Study of Metal Binding Site of Methyl Parathion Hydrolase

A thesis submitted for the degree of Doctor of Philosophy of
The Australian National University

Nur Hafizah Azizan

Research School of Chemistry

Australian National University

June 2015
Declaration

This thesis presents original research and to the best of my knowledge does not contain material that has been previously published, except where specified and referenced within the text.

The work contained in this thesis, except where due acknowledgement has been made, is original work that I carried out during my PhD candidature and has not been submitted previously in whole or in part for any other degree or diploma in any university or tertiary institution.

Contributions of individuals to this thesis are listed below:

- Plasmid of wild-type mpd in pETMCS1 was obtained from Ollis group plasmid stock solution.
- Fourier transform ion cyclotron resonance mass spectroscopy (FTICR-MS) measurements were performed by RSC Microanalytical unit and data analyses were performed by Dr. Joanne Porter and Mr. Samuel Fraser.
- ITC binding simulations described in the introductory chapter were suggested by Emeritus Professor Don Winzor from the University of Queensland.

Nur Hafizah Azizan
Acknowledgements

First and foremost, my thanks to God, for his Humanity and Power to His creature to let me complete this PhD thesis.

My highest gratitude to my supervisor Professor David Ollis for the invaluable guidance, encouragement and generous assistance throughout the completion of this thesis. My thanks to Associate Professor Gary Schenk and Emeritus Professor Don Winzor from The University of Queensland for their knowledge and precious information especially on ITC result analysis. My appreciation also goes to my MPH-mate, Dr. Tee Kheang Ng thanks for all of the knowledge sharing, guidance and assistance especially at the beginning of my study.

My gratitude to the Research School of Chemistry for the instruments and facilities provided. My big thank you also goes to the Ministry of Higher Education Malaysia and the International Islamic University Malaysia for the financial support throughout the study.

A special dedication to my beloved mum, Mrs. Rugaiyah Mohamed Yunus and my siblings Muhammad Falah Azizan, Nur Safiah Azizan, Adriza Jaehzah Jamil and Mohamad Nizar Othman who always inspire and pray for my success, without which this thesis would never have been completed. To my best friend Dr. Zurahanim Fasha Annual, thanks a bunch for the endless support and the absolute assistance throughout the journey. My encouraging clan, the housemates Diana and Aisyah thanks for the overwhelming friendship for the whole three and the half years life in Canberra.

My thanks to Dr, Joanne Porter for the unconditional proofreading services provided. To Miss Tracy Murray and Dr. Bradley Stevenson, thank you for the thoughts, bighearted helps and encouraging words. Last but not least thank you very much to all my lab mates and those who directly or indirectly assisted and supported me in finishing this task.
Abstract

Metal ions are an essential component of numerous proteins. In some cases they have a structural role, but their more important roles are redox proteins and enzymes. Metals interact quite differently with proteins depending upon the function of the protein. In enzymes metals are coordinated to the protein, but maintain vacant coordination sites to allow them to interact with substrates or to activate nucleophiles for attack. In binuclear metalloenzymes, metals have the potential to function in both capacities where they can bind substrate and can also activate water for attack. Most studies of binuclear metalloenzymes focus on the native enzyme how it might interact with the substrate. Clearly, the interaction between protein and substrate depends critically upon the metal centers. This study sets out to study the metal centers, to identify the role of particular residues in binding metals and the effect of perturbing the metal centers on catalytic activity. This study was never intended to make a detailed study of catalytic activity, but rather to provide background information for such studies. It was also hoped that data collected during the course of this study might shed some light on previous mechanistic studies.

To achieve the objective outlined above a suitable enzyme was selected for study, which is methyl-parathione hydrolase (MPH). Its active site residues were mutated and the resulting protein variants were subjected to a variety of experiments. The effects of mutations on metal binding affinity, catalytic activity as well as overall protein stability were determined. Very early in the study, it became clear that metals ions had a significant effect on protein stability and that the apo-protein was much less stable that the fully metal bound form. Some mutations had a profound effect on activity yet caused minor changes in stability while other mutants exhibited the opposite effect. Surprisingly, in some cases removing the
side-chain of a coordinating group had little effect on the affinity of the protein for the metal or on its catalytic activity. However, it was also clear that two metal ions were essential for activity; little activity was observed with a single metal ion bound to the protein.

MPH has evolved to accommodate two metal ions in its active site such that minor alterations to the coordinating residues results in a loss of either stability or activity. These observation suggest that MPH has evolved to optimize activity as well as stability; the latter being important for the long-term ability of the enzyme to be an efficient catalyst.
Table of Contents

Declaration................................................................................................................................i
Acknowledgements ................................................................................................................ iii
Abstract....................................................................................................................................v
Table of Contents ..................................................................................................................vii
List of Figures .........................................................................................................................x
List of Tables ..........................................................................................................................xi
Abbreviations ........................................................................................................................xii

Chapter 1 – Introduction........................................................................................................1
1.1 Preface................................................................................................................................ 1
1.2 Metals in biological systems – function and importance ..............................................2
1.3 Metals in enzymes ............................................................................................................ 3
  1.3.1 Metalloenzymes: hydrolysis, the most common function ............................................5
  1.3.2 Role of metals ............................................................................................................. 8
  1.3.2.1 Structural .......................................................................................................................8
  1.3.2.2 Electron transfer ......................................................................................................... 10
  1.3.2.3 Transport proteins ....................................................................................................... 13
  1.3.2.4 Metals in hydrolytic enzymes ...................................................................................... 16
  1.3.3 Examples of metallohydrolyase-function, structure and metal co-ordination ..........16
    1.3.3.1 Mononuclear enzymes:
      i) Carbonic anhydrase .......................................................................................................... 16
      ii) Carboxypeptidase A ........................................................................................................ 18
      iii) Staphylococcal nuclease .................................................................................................. 19
    1.3.3.2 Binuclear enzymes:
      i) Amino peptidase or pitabread fold ..................................................................................20
      ii) Arginase-like enzymes ..................................................................................................... 21
      iii) Amidohydrolase fold ........................................................................................................ 22
      iv) α/β sandwich poteins or the α/β/β/α fold ......................................................................... 27
  1.3.4 Common features of metalloproteins ...........................................................................29
1.4 Enzymes to be studied.................................................................................................. 30
  1.4.1 MPH – function, structure and metal coordination ....................................................... 30
  1.4.1.1 Reaction catalysed by MPH ...........................................................................................31
  1.4.1.2 Overall structure of MPH ............................................................................................. 32
  1.4.1.3 Metal coordination ........................................................................................................ 32
1.5 Techniques to be used .................................................................................................... 34
  1.5.1 ITC .................................................................................................................................. 34
  1.5.2 Ligand binding – equation ............................................................................................. 35
1.6 Protein stability ............................................................................................................. 39
1.7 Aims ................................................................................................................................... 40
1.8 Reference ......................................................................................................................... 41

vii
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 2 - Materials and Methods</td>
<td>49</td>
</tr>
<tr>
<td>2.1 Preface</td>
<td>49</td>
</tr>
<tr>
<td>2.2 Bacterial strains and their growth</td>
<td>50</td>
</tr>
<tr>
<td>2.2.1 Strains</td>
<td>50</td>
</tr>
<tr>
<td>2.2.2 Growth media and bacterial storage</td>
<td>50</td>
</tr>
<tr>
<td>2.2.3 Preparation of electrocompetent cells</td>
<td>51</td>
</tr>
<tr>
<td>2.2.4 E.coli transformation</td>
<td>51</td>
</tr>
<tr>
<td>2.3 DNA manipulations</td>
<td>58</td>
</tr>
<tr>
<td>2.3.1 Plasmids</td>
<td>52</td>
</tr>
<tr>
<td>2.3.2 Plasmid extraction and purification</td>
<td>52</td>
</tr>
<tr>
<td>2.3.3 DNA visualisation</td>
<td>52</td>
</tr>
<tr>
<td>2.3.4 DNA quantification</td>
<td>53</td>
</tr>
<tr>
<td>2.3.5 DNA amplifications</td>
<td>53</td>
</tr>
<tr>
<td>2.3.6 Restriction digest</td>
<td>54</td>
</tr>
<tr>
<td>2.3.7 DNA ligation</td>
<td>54</td>
</tr>
<tr>
<td>2.3.8 DNA sequencing</td>
<td>55</td>
</tr>
<tr>
<td>2.3.9 Site directed mutagenesis</td>
<td>56</td>
</tr>
<tr>
<td>2.4 Protein manipulations</td>
<td>58</td>
</tr>
<tr>
<td>2.4.1 Protein expression</td>
<td>58</td>
</tr>
<tr>
<td>2.4.2 Cell lysis</td>
<td>58</td>
</tr>
<tr>
<td>2.4.3 Protein purification</td>
<td>59</td>
</tr>
<tr>
<td>2.4.4 Protein quantification</td>
<td>60</td>
</tr>
<tr>
<td>2.5 Molecular weight determination</td>
<td>61</td>
</tr>
<tr>
<td>2.6 Metal analysis</td>
<td>62</td>
</tr>
<tr>
<td>2.6.1 Preparation of apo-MPH</td>
<td>62</td>
</tr>
<tr>
<td>2.6.2 Apo-enzyme reconstitution</td>
<td>63</td>
</tr>
<tr>
<td>2.6.3 ICP-OES</td>
<td>63</td>
</tr>
<tr>
<td>2.7 Protein stability</td>
<td>64</td>
</tr>
<tr>
<td>2.7.1 Thermoflow assay</td>
<td>64</td>
</tr>
<tr>
<td>2.8 Spectrophotometric assay</td>
<td>65</td>
</tr>
<tr>
<td>2.8.1 Activity assay</td>
<td>65</td>
</tr>
<tr>
<td>2.8.2 Kinetic characterisation</td>
<td>65</td>
</tr>
<tr>
<td>2.8.3 pH profile</td>
<td>67</td>
</tr>
<tr>
<td>2.9 Determination of metal binding affinity</td>
<td>67</td>
</tr>
<tr>
<td>2.9.1 Isothermal titration calorimetry (ITC)</td>
<td>67</td>
</tr>
<tr>
<td>2.10 Activity as a function of metal ion concentration</td>
<td>68</td>
</tr>
<tr>
<td>2.11 Computer software programs</td>
<td>69</td>
</tr>
<tr>
<td>2.12 References</td>
<td>70</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1.1 Alcohol dehydrogenase.
Figure 1.2. Zinc finger with C2H2 binding coordination.
Figure 1.3. Plastocyanin.
Figure 1.4. Rubredoxin. A 1Fe-0S centre
Figure 1.5. Ferredoxins
Figure 1.6. Myoglobin
Figure 1.7. Hemocyanin.
Figure 1.8. Myohemerythrin
Figure 1.9. Carbonic anhydrase.
Figure 1.10. Carboxypeptidase A.
Figure 1.11. The catalytic domain of carboxypeptidase G2.
Figure 1.12. Arginase.
Figure 1.13. TIM barrels of Amidohydrolase fold.
Figure 1.14. Figure shows metal coordination in GpdQ.
Figure 1.15. GpdQ fold diagram.
Figure 1.16. General structural formula of organophosphate.
Figure 1.17. Schematic diagram of MPH chemical reaction.
Figure 1.18. Figures show MBL superfamily that shares similar α/β/β/α fold.
Figure 1.19. MPH.
Figure 1.20. Schematic diagram of an ITC instrument.
Figure 2.1. Overlap extension PCR involves the usage of two separate sets of primers for
the reactions.
Figure 2.2. Protein concentration accuracy test.
Figure 2.3. Calibration curve of protein standard.
Figure 2.4. Standard curve of pNP.
Figure 3.1. The amino acids involved in metal ions coordination.
Figure 3.2. Proposed metal ion coordination in Asp151Asn and Asp151Ala.
Figure 3.3. Proposed metal ion coordination in Asp255Asn and Asp255Ala.
Figure 3.4. SDS-PAGE profiles of purified proteins.
Figure 3.5. SDS-PAGE profiles of purified MPH wild-type and its variants.
Figure 3.6. Activity assay for MPH wild-type and its variants using crude lysate.
Figure 3.7. Preliminary activity assay for purified proteins.
Figure 3.8. Deconvoluted FTICR-MS spectrum of MPH wild-type.
Figure 3.9. Chromatographic separation and calibration curve for the protein standard
overlayed with the MPH native elution profile.
Figure 3.10. Remaining activity of MPH wild-type and MPH variants during metal
chelating.
Figure 3.11. Duration of metal reconstitution in MPH native protein.
Figure 3.12. Activity comparison of apo-native, MPH native as purified and Zn(II)-
reconstituted enzyme.
Figure 3.13. Comparison of apo-native activities with foreign metals. The activity was
normalized to WT with native metal activity.
Figure 3.14. Separation of different forms of MPH on 10 % native PAGE.

Figure 3.15. Figure shows the melting temperature of MPH wild-type and all of the variants.

Figure 3.16. The pH rate profiles of MPH wild-type and D151N.

Figure 3.17. Calorimetric titration of Zn$^{2+}$ with apo-MPH native.

Figure 3.18. Reactivation of apo-native with varying amounts of Zn(II).

Figure 4.1. SDS-PAGE profile of MPH purified fractions.

Figure 4.2. Comparison of D151 variant results.

Figure 4.3. Comparison of α variant results.

Figure 4.4. Comparison of β variant results.

Figure 4.5. Comparison of bridging ligand variant results.

Figure 4.6. Comparison of link residue variant results.

Figure 4.7. Comparison of secondary coordination residue variant results.

List of Tables

Table 3.1. Analysis of band’s intensities using ImageJ software.

Table 3.2. Zinc metal content of MPH native protein.

Table 3.3. Kinetic profiles of MPH wild-type and its variants.

Table 3.4. Thermodynamic parameters of Zn$^{2+}$ binding to MPH.

Table 3.5. $K_J$ values (µM) for MPH native and its variants for kinetic as a function of zinc concentration.

Table 3.6. $K_J$ values (µM) for MPH native and its variants for kinetic as a function of cobalt concentration.
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNH</td>
<td>Binuclear hydrolases</td>
</tr>
<tr>
<td>BNM</td>
<td>Binuclear metallohydrolases</td>
</tr>
<tr>
<td>CAPS</td>
<td>ACETATE and 3-(cyclohexylamino)-1-propanesulfonic acid</td>
</tr>
<tr>
<td>CHES</td>
<td>2-(N-cyclo-hexylamino)ethanesulfonic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide Triphosphate</td>
</tr>
<tr>
<td>FTICR-MS</td>
<td>Fourier transform ion cyclotron resonance mass spectroscopy</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-(N)-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Inductively Coupled Plasma – Optical Emission Spectrometry</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal Titration Calorimetry</td>
</tr>
<tr>
<td>(K_b)</td>
<td>Binding constant</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MPH</td>
<td>Methyl Parathion Hydrolase</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>OP</td>
<td>Organophosphate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PTE</td>
<td>Phosphotriesterase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SDM</td>
<td>Site-Directed Mutagenesis</td>
</tr>
<tr>
<td>SOE</td>
<td>Sequence overlap extension</td>
</tr>
<tr>
<td>TB</td>
<td>Terrific broth media</td>
</tr>
<tr>
<td>(T_m)</td>
<td>Melting Temperature</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>(\Delta G)</td>
<td>Gibbs free energy of binding</td>
</tr>
<tr>
<td>(\Delta H)</td>
<td>Enthalpy</td>
</tr>
<tr>
<td>(\Delta S)</td>
<td>Entropy</td>
</tr>
</tbody>
</table>

xii
INTRODUCTION

Studies of Metal Binding in Binuclear Metallohydrolases

1.1. Preface

The aim of this thesis was to gain a better understanding of metal coordination in binuclear metallohydrolases (BNM). These enzymes form a structurally diverse group whose members have a variety of functions. Although a great deal is known about BNM, the details of how they function are still subject to a great deal of controversy.

It was a belief of the candidate that a better characterization and understanding of the metal coordination of the BNM would resolve some of the controversy surrounding them and lead to a better understanding of their functions. More specifically the aim of this thesis was to determine the role of specific amino acids in metal binding — first and second coordination sphere residues. It was hoped that this could be achieved by obtaining binding constants for the two metals in the native and mutant forms of the proteins. In addition, by looking at the activity of the enzyme as a function of metal concentration it was thought that it would be possible to
gain a better understanding of the metal binding as reflected in terms of activity – were two metals required for activity or could partial activity be achieved with a single metal.

This introduction includes a review of metals in biology and more specifically as they are found in proteins. This includes some background on the structure and function of mono- as well as binuclear enzymes. The enzyme selected for this study will also be briefly reviewed.

The binuclear hydrolases (BNHs) were too numerous to allow the proposed experiments to be carried with all of them so that a choice of candidates had to be made. The two enzymes chosen for this study were already being studied in the Ollis laboratory. This choice was in part for convenience - they were easily expressed purified and assayed. They were also typical of the BNMs and studies with them would be relevant to many other enzymes. The more important techniques used in this study are reviewed and finally the specific aims of the thesis are covered in the latter part of the introduction.

1.2. Metals in Biological Systems – Function and Importance

Although metals constitute a small fraction of cellular mass, they are nonetheless essential component. Clearly, a better understanding of how metals function in the biosphere would be useful in terms of dealing with a variety of issues – health and environmentally related issues as well as industrial applications. Some metals are extremely common in bio-systems, sodium and potassium for example while others are rare, but still essential, cobalt being one such. Not all metals are essential for life; in fact a number are toxic – mercury, for example. The interaction of metals with other molecules is also quite variable – some form labile complexes while others form stable complexes. It is not possible to study all aspects of metal chemistry in the biosphere
- indeed much has already been done in this area. One of the more productive areas of research has been the study of metalloenzymes; metallohydrolases in particular – this area will be the focus of the present work.

