of Tris, 0.93 g of disodium EDTA and 5.04 g of boric acid per l. The samples (25 to 100 µg of protein) containing 40% sucrose and bromophenol blue were layered under the buffer on the upper, cathodal gel surface. In addition to the samples of enzyme, samples of five proteins of known molecular weight were usually included in each electrophoretic run. Electrophoresis was carried out at 70 V for 20 min and then at 125 V for 18 hr. The temperature was maintained at 10°C by continuous recirculation of the buffer through a heat exchange bag suspended in a cooling bath. The gels were stained for 45 min in 0.7% Amidoblack in 7% acetic acid and destaining was carried out in a gradipore destaining unit using 7% acetic acid. Electrophoresis for 40 min at 36 V was sufficient to remove most of the dye. The mobility of each samples was calculated as the distance migrated by the protein from the place of application. Finally the log of mobility of each protein of known molecular weight was plotted against the log of the
molecular weight. The resulting curve was used to estimate the molecular weight of the enzyme.

(ii) In sodium dodecyl sulphate

The samples of proteins to be electrophoresed were dialyzed for 4 hr at room temperature against 500 ml of 0.01 M sodium phosphate buffer, pH 7.0, containing 1% β-mercaptoethanol. Sodium dodecyl sulphate was then added to the dialyzed samples to give a final concentration of 1%, and the tubes were immediately placed in a boiling water bath at 100°C for 5 min. The protein samples denatured in this manner were then electrophoresed according to the procedure of Weber et al (1972).

Protein samples (30-100 µg) containing bromophenol blue were electrophoresed at room temperature in 10% gels at a constant current of 4 mA/gel. After electrophoresis, the gels were removed and the length of the gel and the distance moved by the dye measured. Staining was carried out with Coomassie brilliant blue (Weber and Osborn, 1969) and the gels were destained with a mixture of 75 ml of acetic acid, 50 ml of methanol and 875 ml of water. After destaining, the length of the gel and the distance moved by the protein were recorded and the mobility of each protein was calculated as:

\[
\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}
\]

Mobilities calculated as above were then plotted versus the log of the subunit molecular weights of reference proteins and the resulting curve was used to estimate the subunit molecular weight of the enzyme.
(b) Isoelectric focussing

Analytical isoelectric focussing was performed on acrylamide gels according to the procedure of Wrigley (1971). Columns (75 x 5 mm) of 7.5% acrylamide were prepared which contained ampholines in the range of pH 3 to 10. The enzyme (20 µg in 10% sucrose) was layered on top of the gel under a protecting layer of 1% carrier ampholyte solution (in 5% sucrose). A disc electrophoresis apparatus was used for the electrofocussing and the current was maintained at a maximum of 2 mA per tube, while the voltage was gradually raised to a maximum of 400 V. At the end of 2 hr, the gels were removed and one of the set of unstained gels was cut into 15 equal (0.5 mm) slices. The pH of each piece was determined after mixing in a vortex mixer with 1.0 ml of distilled water. A duplicate gel was washed extensively with 5% trichloro acetic acid and stained for 1 hr with 1% amido black in 7% acetic acid. The gels were destained with 7% acetic acid. A pH gradient curve was constructed by plotting the pH measured in the slices versus the distance along the length of the gel. The isoelectric point was then determined by locating the position of the stained region along the pH gradient curve.

(c) Absorption spectra and dry weight determinations

In order to determine the extinction coefficient of the enzyme both in water and in N-ethylmorpholine-HCl buffer (0.1 M, pH 7.5) containing 1 mM EDTA and 10% glycerol (w/v), a solution of the enzyme (3 mg/ml) was dialyzed against 200 volumes of water with four changes at about 5 hr intervals, lyophilized and dissolved in either buffer or water. The solutions were centrifuged at 23,000 x g and the absorption spectra of the supernatant solutions were recorded on a Cary 118 spectrophotometer.
The supernatant solution of the sample in buffer was redialyzed against water after which the concentration of protein in both supernatants was determined by dry weight measurements. In this method, triplicate samples of the protein were heated to $105^\circ C$ over silica gel and were weighed on a microchemical balance (Oertling Model 147) until constant weight was obtained over a 2-day period.

(d) Amino acid analysis

Analysis of acid hydrolysates of the protein were performed essentially by the method of Spackman, Moore and Stein (1958) on a Beckman Model 120B amino acid analyzer. Analysis of protein oxidized with performic acid yielded the estimates for cystine-cysteine as cysteic acid, and methionine as methionine sulphone. The tryptophan content of the enzyme was estimated spectrophotometrically by the method of Beavan and Holiday (1952).

(e) Preparation of peptide maps

The peptide maps were prepared in conjunction with Dr. D. C. Shaw of the Department of Physical Biochemistry, Australian National University. The protein solution (6 mg) was dialyzed exhaustively against 50 mM ammonium bicarbonate solution (pH 7.5) and lyophilized. The lyophilized sample was oxidized with preformed performic acid (Hirs, 1956) and relyophilized. The material was then dissolved in 0.5% ammonium bicarbonate and hydrolyzed by trypsin (used at a ratio of 1:50) at $37^\circ C$ for 4 hr at pH 8.1. The ammonium bicarbonate was removed by lyophilization, the residue dissolved in 1 ml of water and the solution adjusted to pH 4.0 by the addition of glacial acetic acid. This
treatment results in the precipitation of a small amount of 'core' material which was less than 5% by weight of protein. The 'core' material was removed by centrifugation after which the soluble peptides were lyophilized and used for the preparation of peptide maps.

After dissolving in a minimum volume of water, the complete sample was applied to a sheet of Whatman 3 mM paper, and subjected to electrophoresis at pH 4.7 in pyridine-glacial acetic acid buffer for 90 min at 40 V/cm. The relevant strip was cut out, sewn on to a fresh sheet of paper and subjected to a chromatographic separation using the solvent system butanol:acetic acid:pyridine:water (15:3:10:12 v/v). To ensure detection of peptides present in low concentrations, the chromatogram was sprayed with fluram (0.01% in acetone containing 1% pyridine) and after 15 min, the stained peptides were located as fluorescent spots under UV light. The map was then sprayed on the reverse side with ninhydrin (0.1%), allowed to develop overnight and the position of the stained peptides noted. The chromatogram was sprayed with acetone containing 0.5% HCl to remove any traces of colour after which it was stained with specific stains for arginine, histidine and tyrosine.

(f) Analytical ultracentrifugation

Ultracentrifugal analysis was kindly performed by Dr. L. W. Nichol of the Department of Physical Biochemistry, Australian National University. The enzyme, stored frozen as described in Chapter II, was thawed at 30°C and concentrated to a final concentration of 3 mg/ml using an immersible molecular separator (Millipore Corporation). The concentrated sample was dialyzed against 200 volumes of 0.1 N-ethyl-
morpholine-citrate buffer (pH 7.0) containing 1 mM EDTA and 1 mM dithiothreitol, with four changes at about 5 hr intervals, in order to remove all traces of glycerol that had been added to the samples during storage. The dialyzed enzyme was then subjected to a sedimentation velocity run in a Spinco Model E ultracentrifuge equipped with electronic speed control. A conventional 12 mm, 4° single-sector cell (kel-F centre piece) was employed in an An-D rotor. Patterns were recorded on a Kodak metallographic plate and the sedimentation coefficient was calculated from the rate of movement of the maximum ordinate found with a Nikon-two dimensional comparator. A similar cell loaded with a solution of enzyme (0.3% in the same buffer as above) containing 1 mM prephenate was used in place of a counterbalance. This second cell differed from the first only in that the top window was a 1° negative-wedge which displaced the Schlieren pattern downward. This arrangement permitted the direct comparison of the sedimentation behaviour of the protein in the presence and absence of substrate at the same angular velocity (52,000 rpm) and temperature (20°C).

(g) Sucrose density centrifugation

Sucrose density centrifugation was carried out according to the method of Martin and Ames (1961). Linear sucrose gradients of 5 to 20% in a total volume of 4.5 ml were constructed and stored at 4°C for 4 hr before use. Samples containing 100 µl of purified chorismate mutase-prephenate dehydrogenase (0.5 mg/ml), along with 50 µg of yeast alcohol dehydrogenase and 20 µg of beef liver catalase were layered on the gradient. Centrifugation was carried out at 4°C and 35,000 rpm using a swinging bucket rotor SW-56 in a model L Spinco centrifuge. At the end of 12 hr, the tubes were removed, punctured at the bottom and
the samples fractionated into 100 µl fractions using an automatic fractionator. The fractions were assayed for catalase, alcohol dehydrogenase (Martin and Ames, 1961), chorismate mutase, and prephenate dehydrogenase activities. A graph of fraction number versus level of enzyme activity was then plotted for each enzyme. The distance from the meniscus to the activity peak was estimated for each enzyme and the following ratio $R$ calculated.

$$R = \frac{\text{distance travelled from meniscus by sample (mutase-dehydrogenase)}}{\text{distance travelled from meniscus by standard (reference enzyme)}}$$

From the value of $R$, the $s_{20,w}^{0.725}$ value for mutase-dehydrogenase was calculated using the relationship

$$R = \frac{s_{20,w}^{0.725} \text{ of unknown}}{s_{20,w}^{0.725} \text{ of standard}} \quad \text{s}_{20,w}^{0.725} \text{ is defined as } s_{20,w} \text{ calculated on the assumption of a partial specific volume of 0.725 cm}^3 \text{ per g.}$$

Two estimates of the $s_{20,w}^{0.725}$ value for mutase-dehydrogenase were obtained, one using catalase and the other using alcohol dehydrogenase as the standard protein in the above relationship. The reported value is the average of the two estimates.

4. RESULTS

(a) Molecular weight of the native enzyme

The molecular weight of native chorismate mutase-prephenate dehydrogenase was estimated by pore-limit electrophoresis in a gradient of polyacrylamide gel (Slater, 1969). In this method, the protein molecules are forced to migrate through a continuous gradient of
increasing density and consequently, decreasing pore size. As the run progresses, the molecules move more and more slowly, finally reach their 'pore-limit' and come to a virtual stop. If two proteins of known molecular weight are included in the electrophoretic run, then after the 'pore-limit' has been reached, a simple log-log plot can be used to determine the molecular weight of the unknown sample. Using this method, the tendency of proteins to associate or dissociate can also be identified and the molecular weights of the components involved, easily determined.

When a purified preparation of chorismate mutase-prephenate dehydrogenase was electrophoresed in a gradient gel, a single sharp band was seen as shown in Fig.III.1A. The average estimate of the molecular weight of the enzyme obtained from several electrophoretic runs was 88,000 ± 1,000 (Fig.III.1B). No higher molecular weight forms of the enzyme were detected in the range of concentrations used (25-100 µg protein).

(b) Molecular weight of the enzyme in sodium dodecyl sulphate

The method of Weber et al (1972) was used to estimate the molecular weight of the dissociated enzymes by electrophoresis in sodium dodecyl sulphate. Sodium dodecyl sulphate is known for its ability to dissociate oligomeric proteins into their individual subunits. The binding of large amounts of sodium dodecyl sulphate to each subunit also overcomes any charge differences between different polypeptides. As a result, in gels containing sodium dodecyl sulphate, the proteins migrate in proportion to their molecular weights only.
FIG. III.1. ELECTROPHORESIS OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE IN A GRADIENT OF POLYACRYLAMIDE GEL.

A. PATTERN OF ELECTROPHORESIS OBSERVED WITH
   (a) CROSS-LINKED OVALBUMIN POLYMERS.
   (b) & (c) PURIFIED PREPARATIONS OF CHORISMATE MUTASE-
   PREPHENATE DEHYDROGENASE.

B. ESTIMATION OF THE MOLECULAR WEIGHT OF CHORISMATE MUTASE-
   PREPHENATE DEHYDROGENASE. THE PROTEINS USED AND THEIR
   MOLECULAR WEIGHTS ARE:
   1. CATALASE (240,000).
   2. SERUM ALBUMIN DIMER (136,000).
   3. CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE.
   4. OVALBUMIN DIMER (86,000).
   5. SERUM ALBUMIN MONOMER (68,000).
   6. OVALBUMIN MONOMER (43,000).
When chorismate mutase-prephenate dehydrogenase was electrophoresed in gels containing 0.1% sodium dodecyl sulphate, the enzyme gave only a single sharp band of protein (Fig.III.2A). The molecular weight of the enzyme in 0.1% sodium dodecyl sulphate was calculated to be 42,000 (Fig.III.2B).

(c) Isoelectric point of the enzyme

A single band of protein corresponding to an isoelectric pH of 5.3 was observed when the enzyme was subjected to electrophocussing on polyacrylamide gels containing ampholine from pH 3-10 (Fig.III.3). The enzyme appeared to lose a large proportion of its activity during the electrophocussing and very little enzyme activity could be detected in unstained duplicate gels. Since it is known that the enzyme is largely inactive at pH values below 6.0, it must be noted that the isoelectric pH of 5.3 determined under these conditions may reflect the isoelectric point of a largely inactive enzyme.

(d) Absorption spectrum of the enzyme

Since the dithiothreitol present in samples of enzyme undergoes oxidation and the oxidized dithiothreitol contributes to optical density readings at 280 nm, it was necessary to record the absorption spectrum of the enzyme in samples from which dithiothreitol had been removed by dialysis against water as described under 'Methods'. The only significant absorption of the enzyme appeared to be in the ultraviolet range. The spectrum had a maximum at 278 nm, a minimum at 250 nm, and two small shoulders at 283 and 290 nm (Fig.III.4). The values of the extinction coefficient \( \varepsilon_{1\%}^{\text{cm}} \) at 278 and 280 nm were 0.819 and 0.818.
FIG.III.2. POLYACRYLAMIDE GEL ELECTROPHORESIS OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE IN THE PRESENCE OF 0.1% SODIUM DODECYL SULPHATE.

A. 20 MICROGRAMS OF ENZYME.

B. ESTIMATION OF THE MOLECULAR WEIGHT OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE. THE PROTEINS USED AND THEIR MOLECULAR WEIGHTS ARE:

1. SERUM ALBUMIN (68,000).
2. GLUTAMATE DEHYDROGENASE (53,000).
3. CREATIVE KINASE (40,000).
4. CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE.
5. PEPsin (35,000).
6. TRYPsin (23,000).
FIG. III.3. ISOELECTRIC FOCUSING OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE.

The distribution of pH along the length of the isoelectric focusing gel. Arrow indicates the position of the protein band.
FIG. III.3. ISOELECTRIC FOCUSING OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE.

THE DISTRIBUTION OF pH ALONG THE LENGTH OF THE ISOELECTRIC FOCUSING GEL. ARROW INDICATES THE POSITION OF THE PROTEIN BAND.
FIG. III.4. ABSORPTION SPECTRUM OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE (0.61 MG/ML) IN 0.1 M N-ETHYLMORPHOLINE-HCL BUFFER (pH 7.4) CONTAINING 1 mM EDTA AND 10% (w/v) GLYCEROL.
respectively in water and 0.83 and 0.82 respectively in Buffer B. These values appear to correlate well with the tyrosine and tryptophan content of the enzyme.

(e) Amino acid composition, minimum molecular weight and partial specific volume of the enzyme

The amino acid composition of chorismate mutase-prephenate dehydrogenase is given in Table III.1. The results, representing average values obtained from three analyses, were used to calculate the minimum molecular weight of chorismate mutase-prephenate dehydrogenase using the 'FITMOL' computer program of Gibbs and McIntyre (1970). This procedure assesses how well a particular amino acid composition fits every protein within a stipulated size range by calculating an 'integer deviate index' (IDI) for each protein within that size range. A single, well defined minimum observed within this range usually indicates that the amino acid composition best fits the protein size for which the minimum IDI value was calculated.

The amino acid composition of chorismate mutase-prephenate dehydrogenase was tested for a fit to every protein within the size range of 300-600 amino acids. The choice of this size range was based on a molecular weight of 42,000 for the subunits of the enzyme estimated from electrophoresis in sodium dodecyl sulphate. Using the 'FITMOL' computer program, a minimum 'IDI' value was obtained for a protein size of 386 amino acids corresponding to a minimum molecular weight of 43,000, for the enzyme polypeptide. The amino acid composition in Table III.1 has therefore been presented as residues per 43,000 daltons.
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues/43,000 daltons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>17.4</td>
</tr>
<tr>
<td>His</td>
<td>9.5</td>
</tr>
<tr>
<td>Arg</td>
<td>26.9</td>
</tr>
<tr>
<td>Asp</td>
<td>33.6</td>
</tr>
<tr>
<td>Thr</td>
<td>7.2</td>
</tr>
<tr>
<td>Ser</td>
<td>21.4</td>
</tr>
<tr>
<td>Glu</td>
<td>52.5</td>
</tr>
<tr>
<td>Pro</td>
<td>17.5</td>
</tr>
<tr>
<td>Gly</td>
<td>28.2</td>
</tr>
<tr>
<td>Ala</td>
<td>37.6</td>
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<td>Val</td>
<td>28.9</td>
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<td>Met</td>
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<tr>
<td>Ileu</td>
<td>17.1</td>
</tr>
<tr>
<td>Leu</td>
<td>48.1</td>
</tr>
<tr>
<td>Tyr</td>
<td>10.2</td>
</tr>
<tr>
<td>Phe</td>
<td>13.8</td>
</tr>
<tr>
<td>Trp</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*Cysteic acid was estimated in performic acid oxidized samples.*

*Estimated spectrophotometrically.*
Amino acid analysis on pure preparations of the *E. coli* enzymes consistently yielded 16 moles of threonine/mole of enzyme as compared to a value of 36 moles of threonine/mole of enzyme reported by Koch *et al* (1971b) for chorismate mutase-prephenate dehydrogenase from both *E. coli* and *A. aerogenes*. Loss of threonine due to hydrolysis during the analysis appears as an unlikely explanation for the lower yield of threonine observed, as the yield of serine in the same analysis appears to have been unaffected. It is therefore possible that the high threonine content found by Koch *et al* (1971a) was due to the presence of protein impurities in their preparation.

The amino acid composition determined as above was used to calculate the partial specific volume of the enzyme, according to the method of Cohn and Edsall (1943). The value obtained was 0.738 ml/g, when neither the state of amidation of glutamic and aspartic acids nor the possible presence of carbohydrates was taken into account.

(f) Peptide maps of the enzyme

When the peptide maps of tryptic digests of chorismate mutase-prephenate dehydrogenase were stained with fluram, approximately 45-50 peptides were located as fluorescent spots under UV light. The spots were well resolved and the peptides located with fluram were seen to be also reactive with ninhydrin (Fig.III.5). On staining with individual reagents, 25 of the peptides were found to contain arginine, nine contained tyrosine and nine had histidine. A diagram of the peptide map showing the location of the peptides containing each of these amino acids is shown in Fig.III.6.

Similar efforts by Koch *et al* (1971b) to prepare peptide
FIG.III.5. TRYPTIC PEPTIDE MAP OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE.

FIRST DIMENSION - ELECTROPHORESIS AT PH 4.7 IN ACETIC ACID BUFFER FOR 90 MIN AT 40 V PER CM.

SECOND DIMENSION - CHROMATOGRAPHY FOR 18 HR IN BUTANOL-ACETIC ACID-PYRIDINE-WATER (15:3:10:12 V/V).
FIG. III.6. THE ARGinine-, HISTidine- AND TYROSine- CONTAINING PEPTIDES OF THE TRYPTIC PEPTIDE MAP FOR CHORismate Mutase-PREPHENATE DEHYDROGENASE.
maps of tryptic digests of chorismate mutase-prephenate dehydrogenase, using the conventional peptide mapping procedure had been unsuccessful and had resulted in poor resolution of the peptide maps. The extensive smearing of the spots observed by Koch et al (1971b) in their peptide maps is also consistent with the possible presence of contaminating proteins in the enzyme samples used for the preparation of the maps.

\( s_{t,b} \)

\( s_{t,b} = \frac{1}{\omega^2 r} \frac{dr}{dt} = \frac{1}{\omega^2} \frac{d(lnr)}{dt} \)  \hspace{1cm} (III-1)

where \( \omega \) is the radial velocity (radians/sec). Thus, when the \( r \) is determined at various times \( t \), a plot of \( \log_{10} r \) versus time is a straight line of slope \( \omega^2 s_{t,b}/2.303 \). The dimensions of a sedimentation coefficient are sec and usually values are reported in terms of the Svedberg unit, \( S \), where \( 1 \ S = 1 \times 10^{-13} \) sec. For the purpose of comparison between different experiments carried out in different solvents and at different temperatures, values of \( s_{t,b} \) are corrected to \( 20^\circ C \) in water by employing the following relation (Svedberg and Pedersen, 1940):
\[ s_{20,w} = \frac{s_{t,b}}{\eta_{20,w}^t} \times \frac{\eta_{rel}}{\eta_w^t} \times \frac{(1-Vp_{20,w})}{(1-Vp_{t,b})} \]  

(III-2)

where \( \eta_w^t \) is the viscosity of water at temperature \( t \);

\( \eta_{20,w} \) is the viscosity of water at 20°C;

\( \eta_{rel} \) is the viscosity of the solvent used relative to that of water at temperature \( t \);

\( \bar{V} \) is the partial specific volume of the solute;

\( \rho_{20,w} \) is the density of water at 20°C; and

\( \rho_{t,w} \) is the density of water at temperature \( t \).

Values of \( s_{20,w} \) generally depend upon the concentration of the macromolecule and the particular value obtained by extrapolation to infinite dilution is designated \( s_{20,w}^0 \).

The sedimentation coefficient of a molecule at infinite dilution can be used for the calculation of its molecular weight using the following relationship (Svedberg and Pedersen, 1940)

\[ s_{20,w}^0 = \frac{M(1-V)}{Nf} \]  

(III-3)

where \( M \) is the molecular weight of the species, \( f \) is the frictional coefficient per molecule and \( N \) is the Avogadro's number. The molecular weight of a macromolecule cannot be determined from sedimentation velocity studies alone unless its diffusion coefficient at infinite dilution, \( D^0 \), is known, since this enables \( f \) to be computed from the relationship

\[ D^0 = \frac{RT}{f} \]  

(III-4)

On the other hand, if information on the molecular weight is available, then a value for the frictional coefficient of the molecule can be calculated from equation III-3. The molar frictional coefficient of a spherical, unhydrated molecule of the same mass may be calculated from
Stoke's Law (Svedberg and Pedersen, 1940) as

\[ f_0 = \frac{6\pi \eta N}{4N^2} \left( \frac{M}{4\pi N} \right)^{1/3} \]  

(III-5)

where \( N \) is Avogadro's number and 

\( \eta \) is the viscosity of the solvent

The ratio of the actual frictional coefficient \( f \) to that calculated for an idealised spherical molecule \( f_0 \) is termed 'frictional ratio'. It will obviously be 1.0 for an unsolvated spherical molecule and greater than unity for a molecule which is either asymmetric or solvated.

During sedimentation velocity experiments in 0.1 M N-ethylmorpholine-citrate buffer (pH 7.0) containing 1 mM EDTA and 1 mM dithiothreitol, samples of chorismate mutase-prephenate dehydrogenase moved as a single symmetrical boundary both in the presence and absence of 1 mM prephenate. A typical Schlieren pattern is shown in Fig.III.7. Extrapolation to infinite dilution by the least squares method gave a value for \( s_{20,w} \) of 4.8 S. Assuming a molecular weight of 88,000 at infinite dilution, and substituting a value of 0.738 ml/g for the partial specific volume of the enzyme in equations III-3 and III-5, a frictional ratio of 1.4 was calculated for an unsolvated molecule of chorismate mutase-prephenate dehydrogenase. The significant deviation from unity suggested that the molecule of chorismate mutase-prephenate dehydrogenase was either asymmetric or highly solvated. Assuming an average value of 0.37 g of water/g of protein for the degree of hydration, the axial ratio for a protein molecule corresponding to a frictional ratio of 1.4 was obtained from a contour chart prepared by Oncley (1941). The axial ratio \( (a/b) \) thus obtained was either 0.2 for a prolate molecule or 5.0 for an oblate molecule.
FIG.III.7. ULTRACENTRIFUGATION PATTERN OF PURIFIED CHORISMA TE MUTASE-PREPHENATE DEHYDROGENASE.

PHOTOGRAPHS ARE SHOWN OF SCHLEIßREN PATTERNS WHICH WERE OBTAINED AT THE INDICATED TIMES DURING ULTRACENTRIFUGATION. THE CONDITIONS USED ARE DESCRIBED UNDER 'METHODS'.
(ii) In sucrose density gradient centrifugation

A single symmetrical peak of enzyme activity was observed when the enzyme was centrifuged in a linear 5 to 20% gradient of sucrose (Fig.III.8). A constant ratio of mutase and dehydrogenase activity was observed all along the peak confirming the bifunctional nature of the enzyme. A sedimentation coefficient $s_{20,w}^{0.725}$ of 5 S calculated for the enzyme is in reasonable agreement with the value obtained from sedimentation velocity experiments.

5. DISCUSSION

In this chapter are described some of the general properties of chorismate mutase-prephenate dehydrogenase that have been determined using homogeneous preparations of the enzyme. A molecular weight of 88,000 was estimated for the native enzyme and no aggregation of the native enzyme into larger molecular species was detected. Analytical ultracentrifugation revealed a single symmetrical peak corresponding to a sedimentation coefficient of 4.8 S, both in the presence and absence of prephenate. The observation of identical values for the sedimentation coefficients indicated that this substrate does not cause any detectable changes in the molecular size of the enzyme. A frictional ratio of 1.4 was calculated for the enzyme which indicates that the protein is either asymmetrical or highly solvated. These results may be compared with those obtained by Koch et al (1971a) who reported a molecular weight of 82,000 and a sedimentation coefficient of 4.2 S for the *E.coli* enzyme. A frictional ratio, calculated on the basis of the above results, yielded an even higher value of 1.55.

A molecular weight of 42,000 was estimated for the single
FIG. III.C. SUCROSE DENSITY GRADIENT CENTRIFUGATION OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE.

PATTERN OF ENZYME ACTIVITY IN FRACTIONS OBTAINED AFTER CENTRIFUGATION IN A LINEAR GRADIENT OF SUCROSE AS DESCRIBED UNDER 'METHODS'.
band observed on electrophoresis in sodium dodecyl sulphate. The
detection of only a single polypeptide species and the observation that
the molecular weight of the species was almost exactly half that of the
native enzyme suggested that the enzyme is a dimer consisting of subunits
of identical or very similar size. Since the two subunits could be of
similar size and yet different amino acid composition, it was necessary
to employ a method that would distinguish between the two subunits on
the basis of their sequences. Peptide mapping, has therefore been used
for further characterization of the subunits of the enzyme.

The amino acid composition of the enzyme (Table III.1) was
used to predict the total number of peptides expected from a protein of
molecular weight 88,000. For a protein with nonidentical subunits,
this number would be about 80. The actual number of tryptic peptides
observed was only half of the theoretical value indicating that the
enzyme contained two subunits of very similar or identical sequences.
In addition to identifying the total number of tryptic peptides in the
map, efforts have also been made to identify the number of peptides
containing tyrosine, histidine and arginine. For a protein with
identical subunits, the amino acid composition predicts a maximum of
nine histidine, ten tyrosine and twenty-seven arginine containing peptides
which corresponds well with the observation of nine histidine, nine
tyrosine and twenty-five arginine containing peptides in the peptide map.
It appears therefore, that the enzyme chorismate mutase-prephenate
dehydrogenase is made up of two subunits which are identical or very
similar in their sequence.

Similar subunits have also been reported for the bifunctional
enzyme chorismate mutase-prephenate dehydratase which occurs in the
biosynthetic pathway for phenylalanine. Hence it is unlikely that
the two bifunctional enzymes in *E. coli* evolved by the association of
a single type of mutase subunit with the enzymes prephenate dehydrogenase
and prephenate dehydratase respectively. All the same, it would be
expected that the two proteins would have similar evolutionary origins
and hence similar stretches of amino acid sequences. A comparison of
the amino acid composition of the two bifunctional enzymes (Table III.2)
indicates that the two enzymes are almost identical in their amino acid
composition. In addition to the enzyme chorismate mutase-prephenate
dehydratase, two other enzymes were found to possess amino acid
compositions very similar to that of *E. coli* mutase-dehydrogenase. They
were the aromatic amino acid aminotransferase and the aspartate
aminotransferase from *E. coli*. Both enzymes have been purified and
their respective substrate specificities identified (Powell and
Morrison, 1978). The aromatic amino acid aminotransferase catalyzes
the transamination of phenylpyruvate to phenylalanine and the transamination
of hydroxyphenylpyruvate to tyrosine in the biosynthetic pathway of
aromatic amino acids. It therefore occurs in the sequence after the
mutase-dehydratase in the phenylalanine biosynthetic pathway and after
the mutase-dehydrogenase in the tyrosine biosynthetic pathway. The
aspartate aminotransferase is primarily concerned with the transamination
of aspartate but also catalyzes the above two reactions to a lesser
extent than the aromatic amino acid aminotransferase. These enzymes
are listed in Table III.2 and all have similar molecular weights, similar
subunit structures and remarkably similar amino acid compositions.
Similarity of compositions is not proof of sequence homology and such
investigations have yet to be undertaken. However the results could
lend support to the speculation that these enzymes may all have evolved
from a single ancestral protein.
# Table III.2. Amino Acid Composition of Enzymes in the Aromatic Biosynthetic Pathway of *E. coli*.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th><em>E. coli</em> chorismate a mutase-prephenate dehydrogenase (per 86,000 daltons)</th>
<th><em>E. coli</em> chorismate b mutase-prephenate dehydratase (per 80,000 daltons)</th>
<th><em>E. coli</em> aromatic c amino acid aminotransferase (per 90,000 daltons)</th>
<th><em>E. coli</em> aspartate c aminotransferase (per 84,000 daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>71</td>
<td>62</td>
<td>68</td>
<td>66</td>
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<td>Thr</td>
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<td>38</td>
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</tr>
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<td>Ser</td>
<td>42</td>
<td>38</td>
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<td>Glu</td>
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<td>Gly</td>
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<td>74</td>
</tr>
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<td>Ala</td>
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<td>66</td>
<td>74</td>
</tr>
<tr>
<td>Cys</td>
<td>8</td>
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<td>6</td>
</tr>
<tr>
<td>Val</td>
<td>58</td>
<td>48</td>
<td>56</td>
<td>48</td>
</tr>
<tr>
<td>Met</td>
<td>18</td>
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<td>14</td>
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<tr>
<td>Ileu</td>
<td>34</td>
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<tr>
<td>Leu</td>
<td>96</td>
<td>92</td>
<td>66</td>
<td>63</td>
</tr>
<tr>
<td>Tyr</td>
<td>20</td>
<td>20</td>
<td>16</td>
<td>14</td>
</tr>
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<td>Phe</td>
<td>28</td>
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<td>20</td>
<td>24</td>
</tr>
<tr>
<td>Trp</td>
<td>07</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Lys</td>
<td>37</td>
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<td>48</td>
<td>40</td>
</tr>
<tr>
<td>His</td>
<td>18</td>
<td>26</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Arg</td>
<td>50</td>
<td>40</td>
<td>38</td>
<td>32</td>
</tr>
</tbody>
</table>

* from Table III.1.

* Davidson et al (1972)

* Powell and Morrison (1978)

N.A. Not available
It has been determined that chorismate mutase-prephenate dehydrogenase has a molecular weight of 88,000 and is composed of two identical subunits. Further, the enzyme has a sedimentation coefficient of 4.8 S, a frictional ratio of 1.4, an isoelectric pH of 5.3 and an extinction coefficient \( E_{280}^{1\%} \) of 0.82.
CHAPTER IV

KINETIC MECHANISM OF THE PREPHENATE DEHYDROGENASE
REACTION CATALYSED BY CHORISNATE MUTASE-PREPHENATE
DEHYDROGENASE

1. INTRODUCTION

2. MATERIALS

3. METHODS

(a) Preparation of solutions of enzymes
(b) Preparation of solutions of substrate
(c) Preparation of solutions of reactants
(d) Detection of enzyme activity
(e) Measurement of enzyme activity
(f) Analysis of kinetic data

4. RESULTS

(a) Preliminary investigation of the kinetic properties of chorismate mutase-prephenate dehydrogenase

(1) Selection of a suitable pH for kinetic experiments

(2) Effect of buffer type on activity

(b) Steady state velocity studies in the presence of product

(c) Steady state velocity studies in the presence of deadend inhibitors

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6. SUMMARY
CHAPTER IV  
KINETIC MECHANISM OF THE PREPHENATE DEHYDROGENASE  
REACTION CATALYZED BY CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE

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1. **INTRODUCTION**

The dehydrogenase reaction catalyzed by the enzyme chorismate mutase-prephenate dehydrogenase obeys Michaelis-Menten kinetics (Chapter II). It is therefore possible to determine by standard procedures the kinetic mechanism of the dehydrogenase reaction and to evaluate the magnitude of the kinetic parameters associated with the various reactants. Such a study is also a prerequisite for determining the number of active sites involved in the conversion of chorismate to 4-hydroxyphenylpyruvate by the enzyme, using kinetic techniques (Chapter V). This chapter describes the elucidation of the kinetic mechanism of the dehydrogenase reaction using steady state kinetic techniques.

2. **MATERIALS**

NAD, NADH, AMP and cis-aconitate were supplied by P-L Biochemicals while phenylpyruvic acid, phenylacetic acid, p-hydroxycinnamic acid, 3,5-dihydroxybenzoic acid and lyophilized fatty acid free bovine serum albumin were supplied by Sigma. Adamantane 1,3 diacetic acid and adamantane 1,3 dicarboxylic acid were purchased from Aldrich, while succinate, maleate, α-ketoglutarate and morpholinoethane sulfonic acid were products of Calbiochem. Aspartate aminotransferase from *E.coli*, rabbit muscle creatine kinase and bovine liver arginine kinase were gifts from Dr. J. T. Powell, Dr. K. J. Ellis and Dr. J. F. Morrison respectively. Ovalbumin and lysozyme were supplied by Sigma and urease was a product of Nutritional Biochemicals Corporation.
3. METHODS

(a) Preparation of solutions of 4-hydroxyphenylpyruvate

Hydroxyphenylpyruvic acid was dissolved at room temperature in 0.2 M N-ethylmorpholine-morpholinoethane sulfonic acid buffer, initially at pH 7.7, and the pH was adjusted back to pH 7.7 with 0.01 N NaOH. The solutions were then frozen overnight at \(-15^\circ\text{C}\) after which they were thawed at room temperature and kept on ice for two hours before use. The hydroxyphenylpyruvate was present in the keto form as judged from its absorption spectrum (Knox and Pitt, 1956). The thawed samples were used within 24 hr but were not refrozen and reused. The above procedure ensured that hydroxyphenylpyruvate did not decompose under alkaline conditions to hydroxybenzaldehyde as well as other unknown derivatives which may be more inhibitory than hydroxyphenylpyruvate (Doy, 1960).

(b) Preparation of 5,6-epoxychorismic acid

Chorismic acid was transformed into its 5,6-epoxide by reaction with m-chloroperbenzoic acid according to the method of Ife et al (1976). The product isolated after three recrystallizations had a m.p. of 150°C (expected m.p. 149-152°C). Elemental analysis (in a 185-B analyzer) indicated a value for C of 49.4% and H of 4.3% which corresponds well with that expected for 5,6-epoxychorismic acid (for \(\text{C}_{10}\text{H}_{10}\text{O}_7\), expected C, 49.6%, H, 4.1%).

(c) Preparation of stock solutions of reactants

Stock solutions of substrates and inhibitors used in the
kinetic experiments were prepared by dissolving the compounds in water and adjusting the solutions to a pH of 7.7 with NaOH. The solutions were then stored frozen at -15°C. Stock solutions of chorismate (5 mM) and prephenate (5 mM) were stable for up to two months under these conditions. Solutions of the keto form of phenylpyruvate were prepared as described for hydroxyphenylpyruvate and were used within 24 hr.

(d) **Dilution of concentrated enzyme**

Samples of pure enzyme, stored as described earlier (Chapter II), were thawed at 30°C and diluted to the required concentration with 0.1 M N-ethylmorpholine-citrate buffer (pH 7.0) which contained 10% glycerol (w/v), 1 mM EDTA and 1 mM dithiothreitol. Enzyme samples were observed to lose up to 30% of their original activity immediately after dilution but regained complete activity after 2 hr at 4°C. The diluted enzyme was not stable for more than 24 hr at 4°C but could be stored at -15°C for up to two weeks without any loss in activity. The freshly diluted samples were therefore kept at 4°C for at least 2 hr, refrozen in liquid nitrogen and stored at -15°C. They were thawed as required and used within 24 hr.