This present work builds upon a considerable amount of work dedicated to understanding how metals function in enzymes. Much of the early work dealt with mononuclear enzymes while a great deal of the more recent work has been concerned with enzymes that have two metals in their active sites. The present study was concerned with BNM that utilized transition metals, zinc in particular. Despite their differences, information gained in studies of mononuclear enzymes is useful in understanding the bi-nuclear enzymes. For this reason a short review of metalloenzymes will be given. To place much of the chemistry of metalloenzymes in an appropriate context, reference will also be made to a few metalloproteins that do not have an enzymatic activity – this will serve to highlight some of the important properties of metalloenzymes.

1.3. Metals in Enzymes

The treatment given below will rely a great deal on structural information; specifically the structures of the metalloproteins. This is not because of a bias on the part of the candidate, but because structures generally confirm and summarize much of what has been determined using biochemical methods and frequently add a great deal to our understanding of their function. The research presented herein relies a great deal on published structural data, but does not add to this field.
The question often arises: "why do enzymes have metals?" Enzymes are made of predominantly of simple amino acids that can be adapted to perform a variety of functions. The amino acid side chain interactions stabilize macromolecular structure in a specific conformation to form a biologically active molecule. Remarkably, this macromolecule is dependent on a correct three-dimensional structure to function to be able to bind substrate and subsequently execute the catalysis. However, the chemical adaptability of amino acids does have its limits and a great many proteins appear to recruit metals to aid in their function – in some cases, a metal is essential for function while in others the metal offers an alternative means to the use of amino acids. As a result a significant proportion of proteins can be classified as metalloproteins – estimates range from 30 to 40% of all proteins.

The means by which amino acids can interact with metals has been widely studied. The amino and carboxyl termini as well as the atoms in the peptide bond can coordinate metals but in proteins it is more common for metals to be held in place by the suitably placed side chains of particular amino acids. Most of the coordination links are made with the side chains of histidine and acid residues, but other interactions are possible and a summary of these can be found elsewhere (1). Structural studies of metal coordination in small molecule compounds are of fundamental importance to understand the way metals interact with proteins. However, much of the simple elegance observed in coordination compounds is absent from metalloproteins that have evolved for very specific purposes. Wherever it is possible the proteins themselves should be examined and little reference will be made in this thesis to model complexes of metalloproteins.
As noted above, understanding the structure of metalloproteins have provided a great insight of basic information concerning the protein-metal interactions (2, 3). Focus of many of these studies were on the proteins that contain a single metal (4-6) and many have given a very detailed description of the metal centres (7). More recent studies on binuclear metalloenzymes have also been published (7, 8). However, many of these reviews are too specialized for an introduction to the field. In this chapter a brief overview on metalloproteins and metalloenzymes will be given before discussing binuclear metallohydrolases.

1.3.1. Metalloenzymes: hydrolysis, the most common function

One can justifiably ask why a large fraction of the biochemical literature is devoted to hydrolytic enzymes? The answer to this question really goes to the heart of biology and the molecules responsible for life and helps explain why metalloenzymes appeared quite early in the history of structural biology. Vast arrays of compounds are made in the pursuit of life. Since life is transient and resources are limited, recycling is certainly required. Degradation is usually the first step in the recycling process and this typically involves a hydrolysis reaction. This is the reason why hydrolytic enzymes appeared to be the most abundant class of enzymes found in nature. They are the best characterized according to their kinetic properties and three-dimensional structures. The first enzyme to be structurally characterised was lysozyme and this was followed by the first metalloenzyme. The high-resolution structure of carboxypeptidase A was reported in 1967 (9-11) and since then the structures of numerous other metalloenzymes have been determined. The structure of an enzyme with two active site metals became available in the mid seventies. The structure of Cu / Zn superoxide dismutase was first reported in 1975 (12-14). In this protein the metal atoms are separated by about 6.0 Å and are linked by the side chain of a histidine residue (an arrangement that is not observed in hydrolytic enzymes). The
Cu/Zn superoxide dismutase has an overall structure similar to an immunoglobulin and quite different to that of Mn and Fe containing superoxide dismutases (15-17).

The presence of multiple structural forms of specific enzymes raises questions about how these enzymes evolved over time and how they function. Some of the questions are as follows; how do the structures of the binuclear metalloenzymes relate to other proteins? Are they similar to the mononuclear metalloenzymes with a similar function or are they similar to non-metal containing enzymes? How are the metals of binuclear enzymes accommodated into their protein folds? Is there any similarity between the way metals bind in a binuclear enzyme and the way a single metal binds in mononuclear enzymes? Why do some enzymes have two metals while others have just one? These questions are important for our understanding on how metalloenzymes function. In an attempt to answer these questions, some selected proteins will be used as examples to highlight different aspects of mono and binuclear metalloenzymes. An attempt was made to access the literature for answers to some of these questions as a way of providing background to the present study.

Metalloenzymes are sometimes difficult to study. This is usually due to the variability of the metal binding site in having different coordination numbers and geometries as well as different metal preferences and different combinations of amino acid side chain. In a case where one or both metals bind weakly, the crystal structure of the protein by itself may not give a clear indication of how the metals bind. In some cases metalloenzymes use very specific metals while in other cases the enzyme may function with a variety of metals. This question usually arises especially when proteins are expressed using heterologous systems. For example, the crystal structure of *Escherichia coli* methionine aminopeptidase with a binuclear centre, the
protein was known to be active with either Zn$^{2+}$, Mn$^{2+}$, or Co$^{2+}$ ions. However recent studies have shown that the form of the protein present in some cells contains a single metal (18) and that activity within the cell is due to the presence of Fe$^{2+}$ (19). Accordingly, drawing conclusions from a single structure can be troublesome. Therefore it would be instructive to look at how metals bind to a variety of different proteins to gain some insight into how different metal ligands might bind and how these interactions may differ for mono- and binuclear enzymes.

Apart from enzymes, metals are also found in other types of proteins, for instances they are found in binding proteins as well as in electron transfer proteins. In all these protein metals have a functional role where they participate directly in the activity of the protein. In addition, metals are also known to play a structural role in some proteins. In these cases the metal may be essential for the formation of a correct three-dimensional structure of the macromolecule to become a functional protein, but the metal itself does not participate directly in the biological function. This chapter will briefly discuss a few examples of the role played by metals in non-enzymes followed by a brief review of enzymes with a single metal and finally a few examples of BNMs that were not studied as part of the current work, but which were of some relevance to the work are presented herein.
1.3.2. Role of metals

1.3.2.1. Structural

Metals can serve a structural role in proteins. They were first observed bound to enzymes in locations that were well removed from the active site. Alcohol dehydrogenase for instance has one zinc ion in its active site that is clearly involved in the catalytic activity of the protein. In addition, the second metal bound to the protein serves no other function other than stabilizing the structure of the protein (20-22). It has four cysteine ligands that are close in the linear sequence – separated by 2, 2 and 7 residues (Figure 1.1). This close placement of coordinating ligands suggests that the metal is essential for the formation of a local structural element that might be important for protein folding. The ligands coordinating the metal are arranged in a tetrahedral geometry with the binding pocket that is not assessable to the solvent. This is quite different to what was expected of an enzyme active site. A structural metal is also found in the protease thermolysin, but it is difficult to find such structural metals in other enzymes and therefore for this reason they are probably not of great importance.

Figure 1.1 Alcohol dehydrogenase (PDB entry 8ADH) (23). A. Shows the location of catalytic zinc sites; B. Close-up view of catalytic zinc site near the centre of the molecule; C. Close-up view of the structural zinc binding site. Images not drawn on same scale.
Of considerable importance are the many small domains that require zinc to fold and function – binding double stranded DNA in a site-specific manner. The first of these domains to be identified was the “zinc finger” found in transcription factors that contain repeated copies of the domain. The amino acid sequences had metal ligands (consisting of two cysteine and two histidine residues) conserved in repeated sequences of a transcription factor (24, 25). The classic zinc finger consists of two anti-parallel strands that are followed by an alpha helix. The metal is bound between the strands and the helix by four ligands that are tetrahedrally arranged – a cysteine from each of the strands and two histidines from adjacent turns in the helix (Figure 1.2). The small pieces of peptide that make up these domains would not be expected to fold in the absence of metals. They bind one or two zinc ions and are coordinated mainly by cysteine and histidine residues. Unlike the metal binding sites found in enzymes, structural metals are buried inside of proteins and do not have vacant coordination sites for binding ligands or substrates.

![Figure 1.2. Zinc finger with C2H2 binding coordination (PDB entry 1AIL) (26).](image-url)
1.3.2.2. Electron transfer

Electron-transfer function is usually carried out by iron or copper metalloproteins. Copper utilizes the Cu(I) and Cu(II) oxidation states while iron utilizes the Fe(II) and Fe(III) oxidation states. Copper proteins have been studied in detail as can be found in these references (27, 28).

Plastocyanin is a protein with a single polypeptide chain with type I centre. The copper atom for this metalloprotein is coordinated by 4 ligands that consist of the sulfurs of two cysteine residues, the nitrogen of a histidine imidazole and the sulfur of a methionine (29-32) (Figure 1.3). These groups are arranged in a distorted tetrahedron that is midway between the ideal of Cu(I) and Cu(II) geometry so that the energy barrier between the two oxidation states is minimized in accordance with entatic theory (33). Its blue colour is due to an intense absorption at 600nm that arises from an S (cysteine) $\rightarrow$ Cu(II) charge transfer. The overall structure of the protein is a $\beta$ barrel similar to that found in most other proteins containing a type I centre. Of many proteins that contain other types of Cu centres some contain binuclear centres. In these proteins there are no vacant coordination sites and the metal appears to be chosen because of their ability to be stable in more than one oxidation state (28).

Figure 1.3. Plastocyanin. (PDB entry 1BYO) (34). Figure shows the distorted tetrahedral coordination.
There are numerous iron sulfur proteins that fold in different ways. A “cluster” is formed when the iron is coordinated to the sulfur of cysteine and inorganic sulfur. There are different types of clusters that involve a number of iron atoms. Such a centre can be found in the bacterial protein rubredoxin (35, 36) and consists of a single iron bound by four cysteine thiols that form a distorted tetrahedral environment around the iron and is designated as 1Fe-0S (Figure 1.4). Ferrodoxins however have 2Fe-2S and 4Fe-4S centres (37) (Figure 1.5). The 2Fe-2S centre consists of two iron atoms, each bound to the protein with two cysteine thiols. Two inorganic sulphur atoms bridge the two iron atoms in such a way as to produce a tetrahedral environment around the iron atoms (37). Essentially the function of these proteins is to transfer an electron thus access to the metal by a substrate is not required.

**Figure 1.4.** Rubredoxin. A 1Fe-0S centre (PDB entry 2DSX) (38).
Figure 1.5. A.i) Ferredoxins with 2Fe-2S cluster. (PDB entry 1AWD) (39); A.ii) Close-up view of 2Fe-2S cluster; B.i) Ferredoxins with 4Fe-4S cluster. (PDB entry 11R0) (40); B.ii) Close-up view of 4Fe-4S cluster. Images are not drawn to scale.
1.3.2.3. Transport proteins

A very brief review of oxygen transport proteins will be given as an example of a metal-containing transport protein. The first detailed crystal structure of a protein was that of Myoglobin (41, 42) an oxygen-binding protein found in muscle cells of most animals. It is a monomeric protein and has a similar structure to the β subunit of haemoglobin found in the red blood cells used to transport oxygen (43). The iron in myoglobin and haemoglobin is found in a prosthetic group, protoporphyrin IX. The complex formed by porphyrin and iron is referred to as a heme and it is this entity that is inserted into a crevice of the protein (globin). The heme iron atom is coordinated with four nitrogen atoms of porphyrin and the nitrogen of an imidazole residue of the protein (Figure 1.6). This myoglobin binds oxygen through the 6th coordination site of the iron. This shows that myoglobin is capable of binding a ligand, which is similar to most of the metalloenzymes. It should be noted that the active form of myoglobin possesses iron Fe$^{2+}$ that is usually oxidized to Fe$^{3+}$ during the process of purification and crystallization. This instability usually causes a problem that is common to a number of studies of iron containing enzymes and it probably causes failures in identifying this metal as an active component of a number of enzymes. In myoglobin-oxidized form, a water molecule bound to its sixth coordination site and the structure of this form was initially taken to represent the oxygenated form of the protein. The studies done on this structure gives no clear sign of how the water (or oxygen) might gain entry into the heme pocket. The access to the binding pocket is depending upon the dynamic movements of the protein. This feature of the myoglobin structure is quite different to that of most enzymes in a sense of binding larger substrate and having open active sites for the accessibility to the catalytic residues. Cytochrome P450 molecules for instance, utilize a heme group, but it is bound in an active site that allows ready access to a variety of bulky substrates (44, 45).
Figure 1.6. Myoglobin. (PDB entry 2Z6S) (42). Figure shows the iron atom is coordinated by four nitrogen atoms within the planar haem prosthetic group and assembled orthogonally to the NE2 of residue 93 and a diatomic oxygen molecule.

In addition to myoglobin and hemoglobin, there are at least two other ways to bind and transport oxygen. One of them is Hemocyanins, copper-containing proteins that are found in arthropods. The two copper ions are coordinated by three imidazole nitrogens and are separated by 6.0 Å. The separation of these two copper ions is reduced to about 3.6 Å when oxygen binds between the two copper ions (Figure 1.7) (46). Another oxygen transport systems found in invertebrates are hemerythrins and myohemerythrins, the iron-containing proteins that were first found in marine worms (47). Myohemerythrin is an oxygen storage protein that consists of a single subunit while hemerytherin that is capable of reversibly binding dioxygen contains a number of subunits that have the same structure as myohemerythrin. Myohemerythrin consist a single subunit with four helices. These helices are adjacent and positioned approximately antiparallel for the coordination of the two Fe\(^{2+}\) ions that are linked by carboxylates of two bridging acid residues and a bridging hydroxide as shown in Figure 1.8. In addition, one Fe\(^{2+}\) ion also has three imidazole nitrogen ligands to give a total of six ligands that are arranged in an octahedral manner while the second Fe\(^{2+}\) ion has only two additional imidazole nitrogen ligands.
so there is a vacant coordination site that can accommodate an oxygen molecule. The centres observed in myohemerythrin differ from that found in binuclear metalloenzymes. Metalloenzymes frequently have more than one vacant coordination site on each metal ion for two substrates (one of which is water) to act simultaneously.

Figure 1.7. Hemocyanin. (PDB entry 10XY) (46). Binding of oxygen symmetrically between and equidistant from the two copper atoms effects an allosteric change that brings the copper atom from 6.0 Å to 3.6 Å apart.

Figure 1.8. Myohemerythrin. (PDB entry 2MHR) (48). Figure shows the metal ions are coordinated with two bridging carboxylates and 5 imidazole nitrogen ligands (3 on the right ion, 2 on the left ion).
1.3.2.4. Metals in hydrolytic enzymes

Metals play a number of essential roles in catalysis. In hydrolytic enzymes, metal aid in catalysis through substrate binding or by activation of water molecules prior to attack. Their presence also helps to stabilize the transition states and/or intermediates that may be formed during catalysis. Metal requirement depends upon their function. Some enzymes need a single metal for catalysis, some might need two and some other require the participation of a third metal ion.

Most of our knowledge of protein-metal interactions was gained from studies of mononuclear metallohydrolases. Thus, a brief introduction to enzymes that utilize a single metal will be given so that comparisons can be made with the binuclear metalloenzymes later.

1.3.3. Example of metallohydrolases – function, structure & metal coordination

1.3.3.1. Mononuclear enzymes

i) Carbonic anhydrase

Carbonic anhydrase catalyses the formation of bicarbonate from carbon dioxide. The enzyme has been studied from a structural and mechanistic point of view and a detailed review has been published (49). The zinc ion is bound in the active site by the side chains of three amino acids (Figure 1.9). As common occurrence in many other metal coordinating proteins, two of the three ligands originated from a single β strand. The adjacent amino acids side chains of β strand are located on the opposite sides of the β sheet in which the strand is found. Residues different by two in their sequence number can coordinate a metal ion. The third ligand emanates from a residue located in an adjacent strand. These arrangements (the separation of β strands within β sheet) allow the side chains of residues from adjacent strands to coordinate metals. This feature
is recurring in many of the binuclear proteins. Histidine is a common residue found to coordinate metal in most of carbonic anhydrase variants. However a variety of side chains can be used to coordinate metals in some instances \( (49) \). The coordinating side chains are held in place by hydrogen bonds to other residues (the secondary coordination sphere). In the case of the coordinating histidines in carbonic anhydrase, the imidazole side chains form hydrogen bonds with the peptide backbone of the protein and the side chains of residues containing carboxyl groups. Zinc usually has a tetrahedral coordination sphere and in carbonic anhydrase water occupies the fourth coordination site. Apart from the metal this water molecule is linked to the protein by a hydrogen bond with the side chain of a threonine residue. The pKa of water in bulk solution is about 15. However, for the metal coordinated water in carbonic anhydrase this is reduced to \( \text{ca} \ 7 \) due to the combined affects of the metal coordination and hydrogen bonding \( (50) \). This is clearly shows the importance of the metal in carbonic anhydrase. At physiological pH, the water coordinated to the active site metal is activated for attack (mixture of \( \text{H}_2\text{O} \) and \( \text{OH}^- \)). The protein bind carbon dioxide suitably placed for attack by the coordinated hydroxide. The resulting bicarbonate is also bound to the zinc prior to its displacement by water for another round of catalysis \( (51, 52) \).

**Figure 1.9.** Carbonic anhydrase. (PDB entry 1G3Z) \( (53) \). Figure shows histidine side chains from neighbouring strands coordinating zinc ion.
ii) Carboxypeptidase A

Carboxypeptidase A is an example of a well-studied metalloenzymes. A detailed review on this can be found elsewhere (54). This enzyme has a α/β fold that resembles that of the α/β hydrolase fold enzymes (55, 56). The side chains of three amino acids (two histidine and one aspartic acid) as shown in Figure 1.10, coordinate the metal in the active site. In resting state the metal coordinates a water molecule that was displaced by inhibitors in early structural studies (9). The catalytic mechanism according to early structures suggested the peptide substrate bound to the metal through an aspartic acid residue that was appropriately placed to act as a general base for catalysis. However, later studies with inhibitors that were very similar to the physiological substrate of the enzyme did not interact with the metal and bound in such a way that the metal bound a water molecule that was appropriately placed for attack on a peptide substrate (49). As in carbonic anhydrase, the metal bound water also interacts with the side chain of an amino acid. The changes made to the mechanism of carboxypeptidase A illustrates the difficulty of determining the mode of catalysis from a single structure.