(e) **Measurement of enzyme activity**

Prephenate dehydrogenase activity was measured as described in Chapter II. All reaction mixtures contained 50 mM N-ethylmorpholine-morpholinoethane sulfonic acid buffer (pH 7.7), 1 mM EDTA, 1 mM dithiothreitol and 100 µg albumin per ml except where indicated otherwise.
(f) **Analysis of kinetic data**

The velocity data were first plotted graphically in double reciprocal form to check any departure from linearity and to determine the pattern of the plots. An overall fit of each set of data was then made to the appropriate rate equation by using one of the computer programs of Cleland (1963) in conjunction with a Univac 1100/42 computer. In the absence of products, velocity data were fitted to the equation

\[
\frac{V}{V_{AB}} = \frac{V_{AB}}{K_{iA} K_{B} + K_{B} + K_{A} + AB}
\]

while linear competitive inhibition, linear non-competitive inhibition and linear uncompetitive inhibition data were fitted to equation IV-2, IV-3 and IV-4 respectively:

\[
v = \frac{V_{A}}{K(1 + \frac{I}{K_{iA}} + A)} \quad \text{(IV-2)}
\]

\[
v = \frac{V_{A}}{K(1 + \frac{I}{K_{iS}}) + A(1 + \frac{I}{K_{iI}})} \quad \text{(IV-3)}
\]

\[
v = \frac{V_{A}}{K + A(1 + \frac{I}{K_{iI}})} \quad \text{(IV-4)}
\]

The data sets were weighted on the basis of the results of the residual analysis (Ellis and Duggleby, 1978) and fitted to the above equations to obtain the best estimates of the kinetic constants together with their standard errors and the values so obtained were used to draw the lines of the figures. In cases where a particular kinetic constant was determined in several experiments, the weighted mean values of the
apparent constants, together with their standard errors, were calculated according to the formulae:

\[
\text{weighted mean of } x \text{ values } = \frac{\sum W_i x_i}{\sum W_i} \quad \text{and}
\]

\[
\text{S.E. of weighted mean value } = \sqrt{\frac{1}{\sum W_i}}
\]

where

\[
W_i = \frac{1}{(\text{S.E.}(x_i))^2}
\]

The estimates of the true values for the kinetic constants were calculated from the relationships given in Table IV.6 and the standard errors of these estimates were determined using the following expression:

For \( y = f(x_1, x_2, x_3, \ldots) \)

\[
\text{Variance of } y = \left(f'(x_1)^2 \var(x_1) + f'(x_2)^2 \var(x_2) + \ldots \right)
\]

where \( f'(x) = \frac{dy}{dx} \)

\[
\text{S.E. of } y = \sqrt{\text{var}(y)}
\]

4. **RESULTS**

**a) Preliminary investigation of the kinetic properties of chorismate mutase-prephenate dehydrogenase**

**i) Selection of a suitable pH for kinetic experiments**

In preliminary experiments reaction velocities were measured as a function of the concentration of prephenate at different fixed concentrations of NAD using N-ethylmorpholine-morpholinoethane sulfonic acid buffers over the range from pH 6.0 to 8.5. At pH values below 7.0, marked substrate inhibition by prephenate was observed at concentrations...
above 0.1 mM. All primary plots of data obtained above this pH gave families of straight lines which intersected at a point to the left of the vertical ordinate and above the abscissa. It therefore appears that the reaction has a sequential mechanism. This conclusion was further supported by the observation that all sets of data gave good fits to Equation IV-1 when analyzed using the computer program of Cleland (1963). The results indicated that the maximum velocity of the reaction and the Michaelis constant for prephenate increase significantly with increasing pH whereas the Michaelis constant for NAD decreases slightly with increasing pH. These findings led to the use of N-ethylmorpholine-morpholinoethane sulfonic acid buffer (pH 7.7) in all further kinetic experiments. At this pH, no substrate inhibition was observed and the Michaelis constants for prephenate and NAD were in a convenient range. An added advantage was that the product 4-hydroxyphenylpyruvate was more stable at pH 7.7 than at other higher pH values.

(ii) Effect of bovine serum albumin

When the steady state reaction velocities were measured at pH 7.7 in the presence of small concentrations of albumin, there was observed a marked enhancement in the reaction velocity at lower concentrations of substrates. The increase in reaction velocity caused by the addition of albumin varies as a hyperbolic function of the concentration of albumin in the reaction mixture since the double reciprocal plots of the increase in steady state velocity as a function of albumin concentration are linear (Fig.IV.1). At a fixed concentration of 0.05 mM prephenate and 0.20 mM NAD, an apparent activation constant for albumin of 81 nM could be calculated from the reciprocal of the horizontal intercept of the above double reciprocal plot.
**FIG. IV.1.** EFFECT OF VARYING CONCENTRATIONS OF BOVINE SERUM ALBUMIN (BSA) ON THE STEADY STATE VELOCITY OF THE PREPHENATE DEHYDROGENASE REACTION.

The concentrations of Prephenate and NAD were fixed at 0.05 mM and 0.2 mM respectively. \( V_0 \) denotes the velocity in the absence of bovine serum albumin and \( V \) denotes the velocity in the presence of bovine serum albumin. Velocities are expressed as micromoles of NADH formed per min per mg of enzyme.
The steady state velocity pattern observed in the presence of albumin is also of the intersecting type, but the lines intersect much further to the left of the vertical ordinate and virtually on the abscissa (Fig. IV.2). The maximum velocity of the reaction is not affected by the addition of albumin. Values for the kinetic parameters in the absence and presence of various concentrations of albumin are given in Table IV.1 and it will be noted that activation arises through the lowering of the $K_i$ values for the substrates while the Michaelis constants are not significantly affected. In addition to lowering the $K_i$ values for the substrates, albumin has a significant stabilizing effect on the enzyme. It was therefore included in all further kinetic experiments at a concentration of 100 µg per ml of reaction mixture.

In addition to albumin, a number of other proteins were also observed to cause an activation of the dehydrogenase reaction. In each instance, the activation was due to a lowering of the $K_i$ values for the substrates (Table IV.2). The linearity of the double reciprocal plots for activation by albumin suggest that it may interact with the free enzyme to form an E-protein complex with altered kinetic properties. This conclusion is supported by the finding that a single enzymically active peak, corresponding to a molecular weight of 150,000 is observed when chorismate mutase-prephenate dehydrogenase is subjected to density gradient centrifugation in a medium (0.1 M N-ethylmorpholine-citrate, pH 7.7) containing 100 µg/ml of albumin. The expected molecular weight for a 1:1 complex of chorismate mutase-prephenate dehydrogenase with albumin is 156,000.

(b) Steady state velocity studies in the presence of products

The inhibition by NADH was found to be linear competitive with
FIG.IV.2. EFFECT OF THE CONCENTRATIONS OF NAD AND PREPHENATE ON THE STEADY STATE VELOCITY OF THE PREPHENATE DEHYDROGENASE REACTION IN THE ABSENCE (A) AND PRESENCE (B) OF BOVINE SERUM ALBUMIN (100 MICROGRAMS/ML). THE DATA WERE FITTED TO EQUATION (1). VELOCITIES ARE EXPRESSED AS MICROMOLES OF NADH FORMED PER MIN PER MG OF ENZYME.
### TABLE IV.1.

Maximum velocities (V) and kinetic constants for prephenate and NAD at various concentrations of bovine serum albumin added to the reaction mixture.

The data are given as weighted means ± standard errors of means of values obtained by fitting data sets, including that of Fig. IV.2, to Equation (1). $k_a$ and $k_b$ denote the respective Michaelis constants for prephenate and NAD. $k_{ia}$ and $k_{ib}$ represent the kinetic constants associated with the interaction of prephenate and NAD, respectively, with the free enzyme $a$. V is expressed as micromoles of NADH formed per min per mg of enzyme.

<table>
<thead>
<tr>
<th>Kinetic Constant</th>
<th>Concentration of albumin (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (5) b</td>
</tr>
<tr>
<td>V</td>
<td>33.20 ± 1.28</td>
</tr>
<tr>
<td>$k_{ia}$ (mM)</td>
<td>0.090 ± 0.015</td>
</tr>
<tr>
<td>$k_a$ (mM)</td>
<td>0.036 ± 0.005</td>
</tr>
<tr>
<td>$k_{ib}$ a (mM)</td>
<td>0.443 ± 0.127</td>
</tr>
<tr>
<td>$k_b$ (mM)</td>
<td>0.177 ± 0.035</td>
</tr>
</tbody>
</table>

---

*a* Subsequent studies indicated a rapid equilibrium random mechanism for which $(k_{ia}k_a)/k_a = k_{ib}$ in each experiment.

*b* Figures in parentheses indicate the number of experiments performed at each albumin concentration.
TABLE IV.2.  

MAXIMUM VELOCITIES (V) AND KINETIC CONSTANTS FOR PREPHENATE AND NAD AT FIXED CONCENTRATIONS (200 MICROGRAMS/ML) OF VARIOUS PROTEINS ADDED TO THE REACTION MIXTURE.

THE DATA ARE GIVEN AS WEIGHTED MEANS ± STANDARD ERRORS OF MEANS OF VALUES OBTAINED BY FITTING DATA SETS TO EQUATION (1). $k_a$ AND $k_b$ DENOTE THE RESPECTIVE MICHAELIS CONSTANTS FOR PREPHENATE AND NAD. $k_{ia}$ AND $k_{ib}$ REPRESENT THE KINETIC CONSTANTS ASSOCIATED WITH THE INTERACTION OF PREPHENATE AND NAD RESPECTIVELY WITH THE FREE ENZYME a. V IS EXPRESSED AS MICROMOLES OF NADH FORMED PER MIN PER MG OF ENZYME.

<table>
<thead>
<tr>
<th>Kinetic Constant</th>
<th>Protein added (200 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (5) b</td>
</tr>
<tr>
<td>V</td>
<td>33.20 ± 1.28</td>
</tr>
<tr>
<td>$k_{ia}$ (mM)</td>
<td>0.090 ± 0.015</td>
</tr>
<tr>
<td>$k_a$ (mM)</td>
<td>0.036 ± 0.005</td>
</tr>
<tr>
<td>$k_{ib}$ (mM)</td>
<td>0.443 ± 0.127</td>
</tr>
<tr>
<td>$k_b$ (mM)</td>
<td>0.177 ± 0.035</td>
</tr>
</tbody>
</table>

a Subsequent studies indicated a rapid equilibrium random mechanism for which $(k_{ia} k_b) / k_a = k_{ib}$ in each experiment.

b Figures in parentheses indicate the number of experiments.
respect to NAD (Fig.IV.3A,B) as the varied substrate. When NADH was studied as an inhibitor with respect to prephenate as the varied substrate, noncompetitive inhibition was indicated but the slope variations observed over the range of concentrations of NADH used in the experiment were rather small. The data were therefore fitted to equations for both noncompetitive and uncompetitive inhibition. A comparison of the variance values obtained indicated that the inhibition was definitely noncompetitive. 4-hydroxyphenylpyruvate causes linear competitive inhibition with respect to prephenate (Fig.IV.4A,B) and linear noncompetitive inhibition with respect to NAD (Fig.IV.4C). A double inhibition experiment in which the concentration of hydroxyphenylpyruvate was varied at a number of fixed concentrations of NADH showed intercept variation without any change in the slope of the lines. The data were therefore fitted to the equation for uncompetitive inhibition using values for the concentration of 4-hydroxyphenylpyruvate to replace the values for A in the equation and the apparent kinetic constants thus determined were used to draw the lines (Fig.IV.5).

The product inhibition by bicarbonate was very weak and since velocity measurements at sufficiently high concentrations of bicarbonate (above 0.2 M) were hindered by the appearance of bubbles in the reaction mixture, it was not possible to determine the inhibition patterns for CO₂. The apparent inhibition constants obtained from analysis of the experimental data for the inhibitions by NADH and hydroxyphenylpyruvate, using equations IV-2 and IV-3 are given in Table IV.3.

(c) Steady state studies in the presence of deadend inhibitors

AMP functions as a dead-end inhibitor of the dehydrogenase
FIG.IV.3. PRODUCT INHIBITION OF THE PREPHENATE DEHYDROGENASE REACTION BY NADH. (A) NAD VARIED WITH THE PREPHENATE CONCENTRATION FIXED AT 0.1 mM (B) NAD VARIED WITH THE PREPHENATE CONCENTRATION FIXED AT 0.4 mM (C) PREPHENATE VARIED WITH THE NAD CONCENTRATION FIXED AT 0.1 mM. VELOCITIES ARE EXPRESSED AS MICROMOLES OF NADH FORMED PER MIN PER MG OF ENZYME.
FIG. IV. 4. PRODUCT INHIBITION OF THE DEHYDROGENASE REACTION BY 4-HYDROXYPHENYLPyRUVATE (HPP). (A) PREPHENATE VARIED WITH THE NAD CONCENTRATION FIXED AT 0.2 mM. (B) PREPHENATE VARIED WITH THE NAD CONCENTRATION FIXED AT 2 mM. (C) NAD VARIED WITH THE PREPHENATE CONCENTRATION FIXED AT 0.1 mM. VELOCITIES ARE EXPRESSED AS MICROMOLES OF NADH FORMED PER MIN PER MG OF ENZYME.
FIG. IV.5. DOUBLE PRODUCT INHIBITION OF THE DEHYDROGENASE REACTION BY NADH AND 4-HYDROXYPHENYLPYRUVATE (HPP). THE CONCENTRATION OF PREPHENATE AND NAD WERE 0.05 mM AND 0.2 mM RESPECTIVELY. THE DATA WERE FITTED TO EQUATION (4). VELOCITIES ARE EXPRESSED AS MICROMOLES OF NADH FORMED PER MIN PER MG OF ENZYME.
### TABLE IV.3

**APPARENT PRODUCT INHIBITION CONSTANTS, \( \kappa_i \) SLOPE AND \( \kappa_i \) INTERCEPT FOR PRODUCTS NADH AND HYDROXYPHENYLPYRUVATE.**

The values for the apparent inhibition constants are the weighted means of the constants obtained by fitting the data of Figs. IV.3 and IV.4 and similar experiments to Equation (2) or (3). The constants are expressed as mM.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Type of Inhibition (^a)</th>
<th>Varied Substrate</th>
<th>Fixed Substrate</th>
<th>( \kappa_i ) slope (^b)</th>
<th>( \kappa_i ) intercept (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>NC</td>
<td>Prephenate</td>
<td>NAD (0.1 mM)</td>
<td>0.404 ± 0.065</td>
<td>0.075 ± 0.004</td>
</tr>
<tr>
<td>NADH</td>
<td>C</td>
<td>NAD</td>
<td>Prephenate (0.1 mM)</td>
<td>0.062 ± 0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prephenate (0.4 mM)</td>
<td>0.053 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>Hydroxyphenylpyruvate</td>
<td>NC</td>
<td>NAD</td>
<td>Prephenate (0.1 mM)</td>
<td>0.45 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Hydroxyphenylpyruvate</td>
<td>C</td>
<td>Prephenate</td>
<td>NAD (0.2 mM)</td>
<td>0.060 ± 0.004</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NAD (2 mM)</td>
<td>0.040 ± 0.002</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) NC, non competitive; C, competitive.  
\(^b\) Values are the weighted means of values from three experiments.
reaction giving inhibitions which are linear competitive with respect to NAD (Fig.IV.6A) and linear noncompetitive in relation to prephenate (Fig.IV.6B). The apparent inhibition constants, calculated for AMP as a dead-end inhibitor using equations IV-2 and IV-3 are listed in Table IV.4. Various compounds were tested as potential analogues of prephenate but most of the compounds tested caused double reciprocal plots to become nonlinear with prephenate as the variable substrate. Such compounds included 3,5-dihydroxybenzoate, 2,4-dihydroxybenzoate, phenylpyruvate, phenylacetate, p-hydroxycinnamate as well as the 1,3 diacetic and 1,3 dicarboxylic derivatives of adamantane (cf Andrews et al, 1977). On the other hand, 5,6-epoxy chorismate which was tested as a possible transition state analogue of the mutase reaction (Ife et al, 1976) did not inhibit the dehydrogenase reaction up to a concentration of 5 mM. Compounds tested as analogues of the enolpyruvyl side chain of prephenate included succinate, cis-aconitate, maleate and α-ketoglutarate. No inhibition was observed in the presence of these compounds up to a concentration of 20 mM.

5. DISCUSSION

The steady state velocity patterns for the prephenate dehydrogenase reaction in the absence of products, indicated that the kinetic mechanism is sequential. In addition, the absence of any nonlinearity in the double reciprocal plots and the intersection of the lines to the left of the y-axis indicated that the mechanism was not steady state random or rapid equilibrium ordered. It might therefore be of the ordered, Theorell-Chance or rapid equilibrium type. The results of product inhibition studies in which a pair of competitive and noncompetitive inhibitions was observed eliminate from further
FIG IV.6. DEADEND INHIBITION OF THE DEHYDROGENASE REACTION BY AMP. (A) NAD VARIED AND THE PREPHENATE CONCENTRATION FIXED AT 0.1 mM (B) PREPHENATE VARIED AND THE NAD CONCENTRATION FIXED AT 0.05 mM. VELOCITIES ARE EXPRESSED AS MICROMOLES OF NADH FORMED PER MIN PER MG OF EnZyme.
TABLE IV.4. APPARENT INHIBITION CONSTANTS, $k_i$, SLOPE AND $k_i$ INTERCEPT, FOR THE DEADEND INHIBITOR, AMP. 

THE VALUES FOR THE APPARENT INHIBITION CONSTANTS ARE THE WEIGHTED MEANS OF THE CONSTANTS OBTAINED BY FITTING THE DATA OF FIG.IV.6 AND SIMILAR EXPERIMENTS TO EQUATION (2) OR (3). 

THE CONSTANTS ARE EXPRESSED AS mM.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Type of Inhibition $^a$</th>
<th>Varied Substrate</th>
<th>Fixed Substrate</th>
<th>$k_i$ slope $^b$</th>
<th>$k_i$ intercept $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>NC</td>
<td>Prephenate</td>
<td>NAD (0.05 mM)</td>
<td>1.90 ± 0.08</td>
<td>6.2 ± 0.7</td>
</tr>
<tr>
<td>AMP</td>
<td>C</td>
<td>NAD</td>
<td>Prephenate (0.1 mM)</td>
<td>3.11 ± 0.10</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ NC, non competitive; C, competitive.

$^b$ Values are the weighted means of values from three experiments.
consideration any of the ordered mechanisms. A rapid equilibrium random mechanism with no dead-end complexes (in which all the inhibitions would be competitive) and that with only one dead-end complex (in which one noncompetitive inhibition would be observed) are also eliminated. The product inhibition patterns are however, consistent with a rapid equilibrium random mechanism with two dead-end complexes or with a Theorell-Chance mechanism for which either NAD or prephenate is the first reactant. For a Theorell-Chance mechanism in which prephenate adds before NAD and the products dissociate in the order NADH, CO₂ and hydroxyphenylpyruvate, it would be expected that a dead-end inhibitor combining in the sequence after prephenate, would be uncompetitive with respect to prephenate. The noncompetitive inhibition with respect to prephenate, observed in dead-end inhibition studies with AMP, is clearly inconsistent with this mechanism. Thus the prephenate dehydrogenase reaction must conform to either a rapid equilibrium random mechanism with the formation of enzyme-NAD-hydroxyphenylpyruvate and enzyme-NADH-prephenate dead-end complexes or to a Theorell-Chance mechanism in which NAD adds before prephenate and the products dissociate in the order hydroxyphenylpyruvate, CO₂ and NADH. Quantitative analysis of the data showed that the results were inconsistent with this Theorell-Chance mechanism. Further, binding experiments with the enzyme using labelled substrates indicate that both prephenate and NAD can bind to the free enzyme and therefore argue against a Theorell-Chance mechanism. It therefore appears that the reaction conforms to a mechanism involving the random addition of prephenate and NAD and the formation of two dead-end complexes \textit{viz} enzyme-NADH-prephenate and enzyme-NAD-hydroxyphenylpyruvate. In this respect, the enzyme resembles the bifunctional enzyme chorismate mutase-prephenate dehydrogenase from \textit{A.aerogenes} (Heyde and Morrison, 1978).
Conclusions about the order of product release are limited because it was not possible to determine the inhibition patterns with bicarbonate (CO₂) or to study the reaction in the reverse direction. In this connection, it is of interest that although both NADH and hydroxyphenylpyruvate can independently combine to the free enzyme, an enzyme-NADH-hydroxyphenylpyruvate-complex does not form to any significant extent under the conditions used.

When it is assumed that the random addition of prephenate (A) and NAD (B) occurs under rapid equilibrium conditions with the products hydroxyphenylpyruvate (P) and NADH (Q) combining in the same way as inhibitory analogues of prephenate and NAD respectively, the rate equation for an irreversible reaction may be expressed as

\[
V = \frac{V (A) (B)}{K_{ia} K_{ib} p (A) (B) + (P) (Q)} + K_b (A) \left[ 1 + \frac{(P)}{K_{ip}} \right] + K_a (B) \left[ 1 + \frac{(Q)}{K_{iq}} \right] + (A) (B)
\]

where V represents the maximum velocity of the reaction in the forward direction. From the rapid equilibrium assumption, it follows that

\[
K_{ia} K_{ib} = K_a K_b, \quad p (P) (Q) = K_{ip} K_{iq}, \quad K_{ia}, K_{ib}, K_{ip} \text{ and } K_{iq}
\]

are dissociation constants for the respective reactions of A, B, P and Q with free enzyme and the Michaelis constants \(K_a, K_b, K_p\) and \(K_q\), are also dissociation constants for the reaction of A, B, P and Q with \(EB, EA, EQ\) and \(EP\) respectively. The mechanism is illustrated in Fig.IV.7 which also shows the formation of the dead-end complexes E-NADH-prephenate (EAP) and E-NAD-hydroxyphenylpyruvate (EBQ). Again because of the rapid equilibrium assumption, \(K_{ia} K_{ip} = K_a K_{ip}\) and \(K_{ib} K_{iq} = K_b K_{iq}\) where
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\( K_{ia}, K_{ib}, K_{ip}, \text{ and } K_{iq} \) are also dissociation constants.

Equation IV-5 may be modified by setting \( p=q=0 \) to give equations IV-6 and IV-7 which represent the reciprocal forms of the steady state velocity equations for the forward reaction with \( A \) and \( B \) as variable substrates.

\[
\frac{1}{v} = \frac{K_a}{V} \left[ \frac{K_{ib}}{B} + 1 \right] \frac{1}{A} + \frac{1}{V} \left[ \frac{K_b}{B} + 1 \right] \tag{IV-6}
\]

\[
\frac{1}{v} = \frac{K_b}{V} \left[ \frac{K_{ia}}{A} + 1 \right] \frac{1}{B} + \frac{1}{V} \left[ \frac{K_a}{A} + 1 \right] \tag{IV-7}
\]

Results are consistent with these equations since both the slope and vertical intercepts of the double reciprocal plots vary as a linear function of the concentration of the changing fixed substrate, giving intersecting steady state velocity patterns which meet at a common point (Fig.IV.2).

Modification of equation IV-5 by setting to zero, the concentration of product that is not added and rearrangement in double reciprocal form gives equations IV-8 and IV-9 which illustrate the linear noncompetitive and linear competitive product inhibition by NADH that are in accord with the experimental results (Fig.IV.3).

\[
\frac{1}{v} = \frac{K_a}{V} \left[ 1 + \frac{K_{ib}}{B} \left( 1 + \frac{P}{K_{ip}} \right) \right] \frac{1}{A} + \frac{1}{V} \left[ 1 + \frac{K_b}{B} \left( 1 + \frac{P}{K_{ip}} \right) \right] \tag{IV-8}
\]

\[
\frac{1}{v} = \frac{K_b}{V} \left[ \left( 1 + \frac{P}{K_{ip}} \right) + \frac{K_{ia}}{A} \left( 1 + \frac{P}{K_{ip}} \right) \right] \frac{1}{B} + \frac{1}{V} \left[ 1 + \frac{K_a}{A} \right] \tag{IV-9}
\]
Similarly, for hydroxyphenylpyruvate as a product inhibitor, the equations could be rearranged as follows:

\[
\frac{1}{v} = \frac{K_a}{V} \left[ \left( 1 + \frac{Q}{K_{iq}} \right) + \frac{K_{ib}}{B} \left( 1 + \frac{Q}{K_{iq}} \right) \right] \frac{1}{A} + \frac{1}{V} \left[ 1 + \frac{K_b}{B} \right] \quad (IV-10)
\]

\[
\frac{1}{v} = \frac{K_b}{V} \left[ \frac{\frac{K_{ia}}{A}}{\left( 1 + \frac{Q}{K_{iq}} \right)} \right] \frac{1}{B} + \frac{1}{V} \left[ 1 + \frac{\frac{K_a}{A}}{\left( 1 + \frac{Q}{K_{iq}} \right)} \right] \quad (IV-11)
\]

The inhibition patterns predicted by the above equations are in accord with those observed (Fig.IV.4).

On the basis that the reaction catalyzed by prephenate dehydrogenase conforms to a rapid equilibrium random mechanism the \(K_a\) and \(K_b\) values listed in Table IV.5 can be regarded as dissociation constants for the release of prephenate and NAD from the central complexes and \(K_{ia}\) and \(K_{ib}\) as the dissociation constants for their release from the binary complexes. On the same basis, it is possible to derive expressions relating the true inhibition constants associated with the products and the dead-end inhibitor with their apparent values. The relationship thus derived (Table IV.6) from equations IV-8, IV-9, IV-10 and IV-11 were used together with the apparent values for the inhibition constants, the kinetic constants associated with the fixed substrates, and the concentration of the fixed substrates to calculate the true inhibition constants for the products and the dead-end inhibitor, recorded in Table IV.7. It can be seen from Table IV.7 that there is satisfactory agreement between the values for the same constants, as determined in
TABLE IV.5. KINETIC CONSTANTS FOR THE INTERACTION OF NAD AND PREPHENATE WITH CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kinetic Constants</th>
<th>Value (^a) (mM)</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prephenate (A)</td>
<td>(K_{ia})</td>
<td>0.035 ± 0.003</td>
<td>E + A</td>
</tr>
<tr>
<td></td>
<td>(K_a)</td>
<td>0.034 ± 0.002</td>
<td>EB + A</td>
</tr>
<tr>
<td>NAD (B)</td>
<td>(K_{ib})</td>
<td>0.180 ± 0.024</td>
<td>E + B</td>
</tr>
<tr>
<td></td>
<td>(K_b)</td>
<td>0.177 ± 0.016</td>
<td>EA + B</td>
</tr>
</tbody>
</table>

\(^a\) Values are weighted means of values from five experiments.
### TABLE IV.6

RELATIONSHIPS BETWEEN APPARENT INHIBITION CONSTANTS DETERMINED FROM PRODUCT AND DEADEND INHIBITION STUDIES AND TRUE CONSTANTS FOR A RAPID EQUILIBRIUM, RANDOM MECHANISM WITH TWO DEADEND COMPLEXES.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Variable Substrate</th>
<th>Type of Inhibition</th>
<th>Relationship between kinetic constants from</th>
<th>Intercept replot ( (K_{II}) )</th>
<th>Slope replot ( (K_{Is}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>A</td>
<td>NC</td>
<td>[ K_{IP} = K_{is} \left( \frac{K_{ib}}{K_{ib} + B} \right) ]</td>
<td>[ K_{IP} = K_{ii} \left( \frac{K_{b}}{K_{b} + B} \right) ]</td>
<td>[ K_{is} = \left( \frac{1 + \frac{K_{is}}{A}}{A K_{ip} + 1} \right) ]</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td>[ K_{IQ} = K_{is} \left( \frac{K_{ia}}{K_{ia} + A} \right) ]</td>
<td>[ K_{IQ} = K_{ii} \left( \frac{K_{a}}{K_{a} + A} \right) ]</td>
<td>[ K_{is} = \left( 1 + \frac{K_{ib}}{B} \right) ]</td>
</tr>
<tr>
<td>Q</td>
<td>B</td>
<td>NC</td>
<td>[ K_{IQ} = K_{is} \left( \frac{K_{ia}}{K_{ia} + A} \right) ]</td>
<td>[ K_{IQ} = K_{ii} \left( \frac{K_{a}}{K_{a} + A} \right) ]</td>
<td>[ K_{is} = \left( \frac{1 + \frac{K_{ib}}{B}}{B K_{iq} + 1} \right) ]</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>[ K_{I} = K_{ii} \left( \frac{K_{ib}}{K_{ib} + B} \right) ]</td>
<td>[ K_{I} = K_{ii} \left( \frac{K_{b}}{K_{b} + B} \right) ]</td>
<td>[ K_{is} = \left( 1 + \frac{K_{ib}}{B} \right) ]</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td>[ K_{IS} = \left( \frac{1 + \frac{K_{ib}}{B}}{B K_{iq} + 1} \right) ]</td>
<td>[ K_{IS} = \left( \frac{K_{b}}{K_{b} + B} \right) ]</td>
<td>[ K_{is} = \left( 1 + \frac{K_{ib}}{B} \right) ]</td>
</tr>
</tbody>
</table>

\( ^a \) NC, non competitive; C, competitive
TABLE IV.7. TRUE INHIBITION CONSTANTS FOR THE PRODUCT AND DEADEND INHIBITORS OF THE DEHYDROGENASE REACTION.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Varied Substrate</th>
<th>Fixed Substrate</th>
<th>Type of Inhibition</th>
<th>Apparent $K_i$ (mM) $^a$</th>
<th>Kinetic (dissociation) constant $^b$</th>
<th>Value (mM)</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Slope</td>
<td>Intercept</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH (P)</td>
<td>Prephenate (A)</td>
<td>NADH (B)</td>
<td>NC</td>
<td>$0.404 \pm 0.065$</td>
<td>$0.075 \pm 0.004$</td>
<td>$0.260 \pm 0.043$</td>
<td>E + NADH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prephenate (A)</td>
<td>C</td>
<td>$0.056 \pm 0.003$</td>
<td>$(0.061 \pm 0.004)$</td>
<td>$0.048 \pm 0.003$</td>
<td>E-prephenate + NADH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prephenate (A)</td>
<td>C</td>
<td>$0.053 \pm 0.002$</td>
<td>$(0.051 \pm 0.003)$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD (B)</td>
<td>Prephenate (A)</td>
<td>NADH (B)</td>
<td>NC</td>
<td>$0.45 \pm 0.05$</td>
<td>$0.126 \pm 0.006$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prephenate (A)</td>
<td>C</td>
<td>$0.060 \pm 0.004$</td>
<td>$(0.049 \pm 0.003)$</td>
<td>$0.117 \pm 0.015$</td>
<td>E + HPP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prephenate (A)</td>
<td>C</td>
<td>$0.049 \pm 0.002$</td>
<td>$(0.034 \pm 0.002)$</td>
<td>$0.032 \pm 0.002$</td>
<td>E-NAD + HPP</td>
</tr>
<tr>
<td>4-hydroxyphenylpyruvate (Q) (HPP)</td>
<td>Prephenate (A)</td>
<td>NADH (B)</td>
<td>NC</td>
<td>$1.90 \pm 0.08$</td>
<td>$6.2 \pm 0.7$</td>
<td>$1.49 \pm 0.08$</td>
<td>E + AMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prephenate (A)</td>
<td>C</td>
<td>$3.11 \pm 0.10$</td>
<td>$(3.05 \pm 0.19)$</td>
<td>$4.83 \pm 0.55$</td>
<td>E-prephenate + AMP</td>
</tr>
<tr>
<td>AMP (B $^*$)</td>
<td>Prephenate (A)</td>
<td>NADH (B)</td>
<td>NC</td>
<td>$1.90 \pm 0.08$</td>
<td>$6.2 \pm 0.7$</td>
<td>$1.49 \pm 0.08$</td>
<td>E + AMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prephenate (A)</td>
<td>C</td>
<td>$3.11 \pm 0.10$</td>
<td>$(3.05 \pm 0.19)$</td>
<td>$4.83 \pm 0.55$</td>
<td>E-prephenate + AMP</td>
</tr>
</tbody>
</table>

$^a$ Values for the apparent inhibition constants were obtained from Table IV.3 and IV.4.

$^b$ True values for the product and deadend inhibition constants were determined from the apparent values by using the concentration of the non varied substrate and the relationships given in Table IV.6. The values for the kinetic constants of the substrates used in the calculation were as in Table IV.5.

$^c$ Values given in brackets are apparent inhibition constants which were calculated for comparison with the directly determined values for the apparent $K_i$. Calculations were performed using the relationships given in Table IV.6, the values for the kinetic constants of the substrates from Table IV.5, the true inhibition constants given in column 8 of this Table and the fixed concentration of the non varied substrate.
different ways. Thus in each of the five competitive inhibition experiments (Table IV.7), the directly determined values for each of the apparent inhibition constant are in good agreement with the corresponding calculated values which were obtained using relationships given in Table IV.6. It can also be seen from Table IV.7 that the apparent inhibition constant for NADH as a competitive inhibitor with respect to NAD at a high fixed concentration of prephenate, is virtually identical with the true dissociation constant for the release of NADH from the E-prephenate-NADH complex, thus confirming the validity of the mechanism and the formation of such a dead-end complex. A similar correlation was observed when the apparent inhibition constant for hydroxyphenylpyruvate as a competitive inhibitor with respect to prephenate at a high fixed concentration of NAD, was compared with the true dissociation constant for the release of hydroxyphenylpyruvate from the E-NAD-hydroxyphenylpyruvate complex. This result confirms the formation of an enzyme-NAD-hydroxyphenylpyruvate complex.

It is apparent from Tables IV.5 and IV.7 that while the presence of prephenate on the enzyme does not affect the binding of NAD, it considerably enhances the combination of NADH. Similarly, while prephenate binds equally well to the free enzyme and to the E-NAD-complex, hydroxyphenylpyruvate binds more strongly to the E-NAD complex. By contrast, there is no synergistic effect of the binding of AMP to the E-prephenate complex. On the contrary, the presence of prephenate on the enzyme inhibits the binding of AMP as a dead-end inhibitor.

The kinetic data are qualitatively and quantitatively consistent with the reaction conforming to a rapid equilibrium random mechanism. In general the measurement of the rate of isotope exchange
at equilibrium would allow more definitive conclusions to be reached about whether or not the random mechanism was of the true rapid equilibrium type. But for the prephenate dehydrogenase reaction this is not feasible as the reaction is virtually irreversible.

It must be noted that a rapid equilibrium random mechanism is uncommon among NAD-dependent dehydrogenases. Most of the known dehydrogenases conform to an ordered mechanism in which the addition of NAD necessarily precedes the addition of the second substrate. In this respect, chorismate mutase-prephenate dehydrogenase is similar to IMP dehydrogenase (Heyde et al., 1976), isocitrate dehydrogenase (Uhr et al., 1974) and to the bifunctional enzyme chorismate mutase-prephenate dehydrogenase from *A. aerogenes* (Heyde and Morrison, 1978).

5. **SUMMARY**

(1) The dehydrogenase reaction catalyzed by chorismate mutase-prephenate dehydrogenase has been studied kinetically at pH 7.7 in the presence and absence of products. The steady state velocity pattern in the absence of products as well as product and dead-end inhibition patterns have been determined. These are consistent with a random mechanism in which the two dead-end complexes E-NADH-prephenate and E-NAD-hydroxyphenylpyruvate are formed and in which all steps are in rapid equilibrium except that concerned with the interconversion of central ternary complexes.

(2) Values have been determined for the maximum velocity of the reaction as well as for the Michaelis and dissociation constants associated with the combination of each substrate, each product and
the dead-end inhibitor, AMP with various enzyme forms. The kinetic constants indicate that when albumin is present in the reaction mixture, the presence of one substrate on the enzyme does not affect the combination of the second substrate. On the other hand, the binding of hydroxyphenylpyruvate (NADH) is enhanced by the presence of NAD (prephenate) on the enzyme. These results contrast with the finding that the inhibitory analogue, AMP, binds more strongly to the free enzyme than to the E-prephenate complex.
CHAPTER V

THE RELATIONSHIP BETWEEN THE ACTIVE SITES
OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE

RESULTS

(a) Kinetic studies on the mutase reaction
(1) Effect of bovine serum albumin

(2) Effect of prephenate and 4-hydroxyphenyllpyruvate

(3) Effect of NAD and NACT

(b) Effect of chorismate on the dehydrogenase activity of the enzyme

(c) Studies on the overall reaction catalyzed by the enzyme

(d) Binding of prephenate and NAD to the enzyme

DISCUSSION

SUMMARY
CHAPTER V
THE RELATIONSHIP BETWEEN THE ACTIVE SITES OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE

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3. METHODS
(a) Concentration of the enzyme
(b) Measurement of enzyme activity
(c) Binding of labelled substrates to the enzyme
(d) Analysis of data
   (i) Data from kinetic studies
   (ii) Data from binding studies

4. RESULTS
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   (i) Effect of bovine serum albumin
   (ii) Effect of prephenate and 4-hydroxyphenylpyruvate
   (iii) Effect of NAD and NADH
(b) Effect of chorismate on the dehydrogenase activity of the enzyme
(c) Studies on the overall reaction catalyzed by the enzyme
(d) Binding of prephenate and NAD to the enzyme

5. DISCUSSION

6. SUMMARY
1. **INTRODUCTION**

The aim of the studies presented in this Chapter is the elucidation of the relationship between the sites at which the chorismate mutase and prephenate dehydrogenase reactions are catalyzed by the enzyme. Towards this end, steady state velocity studies have been undertaken to determine the values of the kinetic constants for the inhibition or activation of the mutase reaction by the reactants of the dehydrogenase reaction. The values so obtained have been compared with those obtained for the same reactants from studies on the dehydrogenase reaction and the differences observed in their magnitudes have been used to identify differences in the sites catalyzing the two reactions.