Figure 1.10. Carboxypeptidase A (PDB entry 1M4L).
Staphylococcal nuclease is another example of an enzyme for which the mechanism was problematic. This is a well-studied calcium-dependent enzyme whose structure was determined at high resolution in the presence of an inhibitor. According to the structure, a number of acid residues were claimed to be responsible for calcium ion binding. The mechanism of action based on this structure is quite reasonable and suggested that Asp43 functioned as a general base for attack on the phosphodiester substrate (57). However, recent studies, showed that alteration of this residue had little effects on the activity of the protein (58). Furthermore a thorough examination of the structure showed that the inhibitor was stabilized in the active site of the crystalline protein by interactions with lysine residues of symmetry related molecule (59). Other studies suggested that under some conditions two calcium ions bind to the protein and not one as found in the crystal structure (60). In this case, the resting enzyme the metal ions bind loosely and it is not possible to determine how the enzyme might interact with both metals. Mechanistic studies of binuclear metallohydrolases are also problematic in correctly determining the binding sites of weakly bound metals and interpreting inhibitor structures in terms of catalytic mechanism.
1.3.3.2 Binuclear enzymes

i) Amino peptidase or pitabread fold

There are some binuclear metalloenzymes that catalyse reactions similar to carboxypeptidase A. Carboxypeptidase G2 for instance (Figure 1.11), catalyses the hydrolysis of the C-terminal glutamate moiety from folic acid. The peptide forms two domains; one is a catalytic domain while the other forms a dimer interface (61). The catalytic domain binds two metals with a close resemblance to the catalytic domain of *Aeromonas proteolytica* and bovine lens leucine aminopeptidases. Carboxypeptidase G2 also shares less similar features with other enzymes - sharing similar overall fold with methionine aminopeptidase, amino peptidase P and a prolidase. All of these enzymes act on similar substrates. Leucine aminopeptidase and methionine aminopeptidase for instance, cleave N-terminal residues from peptides while prolidase cleave dipeptides that contain a proline residue. A less stringent structural similarity between carboxypeptidase A and leucine amino peptidase has been reported (62). In this case, they differ in detailed connectivity of the secondary structure but have the same number of strands and location of the active site.

![Figure 1.11. The catalytic domain of carboxypeptidase G2. (PDB entry 1CG2) (61). This enzyme is an achetypical aminopeptidase enzyme. The geometry of the metal coordination can be described as distorted tetrahedral.](image-url)
The metals utilized by the binuclear hydrolases are differing from one to another. For example, leucine aminopeptidase requires zinc ions while the prolidase functions with manganese ions. These proteins have similar ligands for metal binding. The two metals of these proteins are bridged with the side chain of an acid residue and a water molecule (μ-hydroxide). In addition, the two metals are also coordinated with the side chains of acid and histidine residues.

ii) Arginase-like enzymes

Arginase plays a role in the urea cycle. It catalyses the hydrolysis of L-arginine to form L-ornithine. The first arginase structure was obtained with crystals of the rat enzyme. This structure has many similar features with other arginase enzymes and other types of enzymes. Arginase shares a very similar fold to the Rossman fold. It has eight parallel strands (strand order 21387456) while the classic Rossman fold has six strands (strand order 321456) (63). Both stands are connected by α helices that cover most of the exterior surface of the molecules. The arginase subunits aggregate to form homotrimers of about 105 kD.

Arginase is a binuclear metalloenzyme, with two manganese ions. According to the crystal structure of arginase, the two Mn$^{2+}$ ions are separated by approximately 3.3 Å with μ-hydroxide and the side chains of two aspartic acid residues bridge the two metal ions together. A histidine side chain and a non-bridging aspartic acid side chain have also helps in the metal coordination. In its resting state metal α has a bound hydroxide ion. The μ-hydroxide is predicted to function as the catalytic nucleophile (Figure 1.12) (64).
iii) The amidohydrolase fold

Amidohydrolases superfamily forms a large family of metalloenzymes that includes mononuclear and binuclear metallohydrolases. They are a type of hydrolase that acts upon amides or related compounds. All of the proteins in this superfamily have a common overall fold that is \((\alpha/\beta)_1\) or TIM barrel. This fold involved an alternating \(\alpha/\beta\) motif with the first \(\beta\) strands forms a single twisted \(\beta\)-sheet and last strands are linked by hydrogen bonds to form a barrel like structure. \(\alpha\) helices form the outside surface of the barrel while the \(\beta\) strands form the inside of the barrel. The active site crevice is located at the C-terminal end of the interior of the barrel and is readily accessible to substrates (66).
Amidohydrolase enzyme can be classified into three sub-families according to the number and type of metal(s) they possessed and the location as well as the type of coordinating residues. Representative structures are shown in Figure 1.13. Urase, dihydrooratase and phosphotriesterases are proteins that are classified under type 1. Enzymes classified under this class posses 2 metals designated as α and β that are coordinated with the same coordinating group. The α site is coordinated by the side chains of two histidines from strand 1 and an aspartic acid side chain from strand 8. Two histidines side chains from strands 5 and 6 coordinate the second metal. The two metals are bridged by a μ-hydroxide and a carboxylated lysine (67).

Two variants of phosphotriesterase have been characterized; PTE enzyme that is from *Pseudomonas diminuta* and OPDA from *Agrobacterium radiobacter*. These enzymes belong to the binuclear metallohydrolase family and share high sequence (90 % identity at amino acid level) and structural homology. The two most significant amino acid sequence differences between OpdA and OPH are (i) 20 additional amino acids at the C-terminus of OpdA, which appear to be irrelevant for catalysis and (ii) variation of three amino acids located in the substrate-binding pocket (Arg^{254}/His^{254}, Tyr^{257}/His^{257} and Phe^{272}/Leu^{272} in OpdA/OPH) (Figure 1.13a). These sequence differences are thought to be responsible for the variation between the substrate specificities of OPH and OPDA as well as the stability of the metal ions (68).

The variability in stability of the metal ions between these two proteins can be examined through their activity. The activity of PTE protein falls off rapidly with time, which can be restored by the addition of metal ions. In addition, a single mutation in the PTE gene – His254Arg – causes a dramatic decrease in the rate at which metal is leached from the protein.
The residue at position 254 does not coordinate the metals, however it is important in stabilizing the bound metals. Nevertheless, the behaviour of OPDA is quite different to PTE. It has an arginine at position 254 and does not show a reduction in activity in the absence of excess metals. However it is different for mutants of OPDA, which have been found to show a rapid decay of activity with time that can be regained by the addition of metals. These unstable variants contain a number of mutations that are found on loops that contain residues responsible for metal binding. Studies on the structure of the mutant protein found to exhibit high thermal parameters in the loops containing these mutations. This observation suggests that labile metal binding sites may result if coordinating peptides are not held firmly in place (69).

There are two alternative proposals to account for phosphotriesterases enzymatic mechanism where one involves the bridging hydroxide to attacks the substrate (70) while the alternative mechanism involves the attacking water to bound to the $\alpha$ metal and is activated by the combined effects of the metal and the bridging hydroxide (71). This latter proposal is supported by the structure of the protein with a poor (slow) substrate trapped in the active site by freezing (72). However, more studies are needed to clarify the catalytic mechanism.

Type II amidohydrolases subfamily has a member that is referred to as phosphotriesterase homology protein and was identified by the similarity of its sequence to PTE. Despite of the significant sequence similarity, this protein exhibits no phosphotriesterase activity. According to its structure, this protein possesses two metals in the active site. The two metals have the same coordinating ligands except one bridging ligand where the bridging carboxylated lysine is replaced by a glutamate side chain. The glutamate side chain is shorter than the carboxylated lysine. The difference in length is compensating by the addition of an additional residue to the
strand so that the carboxylate side chain can bridge the two metals (73). The type II metal binding site, shown in Figure 1.13b, may be an ancestor of that found in the type I enzymes. However, why the carboxylated side chain replaced the much more stable and readily available glutamic acid is not clear.

The type III amidohydrolases enzymes on the other hand contain a single metal that binds in the α site (74-76) (Figure 1.13c). There is no bridging ligand and the lysine residue found on strand 4 of the type I enzymes found in these enzymes. However, other residues that coordinate the β metal in the type I enzymes are conserved in the type III enzyme. In one case, a histidine on strand 4 shows different conformation to the type I enzyme and coordinates the metal in the α site while a histidine on strand 6 is thought to act as a general base during catalysis. It would appear that a catalytic residue in one protein changes during evolution to become a metal ligand in another.

In summary, the overall structures of the Type I and Type III enzymes are similar. They have a number of similar amino acids in their active sites with different functions. The basis of the evolutionary relation of these two protein classes is uncertain, but the similarity of these proteins suggests that there is an evolutionary connection.
Figure 1.13. TIM barrels of Amidohydrolase fold. A. Type I phosphotriesterase (PDB entry 1HZY) (77), coordination by three histidine and two aspartate residues. The two metal ions are bridged by a μ-hydroxide and carboxylated lysine; B. Type II PTE homology (PDB entry 1BF6) (78), showing metal coordination similar to the Type I proteins but the carboxylated lysine is replaced by a glutamate and there is no bridging μ-hydroxide; C. Type III (cytosine deaminase (PDB entry 1K6W) (76), a single ion is bound by histidine and aspartate side chains.
iv) **α/β sandwich proteins or the α/β/β/α fold**

There are many ways in which α/β units can be arranged. DNAse I where the structure obtained in early 1980s exhibited a fold similar to that of TIM barrel with the α helices on the exterior surface of the protein and β sheets on the interior. The sheets were twisted in the usual manner and packed against each other, but did not bend around to form a single closed sheet (79). A zinc binding site was located in between these two sheets. The catalytic domain of *E.coli* exonuclease III later was observed to have a similar structure (80, 81). This basic architecture α/β/β/α was later found to support the binuclear centres for the catalysis of hydrolysis reactions.

Glycerophosphodiesterase from *Enterobacter aerogenes* (GpdQ) is belongs to the diverse family of binuclear metallohydrolases (82). The gene for the GpdQ protein was isolated from a bacterial operon used to import and degrade glycerolphophodiesters with the GpdQ protein responsible for the hydrolysis of the phosphor-diester substrate (83). The structure of the protein was determined (84) and is shown in the Figure 1.14. The location of the metal binding residues and how the secondary structure elements are connected is shown in Figure 1.15. There are a number of active site residues that can be found in the related proteins:

\[ D(8^*) \times H(10^*) (x)^n G(49)D(50^*) (x)^n G(79)N(80^*)H(81)D/E(82). \]

Residues marked with a * coordinate metals. Residues Asp8 and His10 located at the end of strand 1 while Asp50 emanates from strand 2 and links the two metals. Glycine residues at positions 49 and 79 are necessary for sharp turns that position the metal coordinating residues in the right orientation. Based on structural comparison study, this combination of residues can be used to identify similar folds in other proteins such as Mre11 protein, purple acid phosphatase (PAP), 5' nucleotidase and the Ser/Thr protein phosphatases (85). However, it should be stressed that the binding site is one that is conserved in this protein with a
considerable variation in their overall structures and variations in the residues responsible for metal coordination. For example, in the purple acid phosphatase protein a tyrosine replaces one of the histidine residues gives an intense purple colour of the protein (86). In addition to a number of distantly related proteins there is one protein that has been observed to have a very similar structure to that of GpdQ – the Rv0805 protein from *Mycobacterium tuberculosis* – a 3', 5' cyclic nucleotide phosphodiesterase (87). These proteins are having similar catalytic domains and both aggregate to form closely associated dimers by means of a second domain that has only been observed in these two proteins.

Figure 1.14. Figure shows metal coordination in GpdQ (PDB entry 3D03) (88).
1.3.4. Common features of metalloproteins

As is apparent from earlier discussion the structures of binuclear metallohydrolase can usually be placed in structural superfamilies with other metalloproteins that have a single metal and / or no metal. This is also depicted in oxygen binding proteins where at least three structural families and three different metal coordination systems appear to have evolved with time. In this case, binuclear proteins are one example but not the most efficient and it is not surprising that the presence of binuclear proteins in oxygen transport is restricted to a few relatively primitive organisms. Higher organism on the other hand use a heme to hold the oxygen binding metal in place, which offers a better way to bind and transport oxygen. The presence of binuclear metalloenzymes must offer some advantages to their hosts, given the enormous number of proteins with diverse functions that make up this class, yet the nature of that
advantage is uncertain. A detail description of how these metalloproteins evolved is currently not available, but this may be change given the rapidly growing number of sequence and structural studies.

1.4. Enzymes to be studied

There are other binuclear metalloenzymes that have an overall $\alpha/\beta/\beta/\alpha$ structure that was described above. One of them is the enzyme to be studied in this project, methyl parathion hydrolase (MPH).

1.4.1. MPH - function, structure and metal coordination

MPH belongs to a class of enzymes that are usually referred to as phosphotriesterases – they catalyse the hydrolysis of organophosphate pesticides and nerve agents. Given the medical and environmental problems associated with pesticides, it is not surprising that there has been considerable interest in the enzymes used by organisms to degrade them. MPH was first identified in the bacteria *Pseudomonas* sp and was later found in other organisms (89). The sequences for the gene of various MPH along with one X-ray structure have been published so that engineering studies could be initiated. As noted earlier, the aim of the present study was to gain a better understanding of the metal coordination in binuclear metallohydrolases – with MPH being one of the enzymes to be studied. The aspects of MPH that were relevant to the proposed study were 1) the reaction catalysed by MPH, 2) the overall structure of MPH and 3) the metal coordination of MPH.
1.4.1.1. Reaction catalysed by MPH

As noted above, MPH acts on organophosphate pesticides. These are a class of pesticides (and more generally nerve agents) that are tri-esters of phosphoric, phosphonic and phosphinic acid and sulphur containing analogues (90). Figure 1.16 gives the structure of a typical OP that is the preferred substrate of MPH – methyl parathion. The reaction catalysed by MPH is also shown in (Figure 1.17). One product of this reaction is nitrophenolate – a good leaving group with a distinctive colour that can be used for high throughput assays.

Figure 1.16. A. General structural formula of organophosphate. R1 and R2 are alkyl-, alkoxy-, alkylthio- or amido group (91). X is the acyl residue (labile fluorine-, cyano-, substituted or branched aliphatic, aromatic or heterocyclic groups; B. Methyl parathion.

Methyl parathion

Figure 1.17. Schematic diagram of MPH chemical reaction
1.4.1.2. Overall structure of MPH

MPH was found to be a dimer with 2-fold related subunits that were said to have a β-lactamase fold – that is it was similar to the metallo-β-lactamases from *B. fragilis* and *S. maltophilia* and the β-lactamase domains of rubredoxin:oxygen oxidoreductase and human glyoxalase II and a number of other proteins. The β-lactamase fold was described as an αβ/βα-sandwich with three flanking helices and two internal mixed β-sheets (Figure 1.18). The active site was between the two β sheets with the metal binding ligands emanating from loops at the C-terminal end of the strands (90, 92).

![Figure 1.18. Figures show MBL superfamily that shares similar α/β/β/α fold. Prepared with PyMOL. A. MPH (PDB entry 1P9E); B. AIM-1 (PDB entry 4AWY).](image)

1.4.1.3. Metal coordination

A diagram showing the metal coordination of MPH is shown in Figure 1.19. Chemical analysis showed that MPH occurred with Zinc bound when purified after heterologous expression in *E. coli*. However, the crystal structure had two different metals in the active site in one subunit – it is appeared that Cd$^{2+}$ was essential for crystallization and the Cd$^{2+}$ replaced a Zn$^{2+}$ ion in the crystallization medium (90). It is thought that this is a crystallization artefact and that the cell...
enzyme takes up the much more abundant $\text{Zn}^{2+}$. The coordination spheres of both metals were octahedral with a water molecule and Asp255 bridging the two metals. Asp151, His152 and His302 bound the $\alpha$-metal while His147, His149, His234 and terminal water formed coordinate links to the $\beta$-metal. The residues lining the active site were hydrophobic and included Leu65, Leu67, Phe119, Trp179, Phe196, Leu273 and Leu258 (89). Also lining the active site were the aromatic residues Phe119, Trp179 and Phe196.

Figure 1.19. MPH (PDB entry 1P9E) The purple spheres represent two $\text{Zn}^{2+}$ ions.
1.5. Techniques to be used

1.5.1. Isothermal Titration Calorimetry (ITC)

Isothermal titration calorimetry (ITC) is used to directly characterize the thermodynamics of macromolecular binding interactions. This is a technique capable of quantifying binding constant \((K_b)\) and Gibbs free energy of binding \((\Delta G)\) as well as enthalpy \((\Delta H)\) and entropy \((\Delta S)\) \((97)\). The aim in calorimetric titration experiment is essentially to generate a binding isotherm, a curve that represents the degree of saturation in terms of ligand concentration.

ITC experiments are performed by a stepwise titration of a reactant into a sample solution containing the macromolecule necessary for reaction. The heat released or absorbed that is related to the enthalpy and progress of reaction is monitored calorimetrically. In ITC experiment, the colorimeter sample cell is controlled at a constant temperature (operating on heat compensation principle). Any heat response from the chemical reaction will be sensed and the power applied to the control heater will compensate the heat changes in order to keep the temperature difference zero during the course of the titration. The heat change is calculated by integrating the heater power (microcalories) over time (sec) or more specifically the time required for the control heater power to return to a baseline value \((98)\). A typical schematic diagram of ITC is shown in Figure 1.20.

The titration will continue until the results in peaks become smaller, which signify as the macromolecule become saturated. After saturation, the peak sizes remain constant, which give only the heats of dilution. This needs to be subtracted from all injection peaks before analysis. Ultimately the thermodynamic analysis of the observed heat effects permits quantitative characterisation of the energetic processes associated with the binding reaction \((99)\).
Figure 1.20. Schematic diagram of an ITC instrument. The ligand is titrated into the sample cell. Exothermic reaction releases heat and gives negative peaks. The peaks are integrated and the integration data will be used for curve fitting.

1.5.2. Ligand binding – equations

*Generation of stimulated calorimetry profiles*

Evaluation of calorimetric titration data requires the consideration of the extent of heat evolved or absorbed (the interaction of a ligand (X) with a protein (M) bearing n sites for X) as a function of the amount of bound ligand.
The differential heat change ($dQ$) created by each injection is associated with the formation of corresponding amount of complex; the difference in the concentration of bound ligand, the difference between total ([X]$_{tot}$) and free ([X]) ligand concentrations (100). For each injection, the heat released or absorbed is given by:

$$dQ = d([X]_{tot} - [X])VnAH^0$$

where $V$ is the cell volume and $nAH^0$ is the product of reaction stoichiometry ($n$) and the molar enthalpy of binding ($AH^0$) (100). $dQ$ and $d([X]_{tot} - [X])$ are replaced by their difference counterparts $\delta Q$ and $\delta([X]_{tot} - [X])$. This is due to the incremental amount of heat associated with the change in amount of ligand bound during each aliquot of ligand added. Equation 1 can be rewritten in terms of total ligand concentration that is:

$$\frac{\delta Q}{\delta([X]_{tot} - [X])V} = \frac{\Delta Q}{\Delta([X]_{tot} - [X])V} = nAH^0$$

In a binding isotherm curve, $\Delta Q/\Delta([X]_{tot} - [X])V$ is plotted against $[X]_{tot}/[M]_{tot}$. This depends largely upon the dependence of $([X]_{tot} - [X])$ upon $[X]_{tot}$ in a mixture where $[M]_{tot}$ is gradually decreases due the addition of concentrated ligand solution. For simulation purposes, the determination of [X] in the mixture is required which can be used for various models of the protein-protein interaction. This can be done with a defined $[X]_{tot}$ and a prescribed total acceptor concentration $[M]_{tot}$. 

36
Although MPH is a dimer protein with four metal binding site, the symmetrical dimeric structure posse by MPH simplifies the calculation by expressing $[M]_{tot}$ as the subunit concentration. Therefore the binding-sites for ligand now is (n=2) which the two sites exhibit equivalence and independence in their interactions with the metal ions (X).

**Generation of calorimetry patterns for an acceptor with two equivalent and independent sites**

For an equilibrium mixture of a bivalent acceptor and ligand with respective total concentrations $[M]_{tot}$ and $[X]_{tot}$:

$$[M]_{tot} = [M] + [MX] + [MX_2] = [M] + 2K[M][X] + K^2[M][X]^2 \quad [3a]$$

$$[X]_{tot} = [X] + [MX] + 2[MX_2] = [M] + 2K[M][X] + 2K^2[M][X]^2 \quad [3b]$$

Where $K$ is the intrinsic binding constant (101) describing the interaction of ligand with the two equivalent and independent sites on acceptor. The concentration of $[MX]$ is doubled due to the two equivalent ways of binding the first ligand molecule (-M-X and X-M-), but there is only a single saturated complex (X-M-X), irrespective of the order in which the two ligand molecules are attached.

Following this, equation 3b can be simplified as

$$([X]_{tot} - [X]) = 2K[M][X](1 + K[X]) \quad [4a]$$

Whilst the free acceptor concentration $[M]$ can be eliminated from (Eq. 3a)
\[ [M]_{\text{tot}} = [M](1 + K[X])^2 \]

Combination of equation 4a and 4b gives

\[ ([X]_{\text{tot}} - [X]) = \frac{2K[X][M]_{\text{tot}}}{(1 + K[X])} \]

Which can be presented as the following quadratic in \([X]\)

\[ K[X]^2 + (1 + K[2[M]_{\text{tot}} - [X]_{\text{tot}}])[X] - [X]_{\text{tot}} = 0 \]

Subsequently becomes

\[ [X] = \frac{-[1 + K(2[M]_{\text{tot}} - [X]_{\text{tot}})] + \sqrt{[1 + K(2[M]_{\text{tot}} - [X]_{\text{tot}})]^2 + 4K[X]_{\text{tot}}}}{2K} \]

The combination of the \([X]\) value for each \(([X]_{\text{tot}}, [M]_{\text{tot}})\) allows the calculation of \(([X]_{\text{tot}} - [X])\) for an assigned value of \(K\). Hence, the differenc between this quantity for successive steps divided by the corresponding difference in total ligand concentration: successive estimates of the ordinate parameter of the calorimetry plot, \(\Delta Q(T)\Delta[X]_{\text{tot}}\), are then obtained as the product of those values of \(\Delta([X]_{\text{tot}} - [X])\Delta[X]_{\text{tot}}\) for each ligand addition and assigned magnitude for \(n\Delta H^0\)
Referring to the location of the initial transition in heat release at stoichiometric ratio of unity, the MPH binding favours interpretation of the result in terms of site heterogeneity rather than negative cooperativity of equivalent sites. A non-integer value of the experimental transition \([X]_{\text{tot}}/[M]_{\text{tot}}\) \((n_1=0.96; \ n_2=0.7)\) explains the reason of the system not attributed as a cooperative binding.

### 1.6. Protein stability

To assess the change in thermal stability of the MPH variants constructed throughout the course of this work, a thermal denaturation assay was used and melting temperatures were extracted from the denaturation curves. The fluorophore SYPRO Orange (Sigma-Aldrich) binds to the exposed hydrophobic regions of protein upon unfolding. The fluorescence of SYPRO orange is quenched in aqueous solution but upon binding to the hydrophobic regions of protein the fluorescence signal increases and as such can be used to report the folded state of the protein. The melting temperature can be extracted from the data by fitting of the Boltzmann equation to the transition region.
1.7. Aims

Studies on metalloproteins have provided a great deal of information concerning protein-metal interactions. They not only provide a better understanding of metal ions coordination, but also facilitate to gain a better understanding of the essential aspects of catalysis. Besides, this protein-metal interaction studies also provide structural information, which is sometimes important for understanding the function. This project aims to monitor MPH-metal interactions in order to gain some insight on the physicochemical principles governing the protein-metal recognition. Essentially, this project intends to study the importance of the divalent cations and the interaction of those metals to MPH as well as studying the binding strength that could be altered for function. This would certainly involve the alteration of the active site. The specific aims of this study are as follows:

1) To study the influence on metal coordination of the chemical nature and location of the primary and secondary coordinating groups in the active site. Altering these residues is expected to modulate the catalytic function and the reaction mechanism of the protein.

2) To determine the dissociation constants of the metal ions of MPH wild type and its variants.

3) To examine the effect of metal binding on the overall stability of MPH.
1.8. References


Comparisons with the Manganese Enzyme from Thermus-Thermophilus, Biochemistry 34, 1646-1660.


33. Williams, R. J. P. (1971) Entatic State, Cold Spring Harb Sym 36, 53-+


binding of fluoroaromatic inhibitors to carbonic anhydrase II, *Journal of the American Chemical Society* 122, 12125-12134.


89. XY, C., J, T., NF, W., and YL, F. - An intramolecular disulfide bond is required for the thermostability of methyl, *Published*.

MATERIALS & METHODS

2.1. Preface

This chapter discusses all materials and methods used in this project. Techniques used for DNA and protein manipulations are discussed in the earlier part of this chapter. The experimental details involving instrumental techniques like the ICP and ITC are discussed in subsequent sections. Detailed information on suppliers and equipment, kits, chemicals and reagents used in this project are listed in Appendix A.

Distilled water used in this study was purified with a MilliQ reagent system. For molecular biology work, all glassware, microcentrifuge tubes, micropipette tips, growth media, buffers and solutions were sterilized by autoclaving at 121°C for 15 minutes using ASB270BT autoclave machine (Astell Scientific).
2.2. Bacterial Strains and their Growth

Techniques used for BL21 strain handling are described below. Detailed information regarding the recipes for the media and buffers used in this study are presented in Appendix B. Sterile technique on regular laboratory benches was undertaken when handling cultures, and whenever required, a lamina-flow hood was used to prepare agar plates. Most of the methods used were following the procedure obtained from the laboratory manual for cloning by Sambrook (1).

2.2.1. Strains

*E. coli* strain, BL21(DE3) recA' was used throughout this project with the purpose of producing high-level protein expression. This strain is using T7 regulation vectors with λ-lysogen DE3 gene encoding for T7 RNA polymerase, which will be used to transcribe the targeted gene. The T7 RNA polymerase expression is controlled by IPTG-inducible lac UV5 promoter (2). This strain lacks of lom protease and the ompT outer membrane protease that might degrade the target protein during purification (3).

2.2.2. Growth media and bacterial storage

Terrific broth medium (TB) was used for most of the culturing purposes, which includes growing cells for purification. Luria bertani (LB) media and agar was used to culture cells for the purpose of DNA cloning. Terrific broth (TB) medium was used rather than LB for protein expression since it is nutritionally rich media that supports high density cell growth.

Working cultures were stored on LB agar supplemented with ampicillin at 4°C (for less than a fortnight use). For long-term storage, cultures were stored with 20 % glycerol. An overnight culture (80 %) was used for this purpose and was snapped-frozen prior storage at -70°C. This stock culture was recovered by culturing approximately 10 µl of the stock culture onto LBA-
agar. The colonies were ready for subsequent manipulation after 24 hours of incubation at 37°C.

2.2.3. Preparation of electrocompetent cells

A single colony of BL21 was inoculated into 10 ml YENB broth and was incubated overnight at 37°C. As much as 8 ml of the overnight culture was added into a fresh 800 ml YENB and was incubated for 2 hours and 50 minutes to reach OD_{600} 0.35-0.4. The culture was then rapidly cooled to 4°C and the temperature was maintained at 4°C throughout handling. After cooling for 1 hour, the culture was spun at 5,000 rpm (VWR R9A rotor) for 10 minutes. The supernatant was discarded and the cell pellet was resuspended gently in a pre-cooled 300 mL of dH2O. This washing step was repeated for another round before washing with 10 % glycerol. After washing, 1 ml of 10 % glycerol was used for pellet resuspension prior storage in 50 µl aliquots. The cells were kept frozen at -80°C until needed. Control plates were prepared for a contamination check and some aliquots were used to transform pETMCS1M1T plasmid for a competency check.

2.2.4. E.coli transformation

The exogenous DNA (DNA plasmid) that has been purified using PCR purification kit (Qiagen) was transformed into E.coli competent cell by electroporation using MicroPulser™ (Bio-Rad). 0.2 mm electrode-cuvettes were used with setting "EC2" and were performed following the manufacturer's guidelines. Approximately 1 µl of DNA was transformed into 49 µl of competent cells. The mixture was exposed to 2.5 kV within 5 ms prior resuspending in 1 ml of YENB media. The transformant was then incubated for 1 hour at 37°C then was plated on LB agar in the presence of ampicillin.
2.3. DNA Manipulations

2.3.1. Plasmid

T7 pET vector was generated by Studier (4). Target gene was cloned into the MCS of pETMCSI vector; a vector that was constructed by Neylon and co-workers (5). This vector was from the courtesy of Professor Nick Dixon from the University of Wollongong. NdeI restriction site has been introduced into the multiple-cloning site, downstream of the RBS. This vector lacks the lacI repressor gene that allows leaky expression to occur. pETMCSI carries ampicillin resistance for the host.

2.3.2. Plasmid extraction and purification

As much as 5 - 10 ml overnight culture was used for plasmid isolation. Plasmid DNA was isolated using QIAprep Spin Miniprep kit from Qiagen following the manufacturer’s instruction.

DNA purification was done using DNA gel extraction / DNA PCR purification kit using Wizard® SV Gel and PCR Clean-Up System from Promega. The clean-up process was done according the protocol provided by the manufacturer.

2.3.3. DNA visualization

DNA analysis (size determination) was performed with horizontal 1 % agarose (Sigma) gels in a SB-buffered system, supplemented with 1x RedSafe™ DNA Stain. Gels were casted onto a thin glass plate sized 125 mm wide x 80 mm long.
DNA sample was prepared by adding bromophenol blue loading dye at a ratio of 6:1. The DNA electrophoresis was performed at 150 V and 300 mA and was ran for about half an hour. After DNA separation, the DNA was visualized using gel doc, an electronic imaging using UVI Pro system (UVI Tec) or by using UV transluminator. The DNA size was estimated by comparing the DNA sample with the 1 kb ladder (NEB).

2.3.4. DNA quantification

The DNA concentration was determined using NanoDrop® ND-1000 spectrophotometer at absorption 260 nm. $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios were read for any protein and carbohydrate contaminations.

2.3.5. DNA amplification

i) Basic Polymerase chain reaction

Desired genes were amplified by polymerase chain reaction (PCR). Thermocycler used was MJ Mini Personal Thermal Cycler (Bio-rad). PCRs reagents used were 0.5 μl (of stock solution) of Phusion® polymerase (Finnzyme; NEB), 10 nmole of dNTPs (Roche) and 5x Phusion® HF buffer. A total of 50 μl PCR mixture was prepared for each reaction that included 1 μl of miniprep DNA and 5 μl of 10 pmole of forward and reverse specific primers. The reaction was set for 30 cycles of 98°C for 2 mins; 55°C for 30 s and 72°C for 15 s. Final extension was performed at 72°C for 5 mins.
ii) Site-directed mutagenesis Polymerase chain reaction

*Taq* DNA Polymerase (0.25 μl of 1 U/μL, Roche) and polymerase buffer (5 μl of 10x) were used in replace of Phusion® polymerase and Phusion® HF buffer for colony PCR DNA amplification, respectively. The mix for other reagents was similar as described above. For colony PCR, the reaction was set for 35 cycles of 94°C for 30 s, 55°C for 1 min, 68°C of 1 min and 7 min at 72°C.

2.3.6. Restriction digest

Most of the nuclease restriction enzymes were obtained from New England Biolabs (NEB). The restriction enzymes were used at 20 U/μl with 2 μg of DNA, 1x bovine serum albumin and 1x of supplied buffer in 100 μl of total reaction mixture. The mixture was incubated for 3 hours at 37°C prior heat inactivation at 65°C for 20 minutes. Alkaline phosphatase treatment was done on digested vector plasmid to prevent self-ligation of the vector by incubating the vector with 10 U of calf intestinal alkaline phosphatase (CIP, NEB) at 37°C for 1 hour. All products were gel excised and purified according to method described in Section 2.3.2.

2.3.7. DNA ligation

T4 ligase used in this work was supplied by Thermo Scientific, Fermentas and NEB. As much as 1.5 U of T4 ligase was used at 1:1 molar ratio of insert:vector. The reaction mixture was incubated overnight at ≈ 14°C and was purified the following day (see Section 2.3.2 for purification method used).
2.3.8. DNA sequencing

DNA sequencing was done by ACRF Biomolecular Resource Facility (BRF), The John Curtin School of Medical Research (JCSMR), Australian National University. Samples were prepared independently as recommended by BRF. As much as 150-300 ng DNA sample were mixed with 3.2 pmol primer, 1 µL of BigDye® Terminator and 1x of sequencing buffer (provided by BRF) in a final volume of 20 µl. The thermocycling was performed with 30 – 50 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 mins. The products were ethanol precipitated according to the protocol given (BRF). The products were mixed with 62.5 µL of 95 % ethanol, 3 µL of 3 M sodium acetate pH 4.6 and 14.5 µL of distilled water and were left to stand for 15 mins at room temperature. The mixture was spun for 30 mins and the supernatant was then removed as much as possible (discarded by tipping inverted plate and the plate was centrifuged up side down at 300 rpm for 1 min). The pellet was then washed with 200 µL of freshly prepared 70 % ethanol and was centrifuged for 10 mins. Again, the supernatant was removed, and the sample was air-dried for approximately 30 mins before being sent to BRF for sequencing. DNA sequences were analysed using Sequencher 4.10.1 (Gene Codes Corp).
2.3.9. Site directed mutagenesis

i) Primer designs

The primers pair was synthesized with a mismatched random nucleotide sequence in the middle of a target sequence. Primers were synthesised on 25 n mole scale with 30 bases by GeneWorks or Integrated DNA Technologies (IDT). A list of primers that been used in this studies were listed in Appendix C.

ii) Sequence Overlap Extension (SOE)

Sequence overlap extension (SOE) method was adopted to mutate the desired amino acids. In this method, two separate PCR steps were required to amplify two DNA fragments that were overlap at specific codon in a target sequence. Each primer pair was designed to flank both sides that specifically anneal to the target region. After an initial PCR amplification, another PCR reaction was used to reassemble a new version of the original full-length sequence (Figure 2.1) (6, 7). PCRs were performed under standard conditions using Phusion® DNA polymerase (Section 2.3.5 (ii)). The final product of SOE (full-length sequence) undergone the cloning procedure (Section 2.3.6 & 2.3.7) and was verified through sequencing.
Figure 2.1. Overlap extension PCR involves the usage of two separate sets of primers for the reactions. Each pair of B and C primers was used to introduce a single base change into the wild-type sequence. The first set of primers primer A and primer B were used to make an AB fragment. Whereas primers C and D were the second set of primers that were used to a CD fragment. The two resulting fragments were then combined in the third PCR where primers A and D were used to amplify the A and D fragments (6).
2.4. Protein production

2.4.1. Protein expression

The MPH protein was expressed and purified using ion exchange chromatography. WBC3-MPH was expressed in *E. coli* BL21(DE3) 

A 10 ml starter culture was inoculated into 1000 ml fresh Terrific broth (TB) containing 50 µg/ml ampicillin and was grown at 37°C for 5 hours OD$_{600}$ of (0.6 - 0.8). The starter culture was prepared a day earlier by inoculating 1 colony of cells into 10 ml fresh TB. A total of 0.2 mM of IPTG was used for overnight induction at 18°C. Cells were harvested by centrifugation at 5,000 rpm in a VWR R9A rotor for 15 minutes. The pellet obtained was stored at -80°C until needed.