Binding of the substrates prephenate and NAD to the enzyme has also been studied in order to compare the thermodynamic dissociation constants for the substrates with those obtained in kinetic studies and to provide an estimate of the number of moles of prephenate and NAD bound per mole of the enzyme.

2. **MATERIALS**

The ammonium salt of (14C)-NAO (302 mci/mole) was obtained from the radiochemical centre, Amersham. (14C)-prephenate was a gift of Dr. E. Heyde.

3. **METHODS**

(a) **Concentration of the enzyme**

The enzyme used in binding studies was concentrated to a
final concentration of 3 mg per ml as follows: the pure enzyme (10 mg) stored as described in Chapter II was dialyzed for 24 hr against 200 volumes of 0.1 M N-ethylmorpholine-HCl buffer (pH 7.5) containing 1 mM citrate, 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol (w/v) with two changes at 5 hr intervals. The dialyzed enzyme was applied to a column (1.5 x 1.7 cm) of DEAE-cellulose (DE 52) which had been equilibrated with 0.1 M N-ethylmorpholine-HCl buffer (pH 7.5) containing 1 mM citrate, 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol (w/v). The column was washed with 3 ml of the same buffer and eluted with 3 ml of 0.1 M N-ethylmorpholine-citrate buffer (pH 7.7) containing 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol (w/v). The concentrated enzyme, snap frozen in liquid nitrogen and stored at -20°C, was thawed at 30°C when required and used within 48 hr.

(b) Measurement of enzyme activity

Prephenate dehydrogenase activity was measured as described in Chapter IV, by following the increase in absorbance at 340 nm due to the formation of NADH and hydroxyphenylpyruvate from NAD and prephenate. Chorismate mutase activity was measured in reaction mixtures containing 50 mM N-ethylmorpholine-morpholinoethane sulfonic acid buffer (pH 7.7), 1 mM EDTA, 1 mM dithiothreitol and 100 µg albumin/ml. The disappearance of chorismate was measured spectrophotometrically at either 274 nm or 290 nm. The latter wavelength was used when pyridine nucleotides or 4-hydroxyphenylpyruvate were present, to overcome problems in velocity measurements due to the high absorbance of these compounds at 274 nm. The molar extinction coefficients for chorismate were taken to be 2635 and 1960 at 274 and 290 nm respectively. In reaction mixtures containing chorismate and NAD, both the mutase and the dehydrogenase reactions were
followed simultaneously using a Varian 635 spectrophotometer. The instrument had been modified by Dr. K. J. Ellis so that a reaction could be monitored at several wavelengths with independent adjustable back-off and variable scale expansion at each wavelength. The steady state rates for the mutase reaction were calculated after allowing for the changes in absorbance at 290 nm due to the products of the dehydrogenase reaction. The molar extinction coefficient at 290 nm for the conversion of dehydrogenase substrates to products was determined to be 2570, and the change in absorbance at 290 nm due to the mutase reaction was calculated using the relationship

$$ \Delta E_{\text{chorismate}} = -\Delta E_{290} + \Delta E_{340} \times \frac{2570}{6500} $$

In studies of the overall reaction catalyzed by the enzyme, time courses of both reactions were followed in samples containing chorismate and NAD. The dehydrogenase reaction was monitored at 340 nm and the mutase reaction at 282 nm, using the modified Varian 635 spectrophotometer. Since 282 nm is the isobestic point for NAD and NADH, it was not necessary to make any corrections for changes in concentrations of NAD and NADH at 282 nm. However, it was necessary to allow for the changes in absorbance at 282 nm that are due to the production of 4-hydroxyphenylpyruvate in the dehydrogenase reaction. The latter compound had a molar extinction coefficient at 282 nm of 1480. The change in absorbance at 282 nm due to the mutase reaction was calculated using the relationship

$$ \Delta E_{\text{chorismate}} = -\Delta E_{282} + \Delta E_{340} \times \frac{1480}{6500} $$

A molar extinction coefficient of 2235 was used for calculating the
concentration of chorismate. The concentration of prephenate at any
time 't' was obtained from the difference between the change in
concentration of chorismate at time 't' and the concentration of NADH
at time 't'.

(c) Binding of labelled substrates to the enzyme

The binding of 14C-labelled NAD or prephenate to the enzyme
was measured using the method of Paulus (1969) except for the
replacement of the UMIO membrane with a Visking membrane (Heyde, 1973).
The binding was measured at room temperature in volumes of 0.2 ml
containing 0.1 M N-ethylmorpholine-citrate buffer (pH 7.7), 1 mM EDTA,
1 mM dithiothreitol, 10% glycerol (w/v), 20 µg of bovine serum albumin
and enzyme in the range of 100 to 150 µg. The radioactivity associated
with the enzyme-substrate complex deposited on the membranes was
measured using a Packard tri-carb liquid scintillation spectrometer.
Albumin was observed to bind small quantities of prephenate and NAD and
hence blanks containing albumin alone were used at each concentration
of NAD or prephenate used in the experiment.

(d) Analysis of data

(i) Data from kinetic studies

The kinetic data were analyzed as described in Chapter IV.
In the absence of products, velocity data were fitted to the equation

\[ v = \frac{v_A}{k_a + A} \]  

(V-1)
while linear competitive inhibition and slope-hyperbolic activation
were fitted to equations V-2 and V-3 respectively.

\[
V = \frac{V_A}{k(1 + I/k_i) + A} \quad \text{(V-2)}
\]

\[
V = \frac{V_A(1 + I/k_{in})}{k(1 + I/k_{in}) + A (1 + I/k_{in})} \quad \text{(V-3)}
\]

The values of the parameters obtained from the computer analyses were used to draw the lines in the figures. The weighted mean values of the kinetic constants and their standard errors were calculated as described in Chapter IV.

(ii) Data from binding studies

After subtraction of the appropriate blank values, the binding data were converted to moles of ligand bound per mole of enzyme of molecular weight 88,000. The data were fitted to equation V-1 where values of 'V' represented the moles of ligand bound per mole of enzyme and values of 'A' were the corresponding concentrations of ligand used.

4. RESULTS

(a) Kinetic studies on the mutase reaction

(i) Effect of bovine serum albumin

When the steady state velocities of the mutase reaction catalyzed by the enzyme were measured in 50 mM N-ethylmorpholine-morpholinoethane sulfonic acid buffer (pH 7.7) with 1 mM EDTA and 1 mM dithiothreitol, the double reciprocal plots of the steady state velocities as a function of chorismate concentration were linear. The
data could be fitted to equation V-1 and a value of 0.34 ± 0.04 mM was calculated for the Michaelis constant of chorismate. When small concentrations of albumin were included in the reaction mixture at pH 7.7, there was a marked increase in the reaction velocity at lower concentrations of substrate. The double reciprocal plot of the steady state velocity as a function of chorismate concentration was linear in the presence of albumin (Fig.V.1). There was no change in the maximum velocity of the reaction but there was a marked reduction in the slope of the line reflecting a decrease in the Michaelis constant for chorismate in the presence of albumin (Table V.1). More detailed studies using a range of albumin concentrations showed that albumin acts as a non-essential hyperbolic activator of the mutase reaction (Fig.V.2). Analysis of the data using equation V-3 yielded values of 340 ± 70 nM and 60 ± 9 nM for the dissociation of albumin from the E-albumin and E-chorismate-albumin complexes respectively. The dissociation constant for the E-albumin complex calculated as above can be compared with that obtained in the dehydrogenase reaction. On the basis that the dehydrogenase reaction conforms to a rapid equilibrium random mechanism, the apparent activation constant for albumin calculated in the dehydrogenase reaction can be shown to correspond to $\frac{K_{is}}{K_{im}} \frac{1}{K_{im}}$, where $\frac{K_{is}}{K_{im}}$ represents the ratio of the dissociation constants for prephenate (or NAD) from the E-prephenate (or E-NAD) and E-albumin-prephenate (or E-albumin-NAD) complexes and $K_{im}$ is the dissociation constant for the E-albumin complex. Such calculations using values for $K_{is}$, $K_{is}$ and the apparent activation constant from Chapter IV yield a dissociation constant of 230 ± 60 nM for the E-albumin complex which is comparable to the value of 340 ± 70 nM determined in the mutase reaction.
FIG.V.1. EFFECT OF VARYING CONCENTRATIONS OF CHORISMATE ON
THE STEADY STATE VELOCITY OF THE MUTASE REACTION IN THE ABSENCE
(A) AND PRESENCE (B) OF BOVINE SERUM ALBUMIN (BSA, 100 µg/ml).
THE DATA WERE FITTED TO EQUATION V-1. VELOCITIES ARE EXPRESSED
AS MICROMOLES OF CHORISMATE CONVERTED PER MIN PER MG OF ENZYME.
**Table V.1**

**Effect of Bovine Serum Albumin (200 μg/ml) on the Kinetic Parameters of the Mutase Reaction.**

The values are given as mean ± standard error.

Five data sets including that of fig. V.3. to equation V.1. 30 denote the Michaelis-Menten constant for chorismate. Formed per min per mg of enzyme.

<table>
<thead>
<tr>
<th>Kinetic constant</th>
<th>Without albumin</th>
<th>With albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V )</td>
<td>39.2 ± 2.8</td>
<td>38.6 ± 2.3</td>
</tr>
<tr>
<td>( x_m )</td>
<td>0.04 ± 0.04</td>
<td>0.000 ± 0.000</td>
</tr>
</tbody>
</table>
TABLE V.1. EFFECT OF BOVINE SERUM ALBUMIN (100 µg/ml) ON THE KINETIC PARAMETERS OF THE MUTASE REACTION. THE DATA ARE GIVEN AS WEIGHTED MEANS ± STANDARD ERRORS OF MEANS OF VALUES OBTAINED BY FITTING FIVE DATA SETS INCLUDING THAT OF FIG.V.1, TO EQUATION V-1. $k_m$ DENOTES THE MICHAELIS CONSTANT FOR CHORISMATE. $V$ IS EXPRESSED AS MICROMOLES OF PREPHENATE FORMED PER MIN PER MG OF ENZYME.

<table>
<thead>
<tr>
<th>Kinetic constant</th>
<th>Without albumin</th>
<th>With albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V$</td>
<td>$39.2 \pm 2.8$</td>
<td>$38.6 \pm 2.3$</td>
</tr>
<tr>
<td>$k_m$</td>
<td>$0.34 \pm 0.04$</td>
<td>$0.060 \pm 0.008$</td>
</tr>
</tbody>
</table>
FIG. V.2. ACTIVATION OF THE MUTASE REACTION BY BOVINE SERUM ALBUMIN (BSA). THE DATA WERE FITTED TO EQUATION V-3. VELOCITIES ARE EXPRESSED AS MICROMOLS OF CHORISMATE CONVERTED PER MIN PER MG OF ENZYME.
(b) Effect of chorismate on the dehydratase activity of the enzyme

The concentration of BSA was fixed at 0.25 mM and the effect of chorismate on the dehydratase reaction was examined at preliminary concentrations of 0.05 mM (approximately equal to the Michaelis constant) and 0.20 mM (five times greater than its Michaelis constant). In the presence of 0.5 mM chorismate, the rate of the dehydratase reaction was markedly increased (Table V.3). This increase could not be accounted...
(ii) Effect of prephenate and 4-hydroxyphenylpyruvate on
the mutase reaction

Product inhibition of the mutase reaction by prephenate is
linear competitive as expected for a uni-uni reaction (Fig.V.3A), and
4-hydroxyphenylpyruvate, one of the products of the dehydrogenase
reaction, also behaves as a linear competitive inhibitor of the mutase
reaction (Fig.V.3B). However, the inhibition by 4-hydroxyphenylpyruvate
was very much weaker than that observed with prephenate. The inhibition
constants obtained from analysis of the experimental data for the
inhibitions by prephenate and 4-hydroxyphenylpyruvate are given in
Table V.2.

(iii) Effect of NAD and NADH on the mutase reaction

Both the oxidized and reduced pyridine nucleotides function
as activators of the mutase reaction (Fig.V.4A,B). No change in the
maximum velocity of the mutase reaction was observed in the presence
of NAD or NADH and the data fitted well to slope-hyperbolic activation
as described by equation V-3. The values of the kinetic constants for
activation of the mutase reaction by NAD and NADH are given in Table V.2.

(b) Effect of chorismate on the dehydrogenase activity of
the enzyme

The concentration of NAD was fixed at 0.2 mM and the effect of
chorismate on the dehydrogenase reaction was examined at prephenate
concentrations of 0.05 mM (approximately equal to its Michaelis constant)
and 0.20 mM (five times greater than its Michaelis constant). In presence
of 0.5 mM chorismate, the rate of the dehydrogenase reaction was
markedly increased (Table V.3). This increase could not be accounted
FIG. V.3. INHIBITION OF THE MUTASE REACTION BY PREPHENATE (A) AND BY 4-HYDROXYPHENYLPYRUVATE (B). VELOCITIES ARE EXPRESSED AS MICROMOLES OF CHORISMATE CONVERTED PER MIN PER MG OF ENZYME.
A  
Prephenate (mM)

B  
HPP (mM)
FIG.V.4. ACTIVATION OF THE MUTASE REACTION BY NAD (A) AND NADH (B). VELOCITIES IN THE PRESENCE OF NAD WERE CALCULATED FROM RATE MEASUREMENTS AT BOTH 290 AND 340 nm AS DESCRIBED UNDER 'METHODS'; THOSE IN THE PRESENCE OF NADH WERE CALCULATED FROM MEASUREMENTS AT 290 nm. VELOCITIES ARE EXPRESSED AS MICROMOLES OF CHORISMATE CONVERTED PER MIN PER MG OF ENZYME.
<table>
<thead>
<tr>
<th>Reactant</th>
<th>Effect</th>
<th>Kinetic constant</th>
<th>Value (mM)</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prephenate (A)</td>
<td>Inhibition</td>
<td>$K_{ia}$</td>
<td>0.15 ± 0.01</td>
<td>E + Prephenate</td>
</tr>
<tr>
<td>4-hydroxyphenylpyruvate (o)</td>
<td>Inhibition</td>
<td>$K_{iq}$</td>
<td>1.90 ± 0.32</td>
<td>E + 4-hydroxyphenylpyruvate</td>
</tr>
<tr>
<td>NAD (b)</td>
<td>Activation</td>
<td>$K_{ib}$</td>
<td>0.26 ± 0.05</td>
<td>E + NAD</td>
</tr>
<tr>
<td>NADH (P)</td>
<td>Activation</td>
<td>$K_{iaB}$</td>
<td>0.050 ± 0.004</td>
<td>E - chorismate + NAD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{ip}$</td>
<td>0.28 ± 0.03</td>
<td>E + NADH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{iaP}$</td>
<td>0.090 ± 0.007</td>
<td>E - chorismate + NAD</td>
</tr>
</tbody>
</table>

*a* All values reported are weighted mean values from three experiments including those illustrated in Fig. V.3 and V.4.

*b* The values for $K_{ia}$ and $K_{iq}$ were obtained by fitting data to equation V-2. Values of the kinetic parameters associated with NAD and NADH were obtained by fitting data to equation V-3, in which $K_{is}$ is equivalent to the dissociation constant for the interaction of NAD/NADH (r) with free enzyme and $K_{in}$ is the dissociation constant for their interaction with the enzyme-chorismate complex.
TABLE V.3 EFFECT OF CHORISMATE ON THE RATE OF THE PREPHENATE DEHYDROGENASE REACTION

| (Chorismate) (mM) | (Prephenate) (mM) | Rate (μmol min⁻¹ mg⁻¹)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dehydrogenase</td>
</tr>
<tr>
<td>0.0</td>
<td>0.05</td>
<td>6.2</td>
</tr>
<tr>
<td>0.056</td>
<td></td>
<td>6.6</td>
</tr>
<tr>
<td>0.20</td>
<td></td>
<td>9.05</td>
</tr>
<tr>
<td>0.203</td>
<td></td>
<td>9.07 b</td>
</tr>
<tr>
<td>0.5</td>
<td>0.05</td>
<td>15.40</td>
</tr>
<tr>
<td>0.20</td>
<td></td>
<td>17.15</td>
</tr>
</tbody>
</table>

The concentration of NAD was 0.2 mM.

Values were obtained from a double reciprocal plot of the steady state velocity of the dehydrogenase reaction as a function of prephenate concentration. The concentration of NAD was fixed at 0.2 mM.
for by the increase in concentration of prephenate caused by the conversion of chorismate to prephenate in the mutase reaction. From the simultaneous measurement of the mutase reaction velocity in each reaction mixture, it was estimated that the prephenate produced from chorismate over the period of measurement would raise the concentration of prephenate from 0.05 to 0.056 mM or from 0.200 to 0.203 mM in the two reaction mixtures which would result in only small increases in the steady state rate of the dehydrogenase reaction (Table V.3). However, the addition of 0.5 mM chorismate was observed to cause an increase of 133% and 90% of the reaction velocity in reaction mixtures containing 0.05 mM and 0.20 mM prephenate respectively (Table V.3).

(c) Studies of the overall reaction catalyzed by the enzyme

A study of the overall reaction catalyzed by the enzyme was carried out using a concentration of NAD fixed at 0.5 mM (a concentration three times greater than its Michaelis constant) and three different initial concentrations of chorismate viz 0.1, 0.25 and 1 mM. At the lowest concentration of chorismate, both the mutase and the dehydrogenase reactions were followed (Fig.V.5A) but at higher concentrations of chorismate, only the appearance of NADH at 340 nm was recorded (Fig.V.5B). With an initial chorismate concentration of 0.1 mM, a distinct lag period was observed before a steady state rate of 11.4 µmoles min⁻¹ mg⁻¹ was achieved in the dehydrogenase reaction. However, at the end of this lag period, the concentration of prephenate in the medium was only 0.006 mM which would be expected to produce a steady state rate of only 3.7 µmoles min⁻¹ mg⁻¹. The rate of the dehydrogenase reaction was observed to remain unrelated to the concentration of prephenate in the reaction mixture so that no increase in the rate of the reaction was recorded even
FIG.V.5. KINETICS OF THE OVERALL REACTION CATALYZED BY CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE.

(A) THE OVERALL REACTION OBSERVED WITH 0.1 mM CHORISMATE AND 0.5 mM NAD.