2.4.2. Cell lysis

Cell lysis was performed with two different means. Large-scale production was performed by using French Press (French Pressure Cell Press) or sonicator (BMNI sonic ruptor 400). In this method, cells pellet was resuspended in a purification buffer that was 50 mM of MES buffer, pH 5.5 at 7 ml/g. The lysed cell was then pelleted by centrifugation in a VWR R20A2 rotor at 12,000 rpm for 45 minutes.

A smaller-scale production was done with Bugbuster® protein extraction reagent (Novagen) or rLysosome™ (Novagen). This type of cell lysis was done according to the protocol provided. In this method, cell pellet was resuspended in an assay buffer that was 50 mM Tris-HCl, pH 7.0 at 1 g/10 ml. BugBuster® was added to a final concentration of 1x and was let to stand at room temperature for 15 mins and was spun to separate the soluble and insoluble fractions. Lysis with rLysosome™ on the other hand was frozen at -80°C before lysing the resuspended cell at 7.5 kU/ml.
2.4.3. Protein purification

Purification method that has been adopted for this project with some modifications was from Ekkhunnatham and colleagues and Grimsey and colleagues (8, 9). The purification was done using AKTA™ FPLC System. Soluble cell lysate was loaded onto 5 ml SP Sepharose column. A linear NaCl gradient (20 - 35 %) was applied to elute the protein over 3 column volumes and the eluent was collected in 2 ml fractions. The purity of the purification fractions was examined through Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The size (31538.9 Da by ProtParam) was estimated by comparing the protein fraction samples with a low-range protein marker (bio-rad). The targeted protein fractions were combined and buffer was changed to 50 mM of piperazine (pH 10.0). The protein solution was loaded onto a 5 ml Q sepharose column. The proteins were eluted using NaCl gradient (15 - 35 %) over 3 column volumes and were collected in 2 ml fractions. Fractions with the desired products were combined and concentrated prior measuring the concentration calculated using NanoDrop® (Section 2.4.4).
2.4.4. Protein quantification

Protein concentration was determined by measuring the absorbance at $A_{280}$ using NanoDrop®. As much as 2 μl of protein sample was loaded for each measurement. The value obtained from the absorbance reading was used in Beer-Lambert equation (10) that is:

$$A = \varepsilon.l.c$$

Where, $A$: absorbance; $\varepsilon$: molar absorption coefficient (M$^{-1}$cm$^{-1}$); $l$: cell path length (cm); $c$: molar concentration (M). Protein extinction coefficient ($\varepsilon_{280} = 20400$ M$^{-1}$ cm$^{-1}$) was obtained from ProtParam.

The accuracy of the reading was determined by plotting a graph of a few different concentrations (a series of dilution) of the protein on the X-axis and the absorbance measurement on the Y-axis (one of them is as shown in Figure 2.2). Triplicate measurements of each concentration were performed. A best-fit straight line was anticipated, which reflects the accuracy of the reading.

![Figure 2.2](image)

Figure 2.2. Protein concentration accuracy test. Concentration by $A_{280}$ absorbance as a function of [protein] (differ by dilution).
2.5. Molecular weight determination

Protein molecular weight was determined by using size exclusion chromatography. Superdex 200 size exclusion column was calibrated with commercial protein standards with known molecular weights from Gel Filtration Markers Kit for Protein Molecular Weights (Sigma). Calibration curve was prepared by measuring the elution volumes of blue dextran (2 MDa), albumin from bovine serum (66 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa) and cytochrome C from equine heart (12.4 kDa). A calibration graph of $K_{av}$ value versus logarithm of molecular weight was plotted in Figure 2.3 where the $K_{av}$ values for the standards were calculated as follows:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$  [2.2]

Where $V_e = $ elution volume for the protein  
$V_o = $ column void volume = elution volume for Blue Dextran 2000  
$V_t = $ total bed volume

As much as 0.05 mM of purified MPH was used in this experiment. The molecular weight of MPH was determined from the calibration curve once its $K_{av}$ value was calculated from its measured elution volume.
2.6. Metal analysis

2.6.1. Preparation of apo-MPH

Metal free deionised water was prepared by treating it with Chelex® 100 (Bio-rad). Approximately 10 g of Chelex® 100 was added into 1 L deionized water and was stirred overnight. For buffer preparation, the pH was adjusted prior chelex treatment. Chelex was removed by filtration by using Minisart filter pore 0.2 μM (Sigma-Aldrich). The glassware used for any buffer preparation was rinsed with 1 M nitric acid prior to use.

The method used is similar to that described in other studies (11, 12). Approximately 3 mg of purified MPH was incubated with 3 ml chelating solution containing 5 mM EDTA, 5 mM 1,10-phenanthroline, 5 mM 2,6-pyridine dicarboxylic acid, 5 mM 8-hydroxyquinoline-5-sulfonic acid, 5 mM 2-mercaptoethanol, 20 mM HEPES, pH 7.0. After 24 – 27 hours of incubation at 4°C, the remaining activity of the apo-enzyme was analysed by using spectrophotometer (Section 2.8.1). Separation of apo enzyme with the chelating solution was done by loading the
protein-chelating solution mixture onto an Econo-Pac 10DG (Bio-Rad) gel filtration column or a 5 mL HiTrap Desalting column (GE Healthcare). These columns were treated with 1 column volume of chelating solution followed by 1 column volume of chelex-treated de-ionised water and 1 column volume of chelex-treated 50 mM Tris-HCl buffer (pH 7.0) to equilibrate before hand.

As much as 3 ml of the protein-chelating solution mixture was loaded into the Econo-Pac 10DG (Bio-Rad) gel filtration column and the first 4 ml of the buffer elution was collected. Whereas, 2 ml of the protein-chelating solution mixture was needed to load the 5 mL HiTrap Desalting column (GE Healthcare) and 2 ml of the flow through was collected. The column was re-equilibrated with chelex-treated buffer following each sample loading. The apo protein concentration was determined with Nanodrop® (Section 2.4.4).

2.6.2. Apo-enzyme reconstitution

As much as 1 µM of apo-enzyme was incubated independently with 30-fold excess of ZnCl₂, MnCl₂, CuSO₄.5H₂O, CO₃SO₄.7H₂O, NiCl₂.6H₂O and 3CdSO₄.8H₂O overnight at 4°C. The activity of the enzyme was assayed spectrophotometrically at saturating level of substrate. The assay conditions are presented in Section 2.8.

2.6.3. ICP-OES analysis

Metal analyses were performed by Inductively Coupled Plasma – Optical Emission Spectrometer (ICP-OES), an analytical technique that uses inductively coupled plasma to produce excited atoms and ions that emit electromagnetic radiation at specific wavelength. The
intensity of the emission gives indication of the concentration of the element in the samples (13).

As much as 5 - 10 μM proteins were prepared for the analysis. The protein was diluted 10-fold before being subjected to nitric acid treatment. The samples were first treated with 4 % nitric acid for 1 hour and were filtered (Minisart filter pore 0.2 μM) prior to analysis. For enzyme reconstitution test, the apo enzyme was incubated with respective metal (ZnCl₂, COSO₄.7H₂O and MnCl₂) for 24 hours prior to analyses.

For the calibration curves, the standards used were prepared by the micro analytical unit personnel. Standards used for the calibration curves were CCS-6 that contained (Ag, Cd, Co, Cr₃, Cu, Fe, Hg, Mn, Ni, Pb, Ti, V, Zn) at 0.5 ppm, 1 ppm, 2 ppm, 5 ppm and 10 ppm. Metal concentrations of MPH samples were determined at the following wavelengths (nm): Cd (228.802), Co (228.616), Cu (327.393), Fe (238.204), Mn (257.610), Ni (231.604) and Zn (206.200).

2.7. Protein stability
2.7.1. Thermoflour assay
A thermoflour assay has been set up for a protein thermal stability test. The fluorescence transition and the melting temperature were monitored and determined using 7900HT Fast real-time PCR system (Applied Biosystem) from ACRF Biomolecular Resource Facility, John Curtin Medical School. Small amount of protein (5 μM) was prepared in a 50 mM Tris-Hcl, pH 7.0 containing 250 mM of NaCl together with 5x SYPRO orange with 5 % (v/v) DMSO. Five replicates were done on each sample. The denaturation process was done at temperature ranges
10 – 90°C at 1 degree per min. A melting curve was plotted for the average of the fluorescence signal as a function of temperature. The Scidavis program was used for Boltzmann curve fit (14).

2.8. Spectrophotometric assay

2.8.1. Activity assay

Activity assay was done in 200 μL reaction mixture in a 96-well microtiter plate. The reaction was done using Spectramax M2e microplate. The activity of purified protein with methyl parathion was measured spectrophotometrically by monitoring the formation of the product p-nitrophenolate at 405 nm. Reactions were measured to less than 5 % of total substrate hydrolysis for 3 minutes and the assay mix was equilibrated for 2 min before the measurement. Initial rates were linear in the studied time frame. All experiments were done in triplicates at 30°C.

2.8.2. Kinetic characterisation

Purified enzyme (0.025 – 2.5 μM) in 50 mM Tris-HCl solution was assayed at 30°C with varying methyl parathion substrate concentration ranging from 0 to 300 μM. Kinetic measurements were carried out in two different types of samples, one was with protein as purified and the other one was with fully occupied Zn(II) of MPH, where MPH was preincubated with excess zinc acetate (4 mM) at 4°C at least for 2 hours. A standard curve of pNP (Figure 2.4) was constructed to determine the extinction coefficients of pNP by using the Beer-Lambert equation [Eq. 2.1].
The $K_m$ and $k_{cat}$ were obtained by fitting the data to the Michaelis-Menten equation [Eq. 2.2], using curve-fitting software, KaleidaGraph.

$$V_o = \frac{V_{max} [S]}{K_m + [S]}$$  \[2.3\]

Where, $V_o$ : Initial velocity; $V_{max}$ : maximum velocity; $K_m$ : Michaelis-Menten constant; $S$ : substrate concentration and $V_{max} = k_{cat} \cdot E_o$.  

**Figure 2.4.** Standard curve of pNP. Absorbance $A_{405}$ as a function of [p-nitrophenol].
2.8.3. pH-rate profile

The pH-rate profiles were performed for MPH wild type and MPH Asp151Asn mutant over a pH range of 4.0 – 11.0 at 1 unit increment. The multicomponent buffers containing 100 mM of each of 2-(N-morpholino)ethanesulfonic acid (MES), N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), 2-(N-cyclo-hexylamino)ethanesulfonic acid (CHES), ACETATE and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) were prepared and were chelex-treated after the pH adjustment. The Chelex® 100 used was removed by filtration by using Ministart filters pore 0.2 μM (Sigma-Aldrich) before used for assay. Proteins were supplied with excess Zn(CH₃C00)₂.2H₂O and the excess metals were removed using 5 mL HiTrap Desalting column (GE Healthcare) immediately before assay. Method was adopted and modified from Daumann (15). The Vₘₐₓ values at each pH was determined using a saturating amount of substrate in each assay.

2.9. Determination of metal binding affinity

2.9.1. Isothermal Titration Calorimetry (ITC)

All measurements for binding studies were carried out with a VP-ITC calorimeter (Microcal Inc. GE Healthcare) (Section 1.5.1). Both the sample cell and the syringe were cleaned using 5 % Decon, 10 mM EDTA solution and metal-free milliQ water prior to use.

All solutions used were thoroughly degassed under vacuum for 20 minutes with gentle stirring immediately before use. Metal solution (Zn(CH₃C00)₂.2H₂O) (2 - 3 mM) in a 300 μl injection syringe was titrated into 20 μM apo enzyme solution (in 50 mM Tris-Hcl, pH 7.0) that was placed in a 1.5 ml sample cell. The reference cell, which acts as thermal reference to the sample cell, was filled with de-gassed milliQ water. Titration typically consisted of a preliminary 2 μl
injection followed by 40-65 of 3-4 µl injections, at 4 min interval. All experiments were performed at 18°C. For homogeneous mixing during the course of titration, the stirrer was kept constant at 260 rpm. The experiment was designed based on factors described by Grossoehme (16).

The data were fitted using MicroCal® Origin 7.0 software (17). Data from the first 2 µl injection, which were unreliable due to diffusion between the syringe and cell solutions, was discarded before analysis. Background titration (heat dilution) was removed from the integrated data. Mathematical model of nonlinear least square curve two sets of sites model was used to fit the integrated data. The derivations of the mathematical model used for the data fitting were described in the introduction (18, 19).

### 2.10 Activity as a function of metal ion concentration

A series of reactions in which protein concentration is held constant and Zn(CH₃COO)₂⋅2H₂O concentration is varied (from 50 nm to 15000 nM) was set up to determine the effect of metal ion concentration on the activity of MPH. Apo enzyme was used in this experiment, which ranges from 100 nm to 5000 nm (varies among proteins). The apo enzyme was incubated individually at a respective metal concentration on 96 wells plate at 4 °C for at least 2 hours prior to assay. Substrate concentration was held constant at 0.1125 mM.

All data obtained were analysed by nonlinear regression using GraphPad Prism 6 software, Inc. Rate versus zinc concentrations profiles were fitted to one site specific binding with Hill slope model or competitive two sites model (20).
One site specific binding with Hill slope model equation:

\[ Y = \frac{B_{\text{max}} \cdot X^b}{(K_d^b + X^b)} \] \hspace{1cm} [2.4]

2.11. Computer software programs

Sequencher software version 5.0.1 was used to analyse the entire DNA sequences studied in this project. All of the chemical structure figures were produced using CS ChemBioDraw Ultra software. All of the protein structures including MPH and other proteins used for comparison were obtained from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). All protein structures figures were generated from MacPyMOL. Kaleidagraph (version 4.1.3) program was used to fit the kinetic curves. GraphPad prism 6.0 software, Inc. was used to fit the activity as a function of added metal curves. Scidavis (version 0.2.4) program was used to fit melting curves obtained from the thermo flour assay. MicroCal Origin 7.0 software was used to fit the ITC curves.
2.12. References


3

RESULTS

3.1. Preface

This chapter describes the results from a series of experiments investigating the metal coordinating residues of the metallohydrolase MPH. The objective of this work was to garner a better understanding of the role zinc plays in dictating function. To date, information concerning the specific role of the metal ions that form a catalytically active binuclear centre in MPH is relatively scarce. The coordination of the binding sites in MPH and their affinity for \( \text{Zn}^{2+} \) has not been thoroughly characterised with respect to either the effect on activity or the thermodynamic properties for the \( \text{Zn}^{2+} \) binding. Thus, the present chapter contains results from experiments intending to address these unresolved questions.

A series of experiments were conducted to observe the effects of mutations on the primary ligands and the secondary coordination residues. This information was then used to identify the influence and importance of the respective residues on the overall structure conformation, catalytic mechanism and organisation of the metal binding sites. For this purpose, the primary ligands and the secondary coordination residues for the zinc ions in MPH were changed
individually by site-directed mutagenesis. A series of twenty MPH variants with single mutations were generated then purified and characterised. Gel filtration and mass spectrometry (MS) were used for molecular weight determination. The nature and quantity of enzyme bound metal was determined using inductively coupled plasma optical emission spectrometry (ICP-OES). A test was carried out to investigate the effects of amino acid alterations on the stability of MPH. This experiment was carried out using real time PCR as described in Section 2.7.1. Kinetic characterisation and studies into metal binding affinity using isothermal titration calorimetry (ITC) were conducted to assess changes to the zinc coordination as a result of the mutations.

3.2. Rational Selection of Residues for Site-Directed Mutagenesis

Seven amino acid residues – His147, His149, Asp151, His152, His302, Asp255 and His234 – are responsible for coordination the zinc ions in the binuclear metal centre of MPH (Figure 3.1 (A)). The ligating residues at positions 147, 149, 152, 302 and 234 were mutated individually to both glutamic acid and glutamine. Ligands Asp151 and Asp255 on the other hand were changed to asparagine and alanine. Pro150 that links residues 151 and 149 was changed in turn to glycine and alanine, while three secondary coordination sphere residues at positions 112, 146 and 274 were individually changed to alanine (Figure 3.1 (B)).
Figure 3.1. The amino acids involved in metal ions coordination. A. The ligands that coordinate the two metal ions in the active site; B. Diagram depicts how the secondary residues are associated with the metal ions in the active site.
Histidine is a ligand that is commonly found in the metal centre of zinc metalloenzymes (1). Replacing the relevant His residues in MPH would provide insight into the role played by His in the active site of MPH. This would include information on the metal association and its effect on both structural stability and catalytic activity. Replacing these His residues with glutamic acid (a negatively charged polar amino acid having roughly the same size as His) is expected to produce an enzyme with a higher binding affinity for the zinc ions in the active site. Whereas changing His to a Gln (a polar but uncharged amino acid) would be expected to bind metals less tightly.

Aspartate, a negatively charged polar amino acid is a frequent constituent of the active site of metalloenzymes (2). Asp151 in MPH forms a hydrogen bond with the bridging hydroxide in the enzyme active site (Figure 3.1(A)). Substitution of Asp with Asn, a polar amino acid that consists of an amide rather than a carboxylate side chain, is expected to give rise to an additional hydrogen bond (Figure 3.2). Changing Asp151 to Ala, will result in the loss of a negative charge from the carboxylate group that forms a hydrogen bond to bridge the hydroxide (Figure 3.2).
The bridging ligand Asp255 plays an important role in coordination of the metal ions through the carboxylate side chain. Changing this bridging ligand into an Asn and Ala is expected to perturb the coordination of both zinc ions since it plays such a crucial role in maintaining the protein geometry. The protein is predicted to alter the binding capacity for the α and β metals due to the loss of negative charge provided by the Asp side chain. Ala mutant will allow water in so that a second bridging hydroxide will be present in the active site. Asn mutant should bridge the metal, but with reduced affinity (Figure 3.3).
Residue Pro150 plays an important role in metal ion coordination in the active site because of its link with Asp151 and His149, which coordinate the $\alpha$ and $\beta$ metals respectively. Proline is a rigid amino acid and this rigidity is likely important to ensure stability and the correct position of Asp151 and His149. Mutating Pro150 to the more flexible Gly and Ala residues is predicted to alter binding affinity through its effect on the neighbouring coordination residues.

Thr146 forms hydrogen bond with His147 that coordinated to the $\beta$ metal in the active site. It also forms H-bonds with Asp112, which in turn interacts with His152 that coordinates the $\alpha$ metal (Figure 3.1 (B)). Mutation of Thr146 could allow His147 to rotate and affect the stabilising H-bond with Asp112. This would likely effect coordination of the $\beta$ metal considerably and the $\alpha$ metal perhaps more subtly, in both cases causing a change of binding.
affinity. In addition, the effect of mutating Asp112 is of interest. The H-bond between these residues is of appropriate distance but the geometry of the bond is unusual. Thus, one of the interests of this study is to understand the effect of this amino acid on the stability of the coordination site. Asp274 is another secondary coordinating residue that has been mutated and characterised as part of this study. This residue forms a good H-bond with the side chain of His234, which coordinates the β metal. Removing this H-bond could allow more freedom of movement to His234, which could affect coordination of the metal ions in the active site.

3.3. Protein Purification

MPH native and mutants were expressed in *E. coli* BL21(DE3) cells harbouring pETMCS1 plasmid capable of expressing MPH(MIT) sequence. MIT is a MPH truncated sequence designed to remove the first 34 MPH amino acids that code for a signal peptide. This truncated form of MPH expressed in the cytoplasm is more highly expressed compared to the protein that is transported to periplasm. This initial gene modification work was performed by Dr. Ng, T. K (3). Accordingly, the truncated enzyme lacking the signal peptide has been used for the entirety of the work presented in this thesis. The published purification in the candidate’s hands failed to produce protein of high purity. This purification resulted in low levels of contaminating proteins and did not prevent proteolytic breakdown of MPH. The improved method described in Section 2.4.3 was developed.
The purification of MPH was done via two stage process. Firstly cation exchange was used at pH 5.5 followed by anion exchange at pH 10.0. The pooled fractions from each column were analysed by SDS-PAGE alongside the pellet, supernatant and loading effluent. These results showed that most of the preparations were highly pure with MPH the only significant protein present with a subunit molecular weight of approximately 32.0 kDa (Figure 3.4). Nearly 0.5 – 1.0 mM of MPH was successfully isolated from 2 L culture. An elution profile for the native protein is shown in Figure 3.4 (A) while Figure 3.5 shows SDS gel for the native and mutant proteins.

For some of the variants there were some faint bands visible by SDS-PAGE, these were smaller than the main band corresponding to MPH (Figure 3.5). This generally can be seen for mutants that are less stable and precipitation was observed during the purification process. Figure 3.5 shows this degradation can also be observed for the wild-type protein when older samples were tested. However, for freshly purified wild-type protein the degradation was not observed (Figure 3.4).
Figure 3.4 SDS-PAGE profiles of purified proteins. A. MPH wild-type eluted fractions from second column (Q sepharose column); B. MPH wild-type and its variants. S denote as standard.
Figure 3.5. SDS-PAGE profiles of purified MPH wild-type and its variants.
3.4. Preliminary Characterisation

A preliminary activity assay was set up to gain some basic knowledge on the effects of the amino acid alterations on the activity of native MPH and substituted variants. As much as 2 μl of lysed crude lysate (soluble fraction) in the presence of 1 mM of ZnCl₂ was assayed at saturating levels of methyl parathion for 3 min (Figure 3.6).

**Figure 3.6.** Activity assay for MPH wild-type and its variants using crude lysate. The main panel shows the activity for all proteins where all activities were normalized to native activity. The inset shows the protein with low activity level.
Figure 3.6 shows that enzyme activity is considerably affected by the mutations. Mutations made to the primary metal binding ligands greatly reduced the activity of the enzyme. This reduction is also noticeable for two of the MPH secondary residues; Asp112Ala and Asp274Ala mutants. However, mutation to residue Thr146, a secondary coordinating residue, showed 6 % increment in the activity. Mutations to Pro150 to Ala and Gly resulted in the variants with approximately 50 % activity of the native enzyme.

Activity assays using purified protein (Figure 3.7) give similar results to those obtained with crude cell lysate. The ligand alterations demolish activity for most of the MPH variants especially where the mutations are at primary ligand sites. The variant possessing the Asp255Ala mutation showed slightly higher than background activity when purified protein was used but when using crude lysate, the activity was undetectable. Thus, the experiment was repeated with the addition of excess zinc (added in the form of ZnCl₂) to the assay mixture, in order to confirm the previous result. The addition of excess metals increased the activity of Asp255Ala by as much as 3-fold and increased the activity of other mutants such that it was detectable, albeit barely. This effect is prominent in the secondary coordination mutants. The presence of excess zinc significantly increased the activity of MPH variants containing mutations to the secondary coordinating residues, including Asp112Ala. This indicates that Asp112Ala has some very low level of binding capacity. To further validate these finding, full kinetic profiles were obtained for native MPH and the full range of mutated variants.
Figure 3.7. Preliminary activity assay for purified proteins. The activity results were presented in v(munits/min). The main panel (bottom) shows the comparison of the purified protein activities with and without the presence of excess zinc. The inset shows the smaller scale of the activity results.
3.5. Molecular Weight Determination

Molecular weight of MPH wild-type was determined with Fourier transform ion cyclotron resonance mass spectroscopy (FTICR-MS) and size exclusion chromatography. Mass spectrometry was conducted to give an accurate protein mass measurement and to check the variation of MPH molecular weights in the purified variant MPH protein samples. The sample preparation procedure is presented in Section 2.4. This analysis was performed with the assistance of Mr. Sam Fraser and Dr. Joanne Porter.

The deconvoluted mass spectrum shows several species with different molecular weights in the wild-type MPH sample. The peak of highest intensity has a mass of 31405.3 Da (Figure 3.8 (A)) corresponding to the mass of purified protein. When excess zinc was added to the sample, the predominant peak corresponded to the enzyme with full zinc occupancy with a mass of 31534.5 Da (Figure 3.8 (B)). This is an addition of 130.8 mass units, which accounts for two zinc ions (MW = 65.4) per monomer. It is assumed that the zinc ions were lost from the as purified sample (of which the molecular ion contained no zinc ions) during the sample preparation, which involved spin filtration to exchange the buffer for water.

The ProtParam theoretical mass of monomer MPH is 31538.9 Da. The experimentally determined masses correspond to MPH minus the N-terminal methionine (131.04), the theoretical value minus the methionine is 31403.7 Da. This matches the as purified sample (31405.3 Da) and the sample with fully zinc occupancy (31403.7 + 130.8 = 31534.5 Da).
Figure 3.8. Deconvoluted FTICR-MS spectrum of MPH wild-type. A. Spectrum of purified enzyme; B. Spectrum of sample with excess zinc.
Gel filtration chromatography was also used to determine the oligomeric structure of MPH. This involved column calibration with commercial protein standards of known molecular weight and subsequent comparison with the MPH elution profile. The calibration curve was prepared by measuring the elution volumes of blue dextran (2 MDa), albumin from bovine serum (66 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa) and cytochrome C from equine heart (12.4 kDa). The specific details of the method are described in Section 2.5.

MPH was eluted between 82 – 85 ml which is approximately the same volume obtained with albumin that has a molecular weight at 63 kD (Figure 3.9). This is very similar to the predicted molecular weight of dimeric MPH.

Figure 3.9. Chromatographic separation and calibration curve for the protein standard overlayed with the MPH native elution profile.
3.6. Metal Studies

A metal binding study was conducted to characterise the metal centre of MPH wild-type and mutated variants. The study began with measurement of the metal content of wild-type MPH. This was expected to provide insight into the stoichiometry ratio of the metal ion(s) to the protein component in MPH. This metal study also involved the conversion of MPH holoenzyme to its apo forms, followed by the determination of metal ions required to achieve the restoration of catalytic function to the characterised level of the parent protein.

3.6.1. Production of apo-enzyme

Activity assays were routinely performed when preparing the apo-enzyme to assess when the metal ions had been fully removed. Figure 3.10 shows the plots of residual activity versus time for the metal removal of MPH wild-type and some of the selected MPH variants. The selected variants are those with appreciable activity (i.e those variants with very low levels of activity were not subjected to this assay). This experiment has been performed using protein as purified that is the protein not fully occupied with metals. The activity has been normalized to the initial activity of each protein. The results show that more than 24 hours of incubation with the chelator was needed in order to reduce the activity to less than 1 % of the activity of these proteins. For some proteins, the activity rapidly dropped after introduction of the chelating solution. It is however difficult to determine whether the reduction of the activity was due to the loss of both metals or due to loss of just one metal ion. Metal analysis is therefore crucial in the process of apo-protein making.
Figure 3.10. Remaining activity of MPH wild-type and MPH variants during metal chelating. A. De-metallation process of MPH primary ligand; B. De-metallation process of MPH secondary coordination sphere residues.
3.6.2. Zinc reconstitution assay

Similarly, reconstitution of the apo-proteins was monitored to assess the progress and determine the rate of reconstitution. To do this a time-course experiment was conducted on the apo-native. A graph of activity as a function of time was plotted and is shown in Figure 3.11. The reconstitution of apo-enzyme with zinc(II) took at least two hours to be fully reactivated such that the activity was equal to the parent protein.

![Graph showing reconstitution of metal reactivation in MPH native protein](image)

**Figure 3.11.** Duration of metal reconstitution in MPH native protein.

An experiment was set up in order to compare the activity of the purified and reconstituted protein. Excess zinc was incorporated independently in the reaction mixture for both samples. Figure 3.12 shows that the reconstitution of apo-wild-type has fully reactivated with the native metal ions, such that the activity is equal to the purified wild-type.
3.6.3. Reconstitution with other divalent metals assay

Activity assays were also performed for apo-native and the apo-native reconstituted with a selection metal ion (Figure 3.13). This experiment was conducted in order to examine the metal specificity of the active site. This assay was also done to study how the replacement of the naturally occurring zinc(II) metal with other divalent cations, such as manganese(II), copper(II), cobalt(II) and nickel(II) affects the activity of the native MPH and whether or not the apo-native is capable of being reactivated with a variety of different metals.

The cobalt-containing enzyme had the highest activity followed by Zn(II)-, Mn(II)-, Cu(II)- and Ni(II)-substituted enzymes. The activity for apo native was barely above the detection level, as expected. Replacing native metal with Co(II) has increased the protein activity significantly over that of the as purified native and the zinc reconstituted enzyme. The Mn$^{2+}$ give rise to
detectable levels of activity that was about one third that of the Zn$^{2+}$ enzyme. The Cd(II)-substituted enzyme failed to achieve any significant reactivation. Ni(II)- and Cu(II)-reconstituted enzymes gave rise to low levels of activity.

Figure 3.13. Comparison of apo-native activities with foreign metals. The activity was normalized to WT with native metal activity.
3.6.4. Native gel electrophoresis

The preparation of apo- and holo-enzyme (reconstituted) were examined using native polyacrylamide gel electrophoresis (Figure 3.14). This analysis was made with the aim to detect anomalies between different forms of MPH enzyme, which includes the apoenzyme, purified protein, and holoenzyme that was fully occupied by Zn$^{2+}$ (reconstituted), whether or not the metal removing effort has changed the conformation or causes denaturation to the protein.

![Native gel electrophoresis diagram](image)

**Figure 3.14.** Separation of different forms of MPH on 10% native PAGE. A. Apo-enzyme; B. Native as purified; C. Reconstituted protein with ratio 2:2; D. Reconstituted protein with ratio 50:1.

Three different bands were present for each sample. The first fraction (top band) was suspected to be the agglutinated protein where the size invariably hindered its migration down the gel. The second fraction (middle) was assumed to be the dimer and the third band (bottom) was predicted to be the monomer of MPH. To more thoroughly analyse the differences between each sample, the thickness and intensity of each band was determined using ImageJ software (Table 3.1).
Table 3.1. Analysis of band’s intensities using ImageJ software

<table>
<thead>
<tr>
<th></th>
<th>Band intensity</th>
<th></th>
<th>Band size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Middle</td>
<td>Bottom</td>
<td>Middle</td>
</tr>
<tr>
<td>A</td>
<td>312</td>
<td>320</td>
<td>46.39</td>
</tr>
<tr>
<td>B</td>
<td>292</td>
<td>318</td>
<td>47.67</td>
</tr>
<tr>
<td>C</td>
<td>280</td>
<td>296</td>
<td>45.76</td>
</tr>
<tr>
<td>D</td>
<td>285.6</td>
<td>330.5</td>
<td>54.32</td>
</tr>
</tbody>
</table>

Table 3.1 shows the size and intensity on each band determined using ImageJ. It shows only trivial differences between the apo- and holo-enzyme. Thus, insinuating that the apo-enzyme is present primarily in its folded form since denatured protein would be expected to have different exposed charges and migrate differently by native SDS-PAGE.

3.6.5. ICP analysis

To begin with, the total metal content was determined by inductively coupled plasma- optical emission spectroscopy (ICP-OES). Metal standards were used to construct the calibration curve and the metal concentration in each protein sample was determined from the average of triplicate measurements. The method used for this analysis is described in detail in Section 2.6.3. A few different forms of MPH wild-type (apo, holo and as purified) were subjected to the analysis. The data obtained is summarised in Table 3.2.
Table 3.2. The zinc metal content of MPH native protein

<table>
<thead>
<tr>
<th></th>
<th>Zinc</th>
<th>Cobalt</th>
<th>Manganese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native as purified as it is</td>
<td>0.660</td>
<td>0.000</td>
<td>0.030</td>
</tr>
<tr>
<td>Apo native</td>
<td>0.020</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Apo native reconstituted with excess Zn</td>
<td>1.900</td>
<td>0.001</td>
<td>0.006</td>
</tr>
<tr>
<td>Apo native reconstituted with excess Co</td>
<td>0.000</td>
<td>1.490</td>
<td>0.000</td>
</tr>
<tr>
<td>Apo native reconstituted with excess Mn</td>
<td>0.000</td>
<td>0.000</td>
<td>0.670</td>
</tr>
</tbody>
</table>

The metal content of the as purified MPH sample was 0.6 zinc per protein monomer. MPH is expected to contain 2 metal ions per monomer. The metal analysis has also been performed on native apo and apo-reconstituted with excess zinc(II), cobalt(II) and manganese (II) (reconstituted individually). The enzyme preparation is discussed in Section 2.4 and Section 2.6.3. There was a small amount of zinc ion left in the apo-protein however none of the other traces metals were detected in the sample. The ICP-OES results show that Mn-reconstituted protein bound fewer metals than Zn- and Co-reconstituted wild-type that had approximately 1.9 and 1.5 equivalents of metal bound per monomer respectively.

The activity measurements described in the previous section were carried out with protein that was not saturated with metals. Higher levels of activity would be expected of higher concentrations of Mn$^{2+}$ and Co$^{2+}$ used to reconstitute MPH.
3.7. Protein Stability

A Thermal stability experiment was performed for all of the MPH proteins (Figure 3.15). This temperature-based assay provides melting temperature that gives indications of the stability or resistance to instability of the protein. This experiment examined how the amino acid alterations influenced the stability of MPH with and without excess Zn$^{2+}$. The method used for this thermofoflour assay is described in Section 2.7.1. There is a very large temperature difference (20°C) between the purified native enzyme and that obtained with the native enzyme supplemented with excess zinc(II). All the mutated enzymes gave rise to varying levels of stability that were lower than that of the zinc(II) supplemented native enzyme.

![Figure 3.15](image)

*Figure 3.15. Figure shows the melting temperature of MPH wild-type and all of the variants.*
Figure 3.15 shows the melting temperatures determined for the native and variant MPH enzymes both with and without excess Zn$^{2+}$. The stability of the variants without the presence of excess zinc was approximately similar to that of the native and apo-native, which suggests that only scarce amount of indigenous metal were present in each of the purified protein samples. This implies that the zinc ions hold important roles in upholding the structure and stability of MPH as well as the role they play in facilitating catalysis. The MPH native protein with excess metal is more stable than the other variants. Of the α variants (those associated with the α metal - Asp151Asn, Asp151Ala, His152Glu, His152Gln, His302Glu and His302Gln), Asp151Asn and Asp151Ala are the most stable proteins while the other α variants are more structurally affected with the degree of denaturation down by approximately 20 °C compared to the wild-type. The β variants (those associated with the β metal - His147Glu, His147Gln, His149Glu, His149Gln, His234Glu and His234Gln) are generally more stable than the α variants with melting temperatures ranging from 61 – 65 °C compared to less than 55 °C for most of the α variants.

The variants with mutations at position 150 (Pro150Ala and Pro150Gly) have the least change in stability compared to the native enzyme. Thermoflour assay performed on these mutants has indicated that they are as stable as the native with the degree of denaturation ranges from 68 – 69 °C. The bridging ligand mutants, Asp255Ala and Asp255Asn on the other hand are among the less stable proteins as the stability dropped by approximately 15 °C as compared to the native. Comparing the stability of secondary sphere residues, mutant Asp274Ala is the most stable protein compared to the other two secondary variants Asp112Ala and Thr146Ala. The melting temperature for these latter variants 51 °C and 54 °C respectively, which is 18 °C lower than native MPH.
3.8. Kinetics characterisation

A kinetic rate profile was carried out for all of the MPH variants with the aim of providing insight into the affect of the mutations. A series of experiments were set up to determine the Michaelis constant and the turnover rate for each of the proteins. The method is described in Section 2.8. Two sets of experiment were performed, one of which used the enzymes as purified (with no excess zinc) and the other used purified protein with an excess amount of ZnCl₂. The results were analysed by non-linear regression (Kaleidagraph) using the Michaelis-Menten equation (4) (Table 3.3).

The $k_{cat}$ and $K_m$ value for MPH wild-type were experimentally determined to be 3.5 s$^{-1}$ and 115 μM respectively. The presence of excess zinc has increased the $K_m$ slightly but increased the turnover rate by up to 5-fold. These kinetic parameters are slightly different to several previous reports (5, 6). They were however, similar to those reported by Tian et al. (2010) (7), where the $k_{cat}$ and $K_m$ values towards methyl parathion were reported to be 4.2 s$^{-1}$ and 76.3 μM respectively. Previously published kinetic parameters for this enzyme are in a similar range with $k_{cat}$ and $K_m$ values of 27.5 ± 1.8 s$^{-1}$ and 27.3 ± 6 μM respectively (3). The difference is thought to be due to different buffers and pH that was used for analysis.
Table 3.3. Kinetic profiles of MPH wild-type and its variants. All values are expressed as mean ± SD, based on triplicate readings.