(B) THE LAG IN THE APPEARANCE OF NADH OBSERVED WITH THREE DIFFERENT INITIAL CONCENTRATIONS OF CHORISMATE. THE CONCENTRATION OF NAD WAS FIXED AT 0.5 mM.
A

Concentration of Chorismate (mM)

Concentration of NADH/Prephenate (mM)

0.10

0.05

0.01

0.02

0.01

0.005

Time (min)

2

4

6

8

10

2

4

6

8

10

NADH

Prephenate

Chorismate

B

Chorismate (mM)

Concentration of NADH (mM)

0.01

0.02

0.03

0.01

0.02

0.03

0.01

0.02

0.03

Time (min)

0

2

4

6

8

10

0

2

4

6

8

10

0.10

0.25

0.40

0.10

0.25

0.40

0.10

0.25

0.40

0.10

0.25

0.40

0.10

0.25

0.40

0.10

0.25

0.40

0.10

0.25

0.40

0.10

0.25

0.40

0.10

0.25

0.40

0.10

0.25

0.40

0.10

0.25

0.40

0.10

0.25

0.40
when the concentration of prephenate was observed to increase from 0.005 mM to a maximum of 0.02 mM (Fig. V.5A).

In experiments where the initial concentration of chorismate was either 0.25 mM or 1 mM, the rate of NADH formation was again significantly higher than that expected on the basis of the concentration of free prephenate in the medium. The lag period, which can be defined as the time that must elapse before a steady state rate is achieved in the dehydrogenase reaction, was observed to decrease progressively with a rise in the initial concentration of chorismate (Fig. V.5B). The lag could be quantitated for comparative purposes by extrapolation of the steady state rate of the dehydrogenase reaction which yields a horizontal intercept corresponding to the value of \( \tau \). \( \tau \) defines the time required for the concentration of the reactants of the dehydrogenase reaction to reach a level required to maintain the steady state rate of the dehydrogenase reaction. The value of \( \tau \) was observed to decrease from 1.5 min to 1 min when the initial concentration of chorismate was increased from 0.1 mM to 0.25 mM. With a concentration of 1 mM chorismate, the rate of NADH formation was linear for up to 6 min and no lag could be detected at all (Fig. V.5B).

(d) Binding of prephenate and NAD to the enzyme

In binding studies with labelled prephenate and NAD, both substrates were observed to bind to the free enzyme. Binding studies were carried out in the presence of albumin, so that a comparison could be made of the dissociation constants determined in the binding studies with those obtained from kinetic studies. Although the concentration of albumin in the reaction mixture was expected to increase during the
ultrafiltration, this was not likely to affect the comparison of the dissociation constants, since the values obtained in the kinetic studies had been determined in the presence of saturating concentrations of albumin (Chapter IV). The weighted mean of five experimental estimates of the dissociation constants for NAD and prephenate are $0.188 \pm 0.016 \text{ mM}$ and $0.12 \pm 0.03 \text{ mM}$ respectively.

Enzyme preparations used in the binding experiments had a specific activity of only 23-25 mutase units/mg as compared to a specific activity of 35 mutase units/mg usually observed in pure enzyme preparations. Consequently, calculations of the number of moles of substrates bound per mole does not necessarily reflect the true number of moles of substrates bound per mole of enzyme. However, pairs of binding experiments were performed on the same enzyme preparation so that a valid comparison could be made of the relative number of moles of prephenate and NAD bound per mole of enzyme. A typical pair of double reciprocal plots of the number of moles of each ligand bound per mole of enzyme as a function of ligand concentration are shown in Fig.V.6. The weighted mean of three values for the maximum number of moles of each substrate bound per mole of enzyme obtained by fitting data to equation V-1 are $0.64 \pm 0.03$ moles of NAD and $1.26 \pm 0.09$ moles of prephenate. From these values, it appears that the amount of prephenate bound per mole of enzyme is twice that of NAD.

5. DISCUSSION

Since the overall conversion of chorismate and NAD to hydroxyphenylpyruvate, NADH and $\text{CO}_2$ catalyzed by chorismate mutase-prephenate dehydrogenase can occur in two stages, it was of interest to determine whether the individual reactions occur at single active
FIG.V.6. A PAIR OF DOUBLE RECIPROCAL PLOTS OF (A) THE NUMBER OF MOLES OF PREPHENATE BOUND PER MOLE OF ENZYME \(r_m\) AS A FUNCTION OF PREPHENATE CONCENTRATION AND (B) THE NUMBER OF MOLES OF NAD BOUND PER MOLE OF ENZYME \(r_m\) AS A FUNCTION OF NAD CONCENTRATION.
All available evidence indicates the aconitase reaction conforms to a rapid equilibrium \textit{or} reaction mechanism (Chapter 11) and the results of the kinetic studies with the enzyme prephenate and NAD are in good agreement with Michaelis-Menten (K = 0.067 M⁻¹). The dissociation constant for L-prephenate is also comparable to that determined in kinetic studies, although there are certain problems associated with its evaluation from binding studies, which will be discussed subsequently. For example, the rate which is not truly rapid also be observed at if the data with yeast 

**A**

\[
1/r_m \quad 4
\]

\[
1/\text{Prephenate (mM}^{-1})
\]

**B**

\[
1/r_m \quad 8
\]

\[
1/\text{NAD (mM}^{-1})
\]
site or at two separate sites on the enzyme. One approach to this problem is to compare the magnitude of the dissociation constants for reactants as determined in kinetic studies of the mutase and dehydrogenase reactions.

All available evidence points to the dehydrogenase reaction conforming to a rapid equilibrium random mechanism (Chapter IV) and the results of the binding studies with the substrates prephenate and NAD are also consistent with this mechanism. The dissociation constants for E-NAD (0.188 ± 0.016 mM) determined in binding studies is in good agreement with that obtained in kinetic studies (0.18 ± 0.024). The dissociation constant for E-prephenate is also comparable to that determined in kinetic studies, although there are certain problems associated with its evaluation from binding studies, which will be discussed subsequently. For a Bi-Bi mechanism which is not truly rapid equilibrium random, agreements between the kinetic and thermodynamic dissociation constants will not be observed as is the case with yeast hexokinase where the dissociation constant for Mg-ATP as determined in the binding studies is an order of magnitude higher than that determined in kinetic studies (Noat et al, 1969).

The dissociation constant for the E-prephenate complex can be also determined from product inhibition studies of the mutase reaction. Usually such studies yield the Michaelis constant \( k_p \) for the product but when the reaction is irreversible because of the irreversibility of the catalytic step \( k_4 = 0 \), the Michaelis constant for the product represents its dissociation constant as shown below:
Similarly, when the irreversibility of the mutase reaction results from an isomerization of the E-prephenate complex, the Michaelis constant for prephenate again represents its dissociation constant.

Assuming that the irreversibility of the mutase reaction is due to either of the above reasons, the dissociation constant for the E-prephenate complex can be compared in the mutase and dehydrogenase reactions. The data (Table V-4) indicate that there is a four-fold difference in the dissociation constant for prephenate as determined in the dehydrogenase reaction and as determined in the mutase reaction, suggesting that the mutase and dehydrogenase reactions occur at active
sites with different kinetic properties.

The product hydroxyphenylpyruvate behaves as a dead-end inhibitor of the dehydrogenase reaction and hence the inhibition constant for hydroxyphenylpyruvate represents the dissociation constant for the E-hydroxyphenylpyruvate complex in the dehydrogenase reaction. Hydroxyphenylpyruvate is also a linear competitive inhibitor of the mutase reaction and hence its inhibition constant must represent the dissociation constant for the E-hydroxyphenylpyruvate complex in the mutase reaction. A comparison of the two values for the dissociation constant of the E-hydroxyphenylpyruvate complex (Table V.4) indicates a 16-fold difference and confirms the earlier conclusion that the mutase and dehydrogenase reactions are catalyzed at two different active sites. In connection with the above conclusions, it is of interest to note that the dissociation constant for the E-NAD and E-NADH complexes are similar in the mutase and dehydrogenase reaction, suggesting the presence of a single binding site on the enzyme for both pyridine nucleotides. The results are also suggestive of some interaction between the mutase and dehydrogenase catalytic sites, mediated by the binding of either pyridine nucleotide to a single site on the enzyme.

In binding studies, the number of moles of prephenate (1.26 ± 0.09) and NAD (0.64 ± 0.03) bound per mole of enzyme were lower than would be expected for an enzyme with two identical subunits. This may be due to the low specific activity of the enzyme used in the binding studies. In the absence of information on the maximum specific activity of the enzyme, it is not possible to calculate the absolute value for the number of moles of either substrate bound per mole of enzyme. However, if it is assumed that the double reciprocal plot of the number of moles...
<table>
<thead>
<tr>
<th>Reactant</th>
<th>Dissociation constant (mM)</th>
<th>Dehydrogenase $^a$</th>
<th>Mutase $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.180 ± 0.024</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>NAD</td>
<td></td>
<td>0.260 ± 0.043</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>Prephenate</td>
<td></td>
<td>0.035 ± 0.003</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>4-hydroxyphenylpyruvate</td>
<td></td>
<td>0.117 ± 0.015</td>
<td>1.90 ± 0.32</td>
</tr>
</tbody>
</table>

$^a$ as reported in Tables IV.5 and IV.7.

$^b$ as reported in Table V-2.
of prephenate bound per mole of enzyme as a function of prephenate concentration (Fig.V.5A) is truly linear, then it is possible to calculate a ratio of the number of moles of prephenate to the number of moles of NAD bound per mole of a given preparation of enzyme. A ratio of two moles of prephenate to one mole of NAD was observed with three different preparations of enzyme. Such a ratio might be expected for a model in which prephenate binds at both the mutase and dehydrogenase sites of the enzyme while NAD binds only at the dehydrogenase catalytic site. Such a model is consistent with the results of the kinetic studies which indicate an identical site of interaction for NAD in the mutase and dehydrogenase reaction and two nonidentical sites of interaction for prephenate in the two reactions. However, on the basis of the results of the kinetic studies, the double reciprocal plot of the number of moles of prephenate bound per mole of enzyme as a function of prephenate concentration would be expected to be nonlinear. No noticeable nonlinearity was observed in double reciprocal plots of the experimental data for the binding of prephenate (Fig.V.5A). But since kinetic studies indicate only a four-fold difference in the values for the dissociation constants of prephenate from the two E-prephenate complexes, measurements of the binding of prephenate over a number of low concentrations of prephenate (below the smaller of the two values for the dissociation constants of the E-prephenate complex) are required to demonstrate the nonlinearity of the double reciprocal plot and to define the values of the two individual dissociation constants. Since the low specific radioactivity of the prephenate used in the binding experiment precludes such measurements, it is not possible, on the basis of the available data, to obtain good estimates of the binding parameters for prephenate. However, despite these difficulties, the results of the binding studies are
generally consistent with those obtained in kinetic studies as illustrated in Fig.V.7, where the binding data for prephenate can be seen to fit reasonably well to the model which assumes two independent binding sites for prephenate and the formation of two E-prephenate complexes with dissociation constants of 0.035 mM and 0.15 mM.

The presence of two nonidentical catalytic sites on the enzyme raises the question of whether there is any interaction between the two sites and whether any channelling of the intermediate, prephenate occurs between the two sites. Although extensive studies of the overall reaction are required before any conclusions regarding the nature of interaction between the two sites can be reached, it is possible from measurements of the rate of the overall reaction under a variety of conditions to suggest the qualitative nature of some of the interactions that may be involved. One of the main features of the overall reaction catalyzed by chorismate mutase-prephenate dehydrogenase is the presence of a lag period before a steady state rate is achieved in the dehydrogenase reaction, the length of which is related to the initial concentration of chorismate (Fig.V.5B). The second feature is the absence of correlation between the concentration of prephenate in the medium and the steady state rate of the dehydrogenase reaction (Fig.V.5A). At low concentrations of chorismate, the lag period observed before a steady state rate is attained in the dehydrogenase reaction, could represent the time taken for the prephenate concentration in the vicinity of the dehydrogenase site to reach a level required to maintain the steady state rate of the dehydrogenase reaction. The decrease in the lag period observed at higher concentrations of chorismate may be due to an increase in the rate of the mutase reaction resulting in a decrease in the time required for the concentration of prephenate at the
FIG.V.7. THE BINDING CURVE FOR PREPHENATE AS A FUNCTION OF PREPHENATE CONCENTRATION.

The points represent the experimental data, expressed as moles of prephenate bound per mole of enzyme. The lines are simulations of the expected results, assuming either (a) two prephenate binding sites with dissociation constants of 0.035 mM and 0.15 mM, and the total number of moles of prephenate bound/per mole of enzyme being set as equal to twice the number of moles of NAD bound per mole of enzyme or (b) one binding site for prephenate with a dissociation constant of 0.15 mM and the total number of moles of prephenate bound/per mole of enzyme being set as equal to the number of moles of NAD bound per mole of enzyme.
The presence of two catalytic sites on the enzyme chorismate作出的 enzyme. In its ability to channel the intermediate prephenate but the degree of channeling is likely to be small, as a considerable proportion of prephenate is released to the medium during the overall reaction (Fig. 5A).
dehydrogenase site to reach the required level. Alternately, the presence of chorismate on the enzyme may enhance the binding of prephenate to the enzyme for the dehydrogenase reaction, thereby allowing the steady state rate of the dehydrogenase reaction to be reached earlier. A detailed study of the overall reaction is necessary to distinguish between the above possibilities. The absence of correlation between the rate of prephenate release into the medium and the steady state rate of the dehydrogenase reaction suggests that there must be some direct channelling of prephenate from the mutase to the dehydrogenase site or that the Michaelis constant for prephenate must be lowered considerably in the presence of chorismate, such that the steady state rate of the dehydrogenase reaction is reached at very low concentrations of prephenate in the medium. Similar conclusions can be reached regarding the activation of the dehydrogenase reaction by chorismate (Table V.3) which could result from the channelling of some of the prephenate formed from chorismate or the enhancement by chorismate of the binding of prephenate or both.

Channelling of intermediates is known to occur in the bi-enzyme complex carbamylphosphate synthase-aspartate transcarbamylase (Lue and Kaplan, 1970) and in the bifunctional enzyme tryptophan synthase from N.crassa (Gaertner et al, 1970), both of which catalyze sequential reactions with little or no release of the intermediate product. Chorismate mutase-prephenate dehydrogenase from E.coli might resemble these enzymes in its ability to channel the intermediate, prephenate but the degree of channelling is likely to be small, as a considerable proportion of prephenate is released in the medium during the overall reaction (Fig.V.5A).

The presence of two catalytic sites on the enzyme chorismate
mutase-prephenate dehydrogenase indicates that it resembles the bifunctional enzyme chorismate mutase-prephenate dehydratase from *E. coli* for which two distinct catalytic sites have been identified (Gething, 1973; Baldwin, 1974; Duggleby *et al.*, 1978). The results also lend support to the speculation that the two enzymes may have evolved by a process of gene fusion such that the two activities of the enzyme reside on discrete regions of the polypeptide chains. However, no evolutionary advantages are apparent for the bifunctional nature of chorismate mutase-prephenate dehydratase from *E. coli* (Chapter I). For chorismate mutase-prephenate dehydrogenase, the possible channelling of prephenate between the catalytic sites may provide a significant advantage to the organism.

5. **SUMMARY**

(1) Binding studies on chorismate mutase-prephenate dehydrogenase indicate that the dissociation constants for the E-NAD and E-prephenate complexes are comparable to those obtained in kinetic studies and confirm the rapid equilibrium random nature of the dehydrogenase reaction.

(2) The dissociation constant for prephenate as determined in the mutase reaction is four times higher than that determined in the dehydrogenase reaction which indicates that the mutase and the dehydrogenase reactions are catalyzed at different active sites. Confirmation of this conclusion comes from the 16-fold difference observed in the dissociation constant for hydroxyphenylpyruvate as determined from studies on the mutase and dehydrogenase reaction.
(3) The number of moles of prephenate bound per mole of the enzyme exceed the number of moles of NAD bound by a factor of two and this result is consistent with the presence of two binding sites for prephenate on the enzyme.

(4) The activation of the dehydrogenase reaction by chorismate and studies on the overall reaction catalyzed by chorismate mutase-prephenate dehydrogenase indicate that some channelling of prephenate between the mutase and the dehydrogenase site may occur or that chorismate may cause an enhancement of the binding of prephenate to the enzyme.
CHAPTER VI

IMMUNOLOGICAL PROPERTIES OF
CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE

1. INTRODUCTION

2. MATERIALS

3. METHODS
   (a) Preparation of antisera
   (b) Immunodiffusion analysis of the antigen-antibody complex
   (c) Quantitative precipitation of chorismate mutase-prephenate dehydrogenase by antisera

4. RESULTS
   (a) Homogeneity of the antisera
   (b) Quantitative precipitation of chorismate mutase-prephenate dehydrogenase by the antisera
   (c) Inhibition of the mutase and dehydrogenase activities of chorismate mutase-prephenate dehydrogenase by the antisera
   (d) Protection by substrates, inhibitors and other compounds against inhibition of chorismate mutase-prephenate dehydrogenase of A. aegypti by antisera
   (e) Cross-reactivity of chorismate mutase-prephenate dehydrogenase from A. aegypti with antisera
   (f) Cross-reactivity of chorismate mutase-prephenate dehydrogenase from E. coli with antisera
   (g) Cross-reactivity of aromatic amino acid transferase from E. coli with antisera
CHAPTER VI
IMMUNOLOGICAL PROPERTIES OF
CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE

1. INTRODUCTION

2. MATERIALS

3. METHODS
   (a) Preparation of antisera
   (b) Immunodiffusion analysis of the antigen-antibody complexes
   (c) Quantitative precipitation of chorismate mutase-prephenate dehydrogenase by antiserum
   (d) Enzyme assays
   (e) Neutralization of enzyme activity by antiserum

4. RESULTS
   (a) Homogeneity of the antiserum
   (b) Quantitative precipitation of chorismate mutase-prephenate dehydrogenase by the antiserum
   (c) Inhibition of the mutase and dehydrogenase activities of chorismate mutase-prephenate dehydrogenase by the antiserum
   (d) Protection by substrates, inhibitors and other compounds against inhibition of chorismate mutase-prephenate dehydrogenase of *E.coli* by antiserum
   (e) Cross-reactivity of chorismate mutase-prephenate dehydrogenase from *A.aerogenes* with antiserum
   (f) Cross-reactivity of chorismate mutase-prephenate dehydratase from *E.coli* with antiserum
   (g) Cross-reactivity of aromatic amino acid transferase from *E.coli* with antiserum
5. DISCUSSION

The studies on the reactions catalyzed by chorismate mutase-phenylacetate dehydrogenase yielded data which suggested that different active sites may be responsible for the conversion of chorismate to phenylacetate and of prephenate and NAD to hydroxyphenylpyruvate, NADH, and CO₂. The studies described in this chapter were undertaken to investigate further the relationship between the two active sites through determination of their antigenic properties. The presence of one or more of a second chorismate mutase, which is linked to the enzyme prephenate dehydratase to form a bifunctional enzyme, prompted the investigation of whether the chorismate mutase sites or the enzyme bifunctional enzymes shared any structural similarity and, if so, the extent of that homology. Since the chorismate-mutase-phenylacetate dehydrogenase from *Escherichia coli* resembles the enzyme from *E. mult* in many of its properties, it was also of interest to measure the cross-reactivity of this enzyme with the antigenum against the *E. mult* enzyme.