<table>
<thead>
<tr>
<th>Protein</th>
<th>( K_m ) (µM)</th>
<th>( k_{cat} ) (x10^3) (s(^{-1}))</th>
<th>( k_{cat}/K_m ) (x10^3) (M(^{-1})s(^{-1}))</th>
<th>( K_m ) (µM)</th>
<th>( k_{cat} ) (x10^3) (s(^{-1}))</th>
<th>( k_{cat}/K_m ) (x10^3) (M(^{-1})s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>115±2</td>
<td>35±1</td>
<td>30.4±0.9</td>
<td>157±10</td>
<td>174±5</td>
<td>111±3</td>
</tr>
<tr>
<td>α-D151N</td>
<td>31±3</td>
<td>0.042±0.002</td>
<td>0.130±0.006</td>
<td>44±4</td>
<td>0.44±0.01</td>
<td>0.89±0.07</td>
</tr>
<tr>
<td>α-D151A</td>
<td>42±3</td>
<td>0.027±0.001</td>
<td>0.072±0.003</td>
<td>60±3</td>
<td>0.21±0.01</td>
<td>0.336±0.007</td>
</tr>
<tr>
<td>α-H152E</td>
<td>49±22</td>
<td>0.13±0.03</td>
<td>0.20±0.06</td>
<td>68±7</td>
<td>21±1</td>
<td>31±1</td>
</tr>
<tr>
<td>α-H152Q</td>
<td>7±3</td>
<td>0.06±0.01</td>
<td>0.8±0.7</td>
<td>49±2</td>
<td>24.9±0.4</td>
<td>51.3±0.5</td>
</tr>
<tr>
<td>α-H302E</td>
<td>11±9</td>
<td>0.020±0.003</td>
<td>0.18±0.08</td>
<td>49±8</td>
<td>0.23±0.01</td>
<td>0.41±0.03</td>
</tr>
<tr>
<td>α-H302Q</td>
<td>152±36</td>
<td>8±1</td>
<td>5.2±0.4</td>
<td>355±19</td>
<td>13.5±0.1</td>
<td>3.79±0.09</td>
</tr>
<tr>
<td>β-H147E</td>
<td>89±30</td>
<td>0.013±0.001</td>
<td>0.011±0.002</td>
<td>14±6</td>
<td>0.067±0.001</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td>β-H147Q</td>
<td>26±11</td>
<td>0.027±0.004</td>
<td>0.12±0.06</td>
<td>41±4</td>
<td>0.054±0.001</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>β-H149Q</td>
<td>73±2</td>
<td>0.02±0.01</td>
<td>0.03±0.01</td>
<td>162±29</td>
<td>0.99±0.04</td>
<td>0.61±0.04</td>
</tr>
<tr>
<td>β-H149E</td>
<td>5±4</td>
<td>0.02±0.01</td>
<td>0.4±0.3</td>
<td>17±11</td>
<td>0.080±0.006</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>β-H234E</td>
<td>9±0.8</td>
<td>0.016±0.001</td>
<td>0.174±0.005</td>
<td>8±1</td>
<td>0.016±0.001</td>
<td>0.25±0.03</td>
</tr>
<tr>
<td>β-H234Q</td>
<td>67±0.5</td>
<td>0.09±0.06</td>
<td>0.15±0.10</td>
<td>21±5</td>
<td>0.081±0.004</td>
<td>0.38±0.09</td>
</tr>
<tr>
<td>Link-P150A</td>
<td>143±33</td>
<td>44±4</td>
<td>30±4</td>
<td>125±25</td>
<td>54±7</td>
<td>43±2</td>
</tr>
<tr>
<td>Link-P150G</td>
<td>68±11</td>
<td>3.3±0.2</td>
<td>4.4±0.8</td>
<td>102±0.2</td>
<td>3.7±0.1</td>
<td>3.91±0.05</td>
</tr>
<tr>
<td>Bridge-D255A</td>
<td>151±43</td>
<td>33±10</td>
<td>22±1</td>
<td>192±39</td>
<td>49±4</td>
<td>25±1</td>
</tr>
<tr>
<td>Bridge-D255N</td>
<td>127±6</td>
<td>10±1</td>
<td>7.9±0.3</td>
<td>158±31</td>
<td>18±3</td>
<td>11±1</td>
</tr>
<tr>
<td>Secondary-D112A</td>
<td>31±24</td>
<td>0.02±0.01</td>
<td>0.06±0.01</td>
<td>252±11</td>
<td>247±7</td>
<td>98.1±0.9</td>
</tr>
<tr>
<td>Secondary-T146A</td>
<td>128±27</td>
<td>34±3</td>
<td>27±1</td>
<td>168±48</td>
<td>147±30</td>
<td>87±5</td>
</tr>
<tr>
<td>Secondary-D274A</td>
<td>81±14</td>
<td>48±4</td>
<td>59±3</td>
<td>176±25</td>
<td>155±20</td>
<td>87.9±0.8</td>
</tr>
</tbody>
</table>
The presence of excess zinc enhanced the $k_{cat}$ for most of the MPH variants including the native protein. The improvement in catalytic activity is especially noticeable for the Asp112Ala mutant where $k_{cat}$ increased by 12000-fold and the $k_{cat}/K_m$ by 1600-fold with the addition of excess zinc. This $k_{cat}/K_m$ ratio was the highest compared to the other secondary coordinating residues, where the $k_{cat}/K_m$ ratio for Thr146Ala and Asp274Ala increased by only three- and two-fold, respectively. This result corresponds with the preliminary results (Figure 3.7), where the variants possessing the Asp112Ala mutation exhibited the highest activity in the presence of excess zinc. Replacing His to either Glu or Gln at location 234 however does not improve the catalytic activity regardless of the presence of excess metal. The turnover rate remains unchanged and the affinity for the substrate shows only a slight increase, thus suggesting that zinc affinity is greater for these variants.

3.8.1. pH profile

In order to study how the rate of MPH catalysis is affected by variations in pH, a pH rate assay was performed on the wild-type and the Asp151Asn variant. Asp151Asn was chosen to compare with the native protein due to the role of the acid group of the side chain during catalysis. Altering Asp151 to an Asn was proposed to replace the H-bond formed between the acid group and the $\mu$-hydroxo bridge and in doing so hamper catalysis. Thus, assessing the activity of this protein at various pH was hoped to bring insight to these predictions. To perform this assay methyl parathion was used at the saturating amount. Details on this experiment are described in Section 2.8.3.
Figure 3.16 shows the pH profile of MPH wild-type and the Asp151Asn variant. The pattern obtained from the pH-rate profile for Asp151Asn is similar to that of the wild-type enzyme. The $k_c$ for both enzymes was highest around neutral biological pH (pH 7 and pH 9) and then dropped rapidly as the pH was increased to 10 and decreased at pH values below 6. On the basis of observed activities and pH-rate profiles, it indicates that the change of an acidic group to an amide does not alter the mechanism of the hydrolysis although the activity has been reduced by 400-fold. The H-bond disruption does not appear to obliterate catalysis.

![Figure 3.16. The pH rate profiles of MPH wild-type and Asp151Asn.](image)
3.9. Isothermal Titration Calorimetry (ITC)

A series of ITC experiments were conducted to examine the thermodynamic properties of Zn\(^{2+}\) binding to the centre of apo-MPH variants. The trace amount of residual metal in the apo-enzymes was determined by ICP analysis. This measurement was also assumed that the holoenzyme (after the titration) was fully occupied by Zn\(^{2+}\) and in native conformation with no denaturation occurring throughout the course of Zn\(^{2+}\) titration.

ITC was conducted for the native and all mutated MPH variants. MicroCal Origin 7.0 software was used to fit the ITC curves and subsequently extract the thermodynamic and stoichiometric parameters. The fitted isotherm determines the binding constant \((K)\), reaction stoichiometries \((n)\), enthalpies \((\Delta H)\) and entropies \((\Delta S)\) from a single experiment. A representative thermogram for the titration of apo-MPH (native) with Zn\(^{2+}\) is shown in Figure 3.17. Thermograms for the MPH variants are compiled in Appendix D.

The top panel of the thermogram (Figure 3.17) shows the differential power signal recorded in the experiment and the bottom panel shows the heat evolved per mole of titrant (Zn\(^{2+}\)) as a function of the molar ratio of total ligand to total enzyme. Thermodynamic parameters derived from the titration of Zn\(^{2+}\) into the apo-native MHP and apo variants are listed in Table 3.4.
Figure 3.17. Calorimetric titration of Zn$^{2+}$ with apo-MPH native. **A. Raw data; B. Plot of the integrated heats for each injection versus Zn$^{2+}$/protein molar ratio, after subtraction of the heat of dilution of Zn$^{2+}$.** Conditions: 20 μM apo-native titrated with a preliminary 2 μl injection followed by 40-65 of 3-4 μl injections of 2mM (Zn(CH$_3$COO)$_2$.2H$_2$O) in 50 mM Tris-HCl, pH 7.0 at 18 °C.
Table 3.4. Thermodynamic parameters of Zn\(^{2+}\) binding to MPH.

<table>
<thead>
<tr>
<th>Protein</th>
<th>(n_1)</th>
<th>(K_1) ((10^6) M(^{-1}))</th>
<th>(\Delta H_1) (kcal mol(^{-1}))</th>
<th>(\Delta S_1) (cal mol(^{-1}) K(^{-1}))</th>
<th>(n_2)</th>
<th>(K_2) ((10^6) M(^{-1}))</th>
<th>(\Delta H_2) (kcal mol(^{-1}))</th>
<th>(\Delta S_2) (cal mol(^{-1}) K(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.96</td>
<td>9±3</td>
<td>-399±3</td>
<td>-96</td>
<td>0.7</td>
<td>2±0.4</td>
<td>-171±4</td>
<td>-30</td>
</tr>
<tr>
<td>(\alpha)-D151N</td>
<td>0.97</td>
<td>30±20</td>
<td>-1300±8</td>
<td>-413</td>
<td>0.46</td>
<td>1.5±0.3</td>
<td>-843±22</td>
<td>-261</td>
</tr>
<tr>
<td>(\alpha)-D151A</td>
<td>0.95</td>
<td>10±5</td>
<td>-644±7</td>
<td>-180</td>
<td>0.5</td>
<td>0.6±0.1</td>
<td>-542±19</td>
<td>-160</td>
</tr>
<tr>
<td>(\alpha)-H152E</td>
<td>0.98</td>
<td>50±20</td>
<td>-995±4</td>
<td>-297</td>
<td>0.4</td>
<td>0.3±0.1</td>
<td>-56±14</td>
<td>-165</td>
</tr>
<tr>
<td>(\alpha)-H152Q</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.65</td>
<td>0.08±0.01</td>
<td>-96±6</td>
<td>-10.4</td>
</tr>
<tr>
<td>(\alpha)-H302E</td>
<td></td>
<td>1</td>
<td>80±30</td>
<td>-1606±11</td>
<td>0.39</td>
<td>1.9±0.5</td>
<td>-1023±36</td>
<td>-322</td>
</tr>
<tr>
<td>(\alpha)-H302Q</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>0.17±0.03</td>
<td>-79±5</td>
<td>-3</td>
</tr>
<tr>
<td>(\beta)-H147E</td>
<td>0.61</td>
<td>0.1</td>
<td>-2500</td>
<td>-811</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\beta)-H147Q</td>
<td>0.94</td>
<td>0.07±0.01</td>
<td>-940</td>
<td>-291</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\beta)-H149Q</td>
<td>1</td>
<td>9±2</td>
<td>-1283±9</td>
<td>-400</td>
<td>0.19</td>
<td>0.25±0.03</td>
<td>-1435±75</td>
<td>-468</td>
</tr>
<tr>
<td>(\beta)-H149E</td>
<td>0.67</td>
<td>0.06±0.02</td>
<td>-1700</td>
<td>-560</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\beta)-H234E</td>
<td>0.79</td>
<td>0.023±0.004</td>
<td>-1186±29</td>
<td>-378</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\beta)-H234Q</td>
<td>0.93</td>
<td>9±6</td>
<td>-874±12.2</td>
<td>-259</td>
<td>0.58</td>
<td>0.8±0.2</td>
<td>-721±32</td>
<td>-221</td>
</tr>
<tr>
<td>Link-P150A</td>
<td>0.95</td>
<td>2.4±0.8</td>
<td>-506±3</td>
<td>-136</td>
<td>0.6</td>
<td>0.6±0.1</td>
<td>-298±8</td>
<td>-76</td>
</tr>
<tr>
<td>Link-P150G</td>
<td>0.94</td>
<td>40±10</td>
<td>-970±4</td>
<td>-289</td>
<td>0.26</td>
<td>6±1</td>
<td>-666±13</td>
<td>-198</td>
</tr>
<tr>
<td>Bridge-D255A</td>
<td>0.95</td>
<td>80±20</td>
<td>-1241±7</td>
<td>-381</td>
<td>0.26</td>
<td>1.0±0.3</td>
<td>-667±31</td>
<td>-202</td>
</tr>
<tr>
<td>Bridge-D255N</td>
<td>1.63</td>
<td>0.0016±0.000</td>
<td>-80±8</td>
<td>-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary-D112A</td>
<td>1</td>
<td>30±20</td>
<td>-1556±8</td>
<td>-491</td>
<td>0.25</td>
<td>2.4±0.4</td>
<td>-1142±32</td>
<td>-363</td>
</tr>
<tr>
<td>Secondary-T146A</td>
<td>0.97</td>
<td>1.9±0.6</td>
<td>-407±3</td>
<td>-99</td>
<td>0.8</td>
<td>0.6±0.1</td>
<td>-194±6</td>
<td>-38.4</td>
</tr>
<tr>
<td>Secondary-D274A</td>
<td>0.96</td>
<td>10±3</td>
<td>-1254±11</td>
<td>-389</td>
<td>0.26</td>
<td>0.9±0.2</td>
<td>-1002±43</td>
<td>-317</td>
</tr>
</tbody>
</table>
The binding of divalent metal cations to the apo-native and its variants is exothermic (as indicated by the downward peaks in the ITC titration), meaning heat is released upon binding of Zn$^{2+}$ in the MPH active site. Some of the thermograms depict two binding events (Asp151Asn, Asp151Ala, His152Glu, His302Glu, His149Gln, His234Gln, Pro150Ala, Pro150Gly, Asp255Ala, Asp112Ala, Thr146Ala and Asp274Ala) whereas others only detect one binding event (His152Gln, His302Gln, His147Glu, His147Gln, His234Glu and Asp255Asn). In this binding study, the first binding event is stronger than the second. The heat observed in the final titrations was the heat of dilution rather than ligand/metal binding. The curves with two binding events are not well described by single-site binding model but are best fitted by a two-site binding equation, assuming a model of independent, non interacting sites.

The stoichiometry of the binding reaction for the titration of Zn$^{2+}$ is typically between 0.93 - 1.00 for the first binding and 0.19 - 0.80 for the second binding event. The variety in the binding stoichiometry implies structural change in the protein, which causes differences in the binding affinity. Note that the stoichiometry of binding at unity of the binding reaction was fixed during data reduction. This was done since the stoichiometric binding is the simplest factor that is physically realistic to modulate. Furthermore, adjustment of the binding stoichiometry gives no impact on measured binding enthalpies where the values are determined solely by the concentration of the ligand.

It is worth mentioning that the curve fitting for some of the MPH mutants (His152Gln, His302Glu, His302Gln, His147Glu, His234Glu and Asp255Asn) was somewhat ambiguous. This is commonly seen in proteins that appeared to have only one binding event (Appendix D). So in the context of the current work, the absence of one metal binding event caused
ambiguities during curve fitting. The unseen baseline at the initial titration invariably affects the binding enthalpy and is predicted to alter the binding stoichiometry. This can be observed particularly in His302Gln and Asp255Asn variants where the binding stoichiometry is higher than what was expected, 2.00 and 1.63 respectively (i.e. two sites with approximately equal affinity). The uncertainty of the zinc titration curve fitting is similarly effects the His302Glu variant, where the plot of the integrated heat at the transition state between the two binding events is unusual. This affects the fitting process especially for the second binding event. This infrequent occurrence is assumed to be due to the instability of the protein. Several attempts have been made to re-titrage this protein but the effort did not produce and less ambiguous data.

Mutation of α centre ligating residues Asp151Asn, Asp151Ala, His152Glu and His302Glu shows a small increment in the binding affinity for the first metal binding and but second metal binding affinity remains consistent with wild-type MPH. However changing His152 and His302 to the uncharged polar amino acid Gln, has altered one of the binding sites and reduced the binding affinity for the other site. It is suspected that mutation of His152 and His302 to Gln have totally eliminated the strong Zn$^{2+}$ binding to the α centre of the protein while retaining the weaker binding (one-metal-ion fit would reflect binding of the weaker zinc ion) with a significant reduction of binding affinity by 25- and 11-fold, respectively.

Altering β centre residues has shown a significant change in the binding pattern in mutants His147Glu, His147Gln, His149Glu and His234Glu where only one of the Zn$^{2+}$ binding sites can be detected. Mutation of His149 and His234 to glutamine gives MPH variants that retain both Zn$^{2+}$ binding sites with reduction in $K_2$ and $K_1$ values similar to wild-type. Note that His149Gln has small binding stoichiometry for the second site, where the value of $n_2$ is 0.19
molar equivalents of Zn\(^{2+}\) per monomer. This small binding stoichiometry suggests that less Zn\(^{2+}\) binds to the second site for which binding is poorly determined. The \(K_2\) value of this particular mutant is also affected as the value is down by an order of magnitude. This would suggest that this alteration has also distorted the second binding site as observed in other mutants. Following this, it is proposed that alteration on \(\beta\) centre residues has imposed a massive structural alteration on the \(\beta\) binding site, either completely eliminating the \(\beta\) site or altering the \(K_2\). This major destruction would evidently confirm the importance of all of the \(\beta\) centre residues (His147, His149 and H234) in preserving the metal coordination in the binding centres of MPH.