It has been observed (Chapter III) that the aromatic amino acid aminotransferase and aspartate aminotransferase from *E. coli* possess amino acid compositions remarkably similar to that of chorismate mutase-phenylacetate dehydrogenase from *E. coli*. Further, it has been found that the aromatic amino acid aminotransferase associates noncovalently with both the bifunctional enzymes chorismate mutase-phenylacetate dehydrogenase and chorismate mutase-phenylacetate dehydratase from *E. coli* although in the latter case, a reactant must be present (Pewi and Horison, unpublished results). Pure preparations of both aromatic amino acid aminotransferase and aspartate aminotransferase were available in our laboratory and therefore these enzymes have been tested for their cross-reactivity.
INTRODUCTION

Kinetic studies on the reactions catalyzed by chorismate mutase-prephenate dehydrogenase yielded data which suggested that different active sites may be responsible for the conversion of chorismate to prephenate and of prephenate and NAD to hydroxyphenylpyruvate, NADH and CO₂. The studies described in this chapter were undertaken to investigate further the relationship between the two active sites through determination of their antigenic properties. The presence in E.coli, of a second chorismate mutase, which is linked to the enzyme prephenate dehydratase to form a bifunctional enzyme, prompted the investigation of whether the chorismate mutase sites on the two bifunctional enzymes shared any structural homology and, if so, the extent of that homology. Since the chorismate mutase-prephenate dehydrogenase from A.aerogenes resembles the enzyme from E.coli in many of its properties, it was also of interest to measure the cross-reactivity of this enzyme with the antiserum against the E.coli enzyme.

It has been observed (Chapter III) that the aromatic amino acid aminotransferase and aspartate aminotransferase from E.coli possess amino acid compositions remarkably similar to that of chorismate mutase-prephenate dehydrogenase from E.coli. Further, it has been found that the aromatic amino acid aminotransferase associates noncovalently with both the bifunctional enzymes chorismate mutase-prephenate dehydrogenase and chorismate mutase-prephenate dehydratase from E.coli although in the latter case, a reactant must be present (Powell and Morrison, unpublished results). Pure preparations of both aromatic amino acid aminotransferase and aspartate aminotransferase were available in our laboratory and therefore these enzymes have been tested for their cross-reactivity.
with antiserum to chorismate mutase-prephenate dehydrogenase.

2. MATERIALS

Freund's complete adjuvant and Bactoagar were obtained from Difco laboratories, sodium veronal and thiomarsal from LKB Products, and sodium tetraborate from Ajax Biochemicals. Chorismate mutase-prephenate dehydrogenase (specific activity of 35 units/mg) was obtained in pure form from cell extracts of E.coli strain JP2312 using the procedure described in Chapter II. Chorismate mutase-prephenate dehydratase (specific activity of 40 units/mg) was isolated from E.coli strain JP492 using the method of Gething et al (1976). Aromatic amino acid aminotransferase (specific activity 21 units/mg) and aspartate aminotransferase (specific activity of 50 units/mg) from E.coli were gifts from Dr. J. T. Powell. Chorismate mutase-prephenate dehydrogenase from A.aerogenes (specific activity of 30 units/mg) was a gift of Dr. E. Heyde.

3. METHODS

(a) Preparation of antiserum to chorismate mutase-prephenate dehydrogenase from E.coli

Homogeneous antigen (250 µg) in 2 ml of 0.05 M N-ethylmorpholine-citrate buffer (pH 7.0) containing 0.5 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol and 0.85% NaCl, was mixed with an equal volume of Freund's complete adjuvant and injected into three areas in the back of a male New Zealand rabbit. The rabbit was bled from an ear vein after five
weeks and a second intradermal injection of 250 µg of enzyme without
the adjuvant was administered. Further intradermal injections, each
of 500 µg of enzymes, were made at two to three week intervals. Blood
was collected from an ear vein seven to ten days after each of the
latter injections. The blood samples were allowed to clot overnight
at 4°C and then centrifuged at 23,000 x g for 15 min at 4°C. The
antiserum was stored at -20°C in 0.5 ml amounts. Control serum was
obtained from the rabbit before immunization and was prepared in a
similar way.

All antisera were titred by quantitative precipitation and
examined by immunodiffusion. The antiserum used in the studies
reported in this Chapter is described as PEC-234 (pooled preparations
of antisera, to pure E.coli chorismate mutase-prephenate dehydrogenase,
from second, third and fourth bleedings respectively).

(b) Immunodiffusion analysis of antigen-antibody complexes

Immunodiffusion analysis of antigen-antibody complexes were
performed according to the method of Ouchterlony (1962) on microslides
prepared in the following manner:
Bactoagar (1%) in 0.05 M Na-Veronal buffer (pH 8.6) containing 0.01%
thiomarsal was melted, cooled to about 50°C and 4.0 ml were pipetted
onto clean slides. A triangular pattern of three circular wells each
with a radius of 4 mm was punched into the gels. One of the wells was
filled with 20 µl of antiserum while the remaining two wells were
filled with 20 µl of the antigens being tested. The gels containing
the samples were incubated at room temperature and the reactions were
recorded after 30 hr.
Quantitative precipitation of chorismate mutase-prephenate dehydrogenase by antiserum was performed as described by Kabat and Mayer (1961). All operations were carried out at 4°C, unless specified otherwise. The samples of antigen and antiserum used in the precipitation reaction were dialyzed for 24 hr against 200 volumes of 0.01 M sodium-borate buffer (pH 8.0) containing 0.15 M NaCl with two changes at 5 hr intervals and the dialyzed samples were centrifuged at 23,000 x g for 30 min to remove any particulate matter. Sodium-borate buffer (0.01 M, pH 8.5) containing 0.15 M NaCl was used to dilute a stock solution of antigen of known concentration to a series of final concentrations in the range of 30 to 600 µg of protein per ml. A fixed volume (0.5 ml) of each antigen solution was then mixed with 0.5 ml of antiserum (1:2 dilution), incubated for 2 hr at 37°C and then left in the refrigerator for 8 days. At the end of this period, the samples were centrifuged for 30 min at 23,000 x g and the supernatant solutions were carefully decanted. The precipitates were washed three times with chilled 0.15 M NaCl and analyzed for nitrogen by the method of Long (1958) while samples from the supernatant solutions were tested for the presence of excess antigen or antibody by agar diffusion according to the method of Wright (1959). In this method, which is a modification of the method of immunodiffusion analysis described earlier, the diffusion plate contains three rows of circular wells. The first row is filled with a fixed volume of antigen, the third row with the same volume of antiserum, and to the second row is added the same fixed volume of each supernatant solution. The samples of supernatants which form precipitates between the first and second rows contain excess
antibody while those which form precipitates between the second and third rows contain excess antigen.

(d) **Enzyme assays**

The mutase and dehydrogenase activities of chorismate mutase-prephenate dehydrogenase from *E. coli* and *A. aerogenes* were assayed as described in Chapter II except for the inclusion in reaction mixtures of 1 mg/ml of bovine serum albumin which was added to overcome the effect of control serum in activating both the mutase and dehydrogenase activities. The mutase activity of chorismate mutase-prephenate dehydratase from *E. coli* was assayed as described in Chapter II while the prephenate dehydratase was assayed as described by Duggleby et al (1978) and involved measurements of the absorbance at 320 nm of the product phenylpyruvate. Aromatic amino acid aminotransferase and aspartate aminotransferase assays were performed as described by Powell and Morrison (1978). They involved measurements of the absorbance at 331 nm of 4-hydroxyphenylpyruvate which was formed as a result of a transamination reaction involving tyrosine and α-ketoglutarate.

(e) **Neutralization of enzyme activity by antiserum**

'Enzyme neutralization' is the term commonly used to denote inhibition of enzymic activity of an antigen by antiserum. In this Chapter, the term 'neutralization' has been used in a similar sense and the term 'neutralizing antibodies' has been used to refer to the antibody components of the antiserum that are responsible for the inhibition of the catalytic activity of enzyme antigens.
Samples of control serum and antiserum were dialyzed for 24 hr against 500 volumes of 0.01 M Na-borate buffer (pH 8.0) containing 0.15 M NaCl. Varying quantities of the dialyzed antiserum were added to 30 µl of the appropriate enzyme (0.25 mg/ml). Reaction mixtures in which similar volumes of control serum replaced the antiserum were used as controls. The samples were mixed and incubated at 37°C for 30 min to allow the reaction to reach equilibrium. Since the dissociation constants of antigen-antibody complexes are usually in the range of 10^{-8} M (Day, 1963), it is unlikely that the enzyme-antibody complex formed in the above reaction will dissociate to any significant extent on further dilution. Aliquots were therefore removed from the above reaction mixture at the end of 30 min and assayed for enzyme activities. Enzyme neutralization curves were then constructed by plotting the percentage activity remaining versus the amount of antiserum added.

When the effect of substrates, inhibitors and other compounds on the neutralization of enzyme activity by antiserum was studied, the compounds were added to 30 µl of the enzyme (0.25 mg/ml) and the mixture incubated for 10 min at 37°C prior to the addition of 12 µl of antiserum or control serum.

In experiments where the cross-reactivity of heterologous antigens with the antiserum to chorismate mutase-prephenate dehydrogenase from *E. coli* was tested, the antiserum (100 µl) was first reacted with 25 µg of the pure heterologous antigen and incubated at 37°C for 1 hr. The antiserum was then used in neutralization experiments which were performed as described above. Control serums were also reacted with heterologous antigens before use in neutralization experiments.
The initial slope of the neutralization curve for the enzyme chorismate mutase-prephenate dehydrogenase from *E. coli* has been used as an index of the neutralizing antibody titre of the antiserum (Fig. VI.3). These slopes were determined using antiserum both before and after treatment with heterologous antigen. The ratio of the two slopes was used to calculate the percentage of neutralizing antibodies remaining in the antiserum after reaction with the heterologous antigen.

\[
\% \text{ neutralizing antibody in serum after reaction} = \frac{\text{slope of enzyme neutralization curve for antiserum after reaction}}{\text{slope of enzyme neutralization curve for antiserum before reaction}} \times 100
\]

4. **RESULTS**

(a) **Homogeneity of the antiserum**

The antiserum raised to pure chorismate mutase-prephenate dehydrogenase from *E. coli* gave a single sharp precipitation line when tested either with homogeneous preparations of the enzyme or with crude cell extracts of *E. coli* (Fig.VI.1.). This result indicated that the antiserum was homogeneous in that it did not contain antibodies to proteins, other than chorismate mutase-prephenate dehydrogenase, which are also present in crude cell extracts of *E. coli*.

(b) **Quantitative precipitation of chorismate mutase-prephenate dehydrogenase by the antiserum**

Quantitative precipitation of the enzyme was performed by varying the quantity of enzyme added over a range of 15 to 300 µg protein. On the addition of increasing quantities of antigen, the
FIG.VI.1. THE PRECIPITATION PATTERNS OBSERVED IN IMMUNODIFFUSION EXPERIMENTS WITH ANTISERUM PREPARED AGAINST PURE CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE FROM \textit{E.coli}.

WELL CONTENTS: (1) PURE CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE FROM \textit{E.coli}.

(2) CRUDE CELL EXTRACTS OF \textit{E.coli}.

(3) ANTISERUM.
amount of total protein precipitated (antigen and antibody) was observed to increase until it reached a maximum and then declined (Fig.VI.2). From the tests on the supernatant, several regions or zones were recognized: the zone of antibody excess, corresponding to the first three points, a region called the equivalence zone in which neither antigen nor antibody is detected in the supernatant and finally a zone of antigen excess.

In the zone of antibody excess and in the equivalence zone, all the antigen is contained in the precipitate and thus the quantity of antigen protein added can be subtracted from the total protein precipitated to yield values for the quantity of antibody precipitated at each antigen concentration in these regions (Table VI.1). It was not possible to calculate the amount of antibody precipitated in regions of antigen excess, as values for the amount of excess antigen in the supernatant which are required for the calculation, had not been determined. Inspection of the values in column 4 of Table VI.1 shows that the maximum quantity of precipitate is formed in the zone of equivalence. The ratio of antibody (Ab) to antigen (Ag) in the precipitate can be seen to change over the course of the reaction. Conversion of these ratios to molecular ratios by using a value of 140,000 for the molecular weight of immunoglobulin type IgG and 88,000 for the molecular weight of enzyme antigen yields an average molecular composition of the insoluble complex which is $\text{Ab}_6\text{Ag}$ in extreme antibody excess and $\text{Ab}_4\text{Ag}$ at the point of maximum precipitation.

(c) **Inhibition of the mutase and dehydrogenase activities of chorismate mutase-prephenate dehydrogenase by the antiserum**

The antiserum raised to the pure enzyme was observed to inhibit
FIG. VI.2. QUANTITATIVE PRECIPITATION OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE FROM *E. coli* BY ANTISERUM PEC-234.

A FIXED VOLUME (0.5 ML) OF ANTISERUM PEC-234 (1:2 DILUTION) WAS REACTED WITH VARYING QUANTITIES OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE (0.5 ML). THE ZONES OF ANTIBODY EXCESS AND ANTIGEN EXCESS WERE DEFINED BY ANALYSIS OF THE SUPERNATANT AS DESCRIBED UNDER 'METHODS'.

(a) ZONE OF ANTIBODY EXCESS.
(b) ZONE OF EQUIVALENCE.
(c) ZONE OF ANTIGEN EXCESS.
TABLE VI.1. ADDITION OF INCREASING AMOUNTS OF *E. coli* CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE (ANTIGEN) TO 0.5 ML OF A 1:2 DILUTION OF ANTISERUM TO CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE FROM *E. coli* (PEC-234). THE CONDITIONS USED ARE DESCRIBED UNDER 'METHODS'.

<table>
<thead>
<tr>
<th>Antigen protein added (µg)</th>
<th>Antigen protein precipitated (µg)</th>
<th>Total protein precipitated (µg)</th>
<th>Antibody protein precipitated (by difference) (µg)</th>
<th>Ratio antibody protein : antigen protein in precipitate</th>
<th>Molecular ratio antibody : antigen</th>
<th>Tests on Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.9</td>
<td>14.9</td>
<td>143.8</td>
<td>128.9</td>
<td>9:1</td>
<td>6:1</td>
<td>Excess antibody</td>
</tr>
<tr>
<td>29.7</td>
<td>29.7</td>
<td>259.4</td>
<td>229.7</td>
<td>8:1</td>
<td>5:1</td>
<td>Excess antibody</td>
</tr>
<tr>
<td>59.4</td>
<td>59.4</td>
<td>493.8</td>
<td>434.4</td>
<td>7:1</td>
<td>5:1</td>
<td>Excess antibody</td>
</tr>
<tr>
<td>95.0</td>
<td>95.0</td>
<td>700.0</td>
<td>605.0</td>
<td>6:1</td>
<td>4:1</td>
<td>No antibody or antigen</td>
</tr>
<tr>
<td>118.8</td>
<td>118.8</td>
<td>668.8</td>
<td>550.0</td>
<td>5:1</td>
<td>3:1</td>
<td>No antibody or antigen</td>
</tr>
<tr>
<td>158.4</td>
<td>N.D.</td>
<td>625.0</td>
<td></td>
<td></td>
<td></td>
<td>Excess antigen</td>
</tr>
<tr>
<td>237.5</td>
<td>N.D.</td>
<td>353.1</td>
<td></td>
<td></td>
<td></td>
<td>Excess antigen</td>
</tr>
<tr>
<td>316.7</td>
<td>N.D.</td>
<td>305</td>
<td></td>
<td></td>
<td></td>
<td>Excess antigen</td>
</tr>
</tbody>
</table>

*Not determined*
both the mutase and dehydrogenase activities of the enzyme (Fig.VI.3). At low concentrations of the antiserum, the mutase activity of the enzyme was inhibited to a greater extent than the dehydrogenase activity of the enzyme. The inhibition of the enzyme was linear with the concentration of antiserum up to an inhibition of 70% of the mutase activity and up to 55% of the dehydrogenase activity. The addition of 30 µl of undiluted antiserum was observed to completely inhibit 8 µg of the enzyme (Fig.VI.3).

(d) Protection by substrates, products and other compounds against inhibition of chorismate mutase-prephenate dehydrogenase by antiserum

Various compounds were tested for their ability to protect the enzyme against inhibition by antiserum (Table VI.2). For this purpose, conditions of reaction were chosen that facilitated comparison of the relative protection of the mutase and dehydrogenase activity of the enzyme by the compounds added. A concentration of antiserum that was just sufficient to cause nearly maximum inhibition of the enzyme was used and the enzyme samples were preincubated with the antiserum in order to allow the reaction between the enzyme and antibody to reach equilibrium. However, it must be noted that under these conditions, any small transitory effect of reactants would not be observed and the degree of protection of the enzyme by the various compounds may be small.

The substrates prephenate and NAD were partially effective in protecting the enzyme against inhibition by the antiserum. NAD, at a concentration of 1 mM protected both the mutase and dehydrogenase activities to the same extent. Prephenate also protected both activities
FIG.VI.3. INHIBITION OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE FROM \textit{E.coli} BY ANTISERUM PEC-234.

8 µg OF THE ENZYME WAS PREINCUBATED WITH VARYING AMOUNTS OF ANTISERUM AND ALIQUOTS WERE ASSAYED FOR CHORISMATE MUTASE AND PREPHENATE DEHYDROGENASE ACTIVITIES AS DESCRIBED UNDER 'METHODS'.

(■■■■■) CHORISMATE MUTASE ACTIVITY.

(●●●●●) PREPHENATE DEHYDROGENASE ACTIVITY.
Table VI. Effect of Various Compounds on the Inhibition of the Mutase and Dehydrogenase Activities of Chorinate Mutase-Pephenate Dehydrogenase from P. coli in Antiserum PEC 754.

The compounds were added to the enzyme before addition of the antiserum. After incubation of the enzyme with antiserum for 30 min, aliquots were removed from the reaction mixture.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>0</td>
</tr>
<tr>
<td>NADH (1 ml)</td>
<td>75</td>
</tr>
<tr>
<td>NADH (1 ml) +</td>
<td></td>
</tr>
<tr>
<td>4-hydroxyphenylpyruvate (1 ml)</td>
<td></td>
</tr>
<tr>
<td>AMP (2 ml)</td>
<td>10</td>
</tr>
<tr>
<td>Tyrosine (1 ml)</td>
<td>10</td>
</tr>
<tr>
<td>Tyrosine (1 ml) + NAD (0.5 ml)</td>
<td>25</td>
</tr>
<tr>
<td>NADH (1 ml) + 4-hydroxyphenylpyruvate (1 ml)</td>
<td>50</td>
</tr>
<tr>
<td>NADH (1 ml)</td>
<td>75</td>
</tr>
</tbody>
</table>

*corresponds to 95% inhibition of the mutase activity.

*corresponds to 80% inhibition of the dehydrogenase activity.
TABLE VI.2. EFFECT OF VARIOUS COMPOUNDS ON THE INHIBITION OF THE MUTASE AND DEHYDROGENASE ACTIVITIES OF CHORISMA TE MUTASE-PREPHENATE DEHYDROGENASE FROM E.COLI BY ANTISERUM PEC-234.

The compounds were added to the enzyme before addition of the antisera. After incubation of the enzyme with antisera for 30 min, aliquots were removed from the reaction mixture and assayed for mutase and dehydrogenase activities as described under 'METHODS'.

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>% activity protected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mutase</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Prephenate</td>
<td></td>
</tr>
<tr>
<td>(0.1 mM)</td>
<td>9</td>
</tr>
<tr>
<td>(0.5 mM)</td>
<td>21</td>
</tr>
<tr>
<td>(1.0 mM)</td>
<td>34</td>
</tr>
<tr>
<td>NAD</td>
<td></td>
</tr>
<tr>
<td>(0.5 mM)</td>
<td>23</td>
</tr>
<tr>
<td>(1.0 mM)</td>
<td>23</td>
</tr>
<tr>
<td>4-hydroxyphenylpyruvate</td>
<td></td>
</tr>
<tr>
<td>(1 mM)</td>
<td>7</td>
</tr>
<tr>
<td>NADH</td>
<td></td>
</tr>
<tr>
<td>(1 mM)</td>
<td>7</td>
</tr>
<tr>
<td>(2 mM)</td>
<td>9</td>
</tr>
<tr>
<td>NADH (1 mM) +</td>
<td></td>
</tr>
<tr>
<td>Prephenate (0.5 mM)</td>
<td>28</td>
</tr>
<tr>
<td>NADH (1 mM) +</td>
<td></td>
</tr>
<tr>
<td>4-hydroxyphenylpyruvate (1 mM)</td>
<td>6</td>
</tr>
<tr>
<td>AMP</td>
<td></td>
</tr>
<tr>
<td>(2 mM)</td>
<td>0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td></td>
</tr>
<tr>
<td>(1 mM)</td>
<td>2</td>
</tr>
<tr>
<td>Tyrosine (1 mM) + NAD (0.5 mM)</td>
<td>2</td>
</tr>
</tbody>
</table>

*a* corresponds to 95% inhibition of the mutase activity

*b* corresponds to 80% inhibition of the dehydrogenase activity
of the enzyme to the same extent but was more effective at concentrations of 1 mM than at lower concentrations. The product, 4-hydroxyphenylpyruvate (1 mM) and the dead-end inhibitor AMP (2 mM) were poor protecting agents. The product NADH, on the other hand, showed strong protection of the dehydrogenase reaction, while having little effect in protecting the mutase reaction against inhibition by antiserum. Protection, by NADH, of the dehydrogenase reaction was unaffected by the inclusion of prephenate in the incubation mixture while the protection of the mutase reaction by this combination appeared to be a sum of the effects observed when each compound is used alone. On the other hand, a mixture of NADH and 4-hydroxyphenylpyruvate was less effective as compared to NADH in protecting the dehydrogenase reaction and was without any effect on the inhibition of the mutase reaction by the antiserum. Tyrosine, the allosteric effector of the enzyme did not relieve the inhibition of either enzymic activity by the antiserum when tested both in the presence and absence of NAD.

(e) Cross-reactivity of chorismate mutase-prephenate dehydrogenase from *A. aerogenes*

In immunodiffusion experiments, the enzyme chorismate mutase-prephenate dehydrogenase from *A. aerogenes* was observed to cross-react with the antiserum prepared against the *E. coli* enzyme. The precipitation band formed with the enzyme from *A. aerogenes* was found to intersect the band formed with the enzyme from *E. coli* giving rise to a small spur pointing towards the well containing the enzyme from *A. aerogenes* (Fig.VI.4). In neutralization experiments, the enzyme from *A. aerogenes* was observed to behave similarly to the *E. coli* enzyme in that both the mutase and the dehydrogenase activities of the enzyme were completely inhibited at high
FIG. VI.4. IMMUNODIFFUSION EXPERIMENT DEMONSTRATING CROSS-REACTIVITY OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE FROM *A. AEROGENES* WITH ANTISERUM RAISED TO CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE FROM *E. COLI*.

WELL CONTENTS:

(1) CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE FROM *E. COLI*.

(2) CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE FROM *A. AEROGENES*.

(3) ANTISERUM.
concentrations of the antiserum. As observed earlier with the *E.coli* enzyme, the mutase activity of the enzyme from *A.aerogenes* was more sensitive than the dehydrogenase activity, to inhibition by low concentrations of the antiserum (Fig.VI.5). The inhibition of the enzyme was linear with the concentration of antiserum up to a value of 60% inhibition of the mutase activity and 50% inhibition of the dehydrogenase activity. The addition of 40 µl of antiserum was required for the complete inhibition of 8 µg of the enzyme which was higher than that required for the complete inhibition of a corresponding quantity of the enzyme from *E.coli*.

The ability of the enzyme from *A.aerogenes* to cross-react and remove the antibodies responsible for neutralizing the activity of the enzyme from *E.coli* was tested by measuring the titre of neutralizing antibodies before and after reaction of the antiserum with the enzyme from *A.aerogenes*. The slope of the linear region of the neutralization curve for the enzyme from *E.coli* was used as a measure of the neutralizing antibody titre of the antiserum.

The initial slope of the enzyme neutralization curve obtained with the antiserum after reaction with the enzyme/ *A.aerogenes* is much smaller than that for the enzyme neutralization curve obtained with the antiserum untreated with the enzyme (Fig.VI.6). Using the expression described under 'Methods', it can be calculated that the preincubation with chorismate mutase-prephenate dehydrogenase from *A.aerogenes* removes almost 90% of the neutralizing antibodies that react with and inhibit the chorismate mutase-prephenate dehydrogenase from *E.coli*. 
FIG.VI.5. INHIBITION OF CHORISMATE MUTASE-PREPHENATE DEHYDROGENASE FROM _A. AEROGENES_ BY ANTISERUM PEC-234.

8 µg OF THE ENZYME WAS PREINCUBATED WITH VARYING AMOUNTS OF ANTISERUM AND ALIQUOTS WERE ASSAYED FOR CHORISMATE MUTASE AND PREPHENATE DEHYDROGENASE ACTIVITIES AS DESCRIBED UNDER 'METHODS'.

(■ ■■■■) CHORISMATE MUTASE ACTIVITY.

(● ●●●●) PREPHENATE DEHYDROGENASE ACTIVITY.
FIG.VI.6. NEUTRALIZATION OF THE MUTASE ACTIVITY OF \textit{E.coli} CHORISMA TE MUTASE-PREPHENATE DEHYDROGENASE BY ANTISERUM PEC-234.

(■) INHIBITION BY UNTREATED ANTISERUM.

(●) INHIBITION BY ANTISERUM (100 \( \mu l \)) PRETREATED WITH AROMATIC AMINO ACID AMINOTRANSFERASE (25 \( \mu g \)) FROM \textit{E.coli}.

(▲) INHIBITION BY ANTISERUM (100 \( \mu l \)) PRETREATED WITH CHORISMA TE MUTASE-PREPHENATE DEHYDRATASE (25 \( \mu g \)) FROM \textit{E.coli}.

(☺) INHIBITION BY ANTISERUM (100 \( \mu l \)) PRETREATED WITH CHORISMA TE MUTASE-PREPHENATE DEHYDROGENASE (25 \( \mu g \)) FROM \textit{A.aerogenes}.
In immunodiffusion experiments, no cross-reactivity of the choromate mutase-pherophosphate dehydratase was observed with the antiserum to choromate mutase-pherophosphate dehydratase from another source. Further, no cross-reactivity of either the mutase or dehydratase activity could be detected when 100% of the antiserum was incubated with the enzyme. Nevertheless, a significant inhibition of the activity of choromate mutase-pherophosphate dehydratase was observed when 100% of the antiserum was incubated with the enzyme. The results indicated that up to 100% of the neutralizing antibodies in the antiserum had been removed by reaction of the antiserum with choromate mutase-pherophosphate dehydratase from another source.

Cross-reactivity of aromatic and aspartate amino acid aminotransferase from syuts with the antiserum to choromate mutase-pherophosphate dehydratase was observed. No precipitation lines were observed with either the aromatic amino acid aminotransferase of the aspartate aminotransferase in immunodiffusion experiments with the antiserum to choromate mutase-pherophosphate dehydratase.
(f) Cross-reactivity of chorismate mutase-prephenate dehydratase from *E. coli* with the antiserum

In immunodiffusion experiments, no cross-reactivity of the chorismate mutase-prephenate dehydratase was observed with the antiserum to chorismate mutase-prephenate dehydrogenase from *E. coli*. Further, no inhibition of either the mutase or dehydratase activity of the enzyme occurred on reaction of 8 µg of the enzyme with up to seven times the concentration of antiserum that is required for the complete inhibition of an equivalent amount of chorismate mutase-prephenate dehydrogenase. But, when 100 µl of the antiserum was incubated with 25 µg of chorismate mutase-prephenate dehydratase, there was a significant decrease in the neutralizing antibody titre of the antiserum. The slope of the enzyme neutralization curve for chorismate mutase-prephenate dehydrogenase was observed to decrease on treatment of the antiserum with chorismate mutase-prephenate dehydratase from *E. coli* (Fig.VI.6). Calculations (as described under 'Methods') showed that there was a decrease in the neutralizing antibody titre of the antiserum to 35% of the titre observed before reaction with the heterologous antigen. These results indicated that up to 65% of the neutralizing antibodies in the antiserum had been removed on reaction of the antiserum with chorismate mutase-prephenate dehydratase from *E. coli*.

(g) Cross-reactivity of aromatic and aspartate amino acid aminotransferase from *E. coli* with the antiserum

No precipitation lines were observed with either the aromatic amino acid aminotransferase of the aspartate aminotransferase in immunodiffusion experiments with the antiserum to chorismate mutase-
prephenate dehydrogenase. Neither of the aminotransferases (8 µg) was inhibited on reaction with up to seven times the quantity of antiserum required to inhibit 8 µg of chorismate mutase-prephenate dehydrogenase. The enzyme neutralization curve obtained with antiserum after reaction with aspartate aminotransferase was identical to that obtained with unreacted antiserum. On the other hand, antiserum treated with 25 µg of aromatic amino acid aminotransferase gave rise to an enzyme neutralization curve with a slight decrease in slope as compared to that obtained with untreated antiserum (Fig.VI.6). The decrease in slope corresponded to a loss of 14% of the neutralizing antibodies due to reaction with aromatic amino acid aminotransferase.

5. DISCUSSION

The primary purpose of the studies reported in this Chapter was to investigate the immunological properties of chorismate mutase-prephenate dehydrogenase with special reference to the antigenic nature of its catalytic sites. But, in addition, studies have been undertaken on the evolutionary relationships between chorismate mutase-prephenate dehydrogenase and chorismate mutase-prephenate dehydratase from \textit{E.coli} and between the chorismate mutase-prephenate dehydrogenases from \textit{E.coli} and \textit{A.aerogenes}.

The antiserum to chorismate mutase-prephenate dehydrogenase from \textit{E.coli} affects the mutase and dehydrogenase activities of the enzyme to different extents. The differences in initial slopes of the neutralization curves (Fig.VI.3) may be due to the presence of a single antibody which exhibits different inhibition constants for the mutase and for the dehydrogenase reaction because of its binding to different
antigenic determinants associated with the two active sites of the enzyme. Alternately, there may be two different types of antibodies involved in the inhibition of the two activities of the enzyme and the differential inhibition may reflect differences either in their inhibition constants or in their relative concentrations in the antiserum. It is generally accepted that each antibody is highly specific for a particular antigenic determinant (Landsteiner, 1962; Kabat, 1968) and therefore, the differential inhibition of the two reactions by the antiserum implies that the antiserum contains two different antibodies which arise because of differences in the antigenic determinants associated with the two catalytic sites.

The observation that NADH protects only the dehydrogenase reaction against inhibition by the antiserum is consistent with the results of the inhibition studies which suggest that different antigenic determinants are involved in the inhibition of the mutase and dehydrogenase reactions. Thus the presence of NADH must affect the two antigenic determinants to different extents. In this connection, it is of interest that in kinetic studies, the binding of NADH at a single site is observed to affect both the mutase and the dehydrogenase activities of the enzyme (Chapter V). The protective effects of NADH must therefore be different from its kinetic effects. However, in the absence of more information on the enzyme-antibody interaction, it is not possible to draw any further conclusions from a comparison of the two effects.

The differential inhibition by antiserum of the two reactions catalyzed by chorismate mutase-prephenate dehydrogenase can be compared with that observed with the bifunctional enzyme DAHP synthase-chorismate
mutase from \textit{B. subtilis}. This enzyme catalyzes the synthesis of 3-deoxy-D-arabinoheptulosnate 7-phosphate (DAHP) from phosphoenolpyruvate and erythrose-4-P, in addition to catalyzing the conversion of chorismate to prephenate. Biochemical and genetic studies on the enzyme have shown that its two activities are catalyzed by independent regions along the polypeptide chain (Nester \textit{et al}, 1967; Huang \textit{et al}, 1974b). Under particular conditions, the antiserum against the pure bifunctional enzyme was observed to inhibit about 80\% of the mutase activity and about 20\% of the DAHP synthase activity of the enzyme (Huang \textit{et al}, 1974b). Such results would be expected for a multifunctional enzyme catalyzing significantly different reactions at two catalytic sites. Thus, the findings with chorismate mutase-prephenate dehydrogenase from \textit{E.coli} and DAHP synthase-chorismate mutase draw attention to the potential that immunological studies may have in elucidating the number of catalytic sites on a multifunctional enzyme.

Immunodiffusion experiments indicated that chorismate mutase-prephenate dehydrogenase from \textit{A.aerogenes} cross reacts with the antiserum prepared against the enzyme from \textit{E.coli} and in so doing gives rise to a small spur (Fig.VI.4). The presence of a spur indicates that the chorismate mutase-prephenate dehydrogenase from \textit{E.coli} has at least one antigenic determinant not shared by the enzyme from \textit{A.aerogenes} (Gasser and Gasser, 1971). But since at least three antigenic determinants are needed to form a sufficiently large lattice with antibodies for precipitation to occur, the cross-precipitation of the enzyme from \textit{A.aerogenes} indicates a high degree of structural homology between the two enzymes. This conclusion is supported by the observation that the enzyme neutralization curves for the two enzymes are almost identical.
(Fig.VI.3, VI.4) and by the finding that the enzyme from *A. aerogenes* is capable of cross-reacting with almost 90% of the neutralizing antibodies present in a volume of antiserum just sufficient to neutralize an equivalent amount of the *E. coli* enzyme (Fig.VI.6).

The failure to observe any cross-reaction between chorismate mutase-prephenate dehydratase from *E. coli* and the antiserum to chorismate mutase-prephenate dehydrogenase from *E. coli* indicates that the two enzymes do not share a great degree of structural homology. But it is possible that the two proteins share a smaller number of common antigenic determinants that are not detectable by immunoprecipitation techniques. Enzyme neutralization has therefore been used to detect homologies in those determinants located at or near the active site. No inhibition of either enzymic activity of chorismate mutase-prephenate dehydratase has been observed with antiserum to chorismate mutase-prephenate dehydrogenase from *E. coli*. But it is possible that the neutralizing antibodies in the antiserum interact with chorismate mutase-prephenate dehydratase without modifying either of its catalytic activities. As chorismate mutase-prephenate dehydratase removes 65% of the neutralizing antibodies present in a volume of antiserum just sufficient to neutralize an equivalent amount of chorismate mutase-prephenate dehydrogenase from *E. coli* (Fig.VI.6), it follows that considerable homology does exist between the two enzymes. However, the homology is limited to those antigenic determinants that react with the neutralizing antibodies. In contrast to chorismate mutase-prephenate dehydratase, the aromatic amino acid aminotransferase and the aspartate aminotransferase exhibit no significant cross-reactivity with the antiserum to chorismate mutase-prephenate dehydrogenase from *E. coli*. 
The antigenic homology between the enzyme chorismate mutase-prephenate dehydratase and chorismate mutase-prephenate dehydrogenase has a number of interesting evolutionary implications. The two enzymes have similar molecular weights, subunit structure and amino acid compositions. The structural gene for the two enzymes are also adjacent to each other on the E.coli chromosome. It can therefore be speculated that the antigenic homology observed between the two enzymes reflects their evolution from a single chorismate mutase gene. Duplication followed by divergent evolution could have resulted in the two bifunctional proteins. Similar speculations have been made about the evolution of the three aspartokinases in E.coli K12 (Cohen et al., 1977). Two of these enzymes are bifunctional and catalyze the dehydrogenation of homoserine in addition to the phosphorylation of aspartate. The three enzymes differ in their regulatory properties and each is subject to regulation by either threonine, methionine or lysine. Although no cross-reaction between the three enzymes has been observed in immunodiffusion reactions (Kaminsky et al., 1969), immunoabsorbents prepared with antiserum against the threonine-sensitive aspartokinase-homoserine dehydrogenase has been shown to be capable of binding the methionine-sensitive aspartokinase-homoserine dehydrogenase and the lysine-sensitive aspartokinase (Truffa-Bachi et al., 1975). This homology is in accord with the idea that the three aspartokinases derive from a common ancestral protein and are not products of entirely distinct evolutionary pathways.

6. **SUMMARY**

(1) Antiserum has been prepared against pure E.coli chorismate mutase-prephenate dehydrogenase and has been shown to be homogeneous
in immunodiffusion experiments.

(2) Studies have been made of the inhibition by antiserum of the mutase and dehydrogenase activities of chorismate mutase-prephenate dehydrogenase and the effect of various reactants in protecting against the inhibition. The results have led to the conclusion that the antiserum contains two types of antibody which are produced because of different antigenic determinants associated with the two catalytic activities of the enzyme.

(3) The antiserum to *E. coli* chorismate mutase-prephenate dehydrogenase shows a high degree of cross-reactivity with chorismate mutase-prephenate dehydrogenase from *A. aerogenes* and a lesser degree of cross-reactivity with chorismate mutase-prephenate dehydratase from *E. coli*. No significant cross-reaction of the antiserum occurs with either the aspartate or aromatic amino acid aminotransferase from *E. coli*. 

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