Alteration of link residues, the Pro150 to a glycine has increased the \(K_I\) by an order of magnitude but cause no appreciable change in \(K_2\). Changing Pro150 to an alanine on the other hand has retained the \(K_I\) but reduced the \(K_2\) by an order of magnitude. It is therefore assumed that Pro150Gly affects the Asp151 ligand that coordinates the \(\alpha\) metal whereas Pro150Ala affects His149 ligand that coordinates the \(\beta\) metal. Modification of bridging ligand (Asp255) to an alanine has increased the \(K_I\) by an order of magnitude and retained the \(\beta\) metal binding affinity. Changing this Asp to Asn however has totally demolished one of the binding sites.

To investigate whether the metal ion binding can be modulated without directly altering the primary coordination residues, Asp112, Thr146 and Asp274 that form H-bonds to the primary ligands (His152, His147 and His234), were mutated using site directed mutagenesis. Alterations of these secondary coordination residues neither eliminate the binding of Zn\(^{2+}\) for either binding site nor provide a pronounced effect on the binding constants. However the binding stoichiometry does differ from the wild-type with \(n_2\) values of 0.25 and 0.26 molar.
equivalent of Zn$^{2+}$ per monomer for Asp112Ala and Asp274Ala respectively. This however does not affect Asp112Ala $K_2$ where the second binding affinity has remained unchanged. The change (Asp112Ala) instead has increased the $\alpha$ metal binding by an order of magnitude. The Asp274Ala mutation only causes changes to $K_2$ with reductions of 2-fold. The alteration made to Thr146 on the other hand affects the $\alpha$ and $\beta$ centres at more or less at the same level with 4-fold and 3-fold reduction in the $K_1$ and $K_2$ values respectively.

3.10. Activity as a function of metal ion concentration

Studies of kinetic as a function of metal ion concentration were performed to determine the roles of the divalent cations in the catalytic activity of the MPH. In other words, this study was conducted to determine how enzyme catalysis was affected by the binding of metals. The strategy involved the determination of metal binding parameters from kinetic experiments that were performed by adding metals to apo protein. A series of plots of initial velocity versus metal ion concentration for Zn(II) were made for the wild-type and some selected variants in order to enable comparison between the graphs of the selected MPHs. The method used for this experiment is described in Section 2.10. The Data obtained was analysed by non-linear regression using GraphPad Prism 6 software.

Figure 3.18 shows a titration curve for the reactivation of MPH apo-native with zinc. According to the graph, two zinc ions are essential for the enzymatic activity. This is because the enzyme reactivation involved the addition of 2 stoichiometric equivalents of metal to protein. Addition of higher levels of zinc (micromolar) failed to provide any significant additional activity. The $V_{max}$ was calculated as 103.5 and $K_d$ was 2.5 $\mu$M. The $K_f$ (2.5 $\mu$M)
obtained from this assay is similar to the $K_d$ value obtained from the second binding site of native protein obtained with ITC (which inverse this $K_d$).

Figure 3.18. Reactivation of apo-native with varying amounts of Zn(II). The inset shows the initial velocity with the metal ranging from 0 to 1000 nM. Apo-enzyme (100 nM) was incubated independently overnight at different zinc ion concentrations. Fitted with one site-specific binding with Hill slope. $V_{max}$: 115.1 ± 4.7; $K_j$: 2.8 ± 0.1; $R^2$: 0.9745.

Table 3.5 shows the $K_d$ of native, Pro150Ala, Pro150Gly, Asp151Asn, Asp112Ala, Thr146Ala and Asp274Ala. The ligand binding plots for the respective proteins are presented in Appendix E. Only one binding event showed in all of the reactivation profiles, which presumes to be the weaker metal ion binding event. This is probably due to the need of the lower-affinity metal to bind to the protein for a catalytic turnover to occur.
Table 3.5. $K_d$ values (µM) for MPH native and its variants for kinetic as a function of zinc concentration.

<table>
<thead>
<tr>
<th></th>
<th>$K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>2.8±0.1</td>
</tr>
<tr>
<td>Asp151Asn</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>Pro150Ala</td>
<td>3±1</td>
</tr>
<tr>
<td>Pro150Gly</td>
<td>4±1</td>
</tr>
<tr>
<td>Asp112Ala</td>
<td>2.4±0.1</td>
</tr>
<tr>
<td>Thr146Ala</td>
<td>2.9±0.1</td>
</tr>
<tr>
<td>Asp274Ala</td>
<td>3.3±0.1</td>
</tr>
</tbody>
</table>

According to the metal binding study, Asp151Asn was reactivated much faster compared to native protein. Pro150Ala reactivated a little faster compared to Pro150Gly variant. Comparing these secondary coordination sphere mutants, apo-Asp112Ala reactivation shows that it needs lesser protein compared to Thr146Ala and Asp274Ala. In other words it needs 24 equivalents of zinc/protein to saturate half of apo-Asp112Ala. Apo-Thr146Ala and apo-Asp274Ala on the other hand, required 29 and 33 equivalents of metal/protein to reactivate half of the total apo-enzyme.

The experiment was further extended to study the binding kinetics of MPH-native and its few selected variants with different metal. The activity assay with foreign metals showed the cobalt containing enzyme showed the greatest activity compared to the other variants that were reconstituted with Zn(II), Mn(II), Cu(II) and Ni(II). Accordingly, the metal binding kinetic assay was performed for Co(II) reconstitution akin to the Zn(II) reconstitution assay to evaluate the catalysis rate of these enzymes. The binding kinetic data generated from this assay was then compared to the Zn$^{2+}$ binding kinetic that was generated earlier. Data obtained was analysed by non-linear regression using GraphPad Prism 6 software.
Table 3.6 shows the $K_d$ values obtained from the titration plot of Co(II) to apo-MPH native, Pro150Ala, Pro150Gly, Asp151Asn, Asp112Ala, Thr146Ala and Asp274Ala. According to the values, native MPH needs $10.47 \mu M$ of Co(II) to reactivate the apo-native.

Table 3.6. $K_d$ values (μM) for MPH native and its variants for kinetic as a function of cobalt concentration.

<table>
<thead>
<tr>
<th></th>
<th>$K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>10±1</td>
</tr>
<tr>
<td>Pro150Ala</td>
<td>23±1</td>
</tr>
<tr>
<td>Pro150Gly</td>
<td>12±1</td>
</tr>
<tr>
<td>Asp112Ala</td>
<td>9±1</td>
</tr>
<tr>
<td>Thr146Ala</td>
<td>13±1</td>
</tr>
<tr>
<td>Asp274Ala</td>
<td>5±1</td>
</tr>
</tbody>
</table>

The cobalt(II) binding kinetics conducted on link residue Pro150 revealed that Pro150Ala reactivation started at approximately $23 \mu M$ Co$^{2+}$ per protein molecule. The Pro150Gly variant was reactivated after binding $12 \mu M$ Co$^{2+}$ per protein. This observation clearly demonstrates that modification of a rigid residue (Pro) to a smaller amino acid (Ala) has weakened one or both of the metal binding in the active site. Alteration of this residue to a more flexible amino acid (Gly) nevertheless has retained the binding stoichiometry similar to the native.

Associating these observations with the secondary coordination residue mutants, Asp112Ala and Thr146Ala, reactivation was attained after the addition of $9 \mu M$ and $13 \mu M$ Co$^{2+}$ per apo-protein molecule respectively. Mutant Asp112Ala stoichiometry numbers are more or less similar to the native protein binding stoichiometry for the reactivation but it is a little higher for Thr146Ala. Alteration of Asp274 (secondary residue) to an Ala on the other hand needed less Co$^{2+}$ to reactive the protein.
3.11 References


DISCUSSION

4.1. Preface

This chapter discusses the results from experiments conducted to obtain insight into the metal centre of the metalloenzyme MPH. More specifically, it discusses the roles of the respective ligands in metal coordination, protein structure and catalytic function. A variety of experimental data has been compared to achieve this. This data includes a mutagenesis study of the primary and secondary coordination residues and the effect of the mutations on protein stability, metal binding affinity and enzyme function relative to metal concentration. It was believed that the nature and location of primary and secondary coordinating residues would influence the strength of the interaction between the protein and the metal ions.
4.2. Overall discussion

4.2.1. Protein purification

Ion exchange chromatography is chosen over His tag affinity column essentially to avoid protein stability interference. Association of MPH N-terminal of each subunit that binds into the groove on the surface of adjacent subunit is thought to cause instability of the oligomeric structure of the protein upon the incorporation of the polyhistidine-tag. The purification method (ion exchange chromatography) has successfully eliminated additional bands that were observed using a purification method previously established for MPH by Dr. Tee-Keang Ng (7). As can be seen in Figure 4.1 (A), the additional bands were thought to be the degradation products. The previously established method used cation exchange followed by size exclusion chromatography. The method devised and used throughout the course of this thesis consisted of two ion exchange columns used in tandem. Cation exchange chromatography was used first, followed by anion exchange chromatography. The combination of two ion exchange columns is unusual compared to other established protein purification methods (2, 3). However, here it has been used to successfully produce protein with a greater level of purity than previously achieved using the combination of cation exchange and gel filtration chromatography (Figure 4.1).

Application of the soluble lysate of MPH expressed in BL21 cells onto an SP sepharose cation exchange column removed a large number of proteins from the sample while retaining the target protein. The pooled fractions from the first column were passed through a Q sepharose anion exchange column. MPH was not retained strongly on this column and eluted early in the salt gradient, while other unwanted proteins were retained and subsequently separated to yield pure MPH. The pH was adjusted between the two columns, in the first a low pH of 5.5 (which
is below the pI of 7.8) was used such that the protein carried a positive charge. For the second column the buffer was changed to piperazine at pH 10, which is above the pI giving the enzyme an overall negative charge. The application of a step-wise salt gradient for the elution of proteins from each column was optimised by lengthening and narrowing the salt elution gradient to achieve maximum purity. Many proteins native to E. coli have a similar pI, as such the separation of MPH needed to be as precise as possible to successfully separate.

MPH is a dimeric protein that is extracted that exhibited little aggregation according to the gel filtration chromatography analysis. The dimeric form was catalytically active. MPH experimentally determined masses (Mass Spectroscopy) corresponds to the theoretical mass of MPH monomer with fully zinc occupancy which is 31534.5 Da and 31538.9 Da, respectively.
Figure 4.1. SDS-PAGE profile of MPH purified fractions. A. Fractions obtained after passing through SP Sepharose column and Superdex 200 column (/); B. Fractions eluted from SP Sepharose and Q Sepharose column. S denotes the standard marker, from top to bottom: 97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa. Numbers across the gel represents the fraction numbers.
4.2.2. Metal studies

Results from the metal analysis (Section 3.6.5) performed on native MPH (protein as purified) using ICP-OES indicated that the purified protein did not have full zinc occupancy. It is suggested that one of the metal ions was lost at some point during the purification process. Apart from that, MPH was expected to express at a very high level and there may not have been sufficient zinc(II) in the cell to fully form the binuclear centre. In addition, the metal binding results suggested that at least one of the sites did not bind metal tightly.

In the apo-making experiment result, removing secondary coordination sphere residues seems to allow metals (at least the weaker binding metal) to be removed more quickly (Figure 3.10), while removing the primary coordination ligands (Asp151Asn, Asp255Ala, His302Glu and His302Gln) seems to be slower or similar to that of native.

Reconstitution of the apo wild-type with native zinc metal successfully re-accommodated full metal ion (two zinc ions per monomeric unit) in the active site whereas cobalt-reconstitution re-accommodated one and a half cobalt ions per single subunit. This observation of incomplete cobalt re-constitution is surprising since the activity assay of the apo-native with foreign metals revealed that cobalt-containing enzyme showed the greatest activity. This is in comparison with the activity of MPH reconstituted with Zn(II), Mn(II), Cu(II) and Ni(II). In addition, the apo-enzyme reactivation profile shows that the second binding site of MPH does have tighter binding affinity towards cobalt ions. However, cobalt(II) occurs only rarely in enzymes and are probably only found in very low levels in *E.coli*.
Based on the activity of the apo-reconstituted enzyme, it is believed that two metal ions are required for catalysis. This is consistent with the previously mentioned observation whereby the activity of Co(II) and Zn(II)-reconstituted proteins showed a high level of activity when full (or almost full in the case of cobalt) occupancy was reached. In agreement with this statement, the low activity of the Mn(II)-substituted enzyme is explained by the lower amount of manganese, such that full occupancy was not reached.

4.2.3. Protein stability
As with the activity measurements, protein stability was assessed using both 'as purified' protein and protein with the addition of excess zinc. Mutation in the metal binding site caused significant change in the protein stability (Figure 3.15). The protein as purified exhibited stability similar to that of the apo-enzyme. It was noted that the addition of excess zinc(II) to saturate the two binding sites caused a significant increase in the stability of the protein. These observations imply that the metal ions render some assistance in the protein stability. This is in agreement with study performed on PTE where the protein maximum stability was achieved only with the presence of $\text{Zn}^{2+}$ (4).

Of the MPH variants with mutations to residues coordinating the $\alpha$ metal (Asp151Asn, Asp151Ala, His152Glu, His152Gln, His302Glu and His302Gln), the Asp151Asn and Asp151Ala mutations yield the most stable proteins. These proteins (Asp151Asn and Asp151Ala) have very low overall catalytic rate but high substrate binding affinity. To summarise, it can be seen that these proteins are essentially inactive but still hold their structural configuration and have comparatively high melting temperatures. The inactivity of these proteins probably could be explained by the role played by the acid group in the active
The native aspartic acid forms a hydrogen bond with the bridging hydroxide that is thought to act as a nucleophile toward the substrate and facilitate substrate hydrolysis. Replacing this acid group is predicted to replace the H-bond, which would simultaneously perturb the hydrolysis. Although activity is lost with mutation to either alanine or asparagine, the negligible effect on stability indicates that the structure and metal binding is not affected. This suggests that this amino acid (Asp151) is directly involved in the catalytic mechanism rather than playing a purely structural role. The Ala mutation is surprising, removal of Asp151 from the α centre does not perturb the metal binding as the protein still binds two metals and the stability is similar to the Asp151 Asn mutant.

Mutation to any of the other residues (aside from Asp151 Ala/Asn) responsible for ligation of the α metal (His152Glu, His152Gln, His302Glu and His302Gln) causes significant reduction in protein stability. The significant drop in stability for His152Gln and His302Gln mutants is possibly due to the distortion of one of the metal ions in the active site (ITC detects only 1 metal binding). Mutants His152Glu and His302Glu also show a significant drop in stability even though the ITC binding studies showed that these mutants bound two zinc ions. It is thought that the amino acid alteration caused some distortion to the α binding site and although the metal still binds the stability of the protein is affected. However the binding affinity for these mutants determined by ITC showed an increase by one order of magnitude to the affinity for the α site and a binding affinity for the β site of more or less the same as wild-type. This presumably explained that the increase in α binding affinity has retained the capability of holding the metal ions in the active site yet the tighter binding has also perturbed the stability of those proteins.
Mutant forms of residues 152 and 302 are among the least stable of all the variant forms of MPH. The stability (as shown in Figure 3.15) is only slightly higher than that of the protein as purified when it has less than one metal ion per subunit. The stability of these mutants is significantly lower that that of mutant forms in which changes to the coordination of the β site have been made. Mutation of residue 255 that coordinates the α site (and bridges the β site) also causes a greater drop in stability compared mutation affecting the α site. In addition, residues 112 and 146 that are found in the secondary coordination sphere of the α metal also cause a much more significant drop in the stability of the protein than do mutations to β site ligands. However, residue 274 that is linked to a ligand of the β site shows a relatively small drop in overall stability. All the above observations are consisted with the idea that modification of the α site causes a much greater drop in stability than do modifications to the β site. However, this is clearly not true for the mutations of residue 151 that causes a similar drop in stability as the mutations to the β site. It would appear that alteration of residues 152 and 302 cause structural changes that alter the metal coordination and leave the protein with much reduced stability while changes to residue 151 cause minor changes. The mutation at position 150 has a relatively minor effect on the overall stability of the protein. This residue links two residues that coordinate centre site metals and alteration have a significant effect on activity, but these mutations do not have a dramatic effect on metal binding and hence stability.

Mutation of His residues deserves a special note. The initial intention was to substitute these residues with amino acids that could also coordinate metal – acids and amides with a reach similar to that of the ε nitrogen of His. In some cases it is clear that this substitution has succeeded while in others it is not clear that this has happened (as will become clear in the discussion of the ITC results). In some instances these mutants have bound two metal ions with
an affinity similar to that of the native. However, the effects of changes to residues coordinating the α metal are quite different to one that coordinates the β metal. Changes to His residues coordinating the β metal have significantly higher stability than those that coordinate the α metal as is very clear from an inspection of Figure 3.15. The stabilities of mutations to residue 152 and 302 are much lower than those for 147, 149 and 234.

In closing this section it is worth noting that none of the mutant proteins have stabilities that match that of the native protein. Even minor changes to the secondary coordination sphere have a significant impact on the overall instability of the protein. All the data are consistent with the idea that native has used metals and stabilizes the overall structure of the protein – the stability is clearly linked to the metal content.

4.2.4. Kinetic characterisation

Site directed mutagenesis caused major changes to the catalytic activity of MPH. Modification performed on primary ligands (ligands that coordinates metal ions in the active site) imparts a more detrimental effect than do modifications of the link residues or the secondary coordination sphere residues. The loss of activity highlights the importance of the coordinating ligands in structural conformation and the placement of the metal ions in the retention of enzyme catalytic activity.

Kinetic study performed on MPH native and its variants revealed that the presence of excess zinc significantly enhances the $k_{cat}$ and $K_m$ for most of the enzymes. The activity of the purified protein was obtained as purified and also in the presence of excess zinc. The latter protein had greater activity. This indicates that there is insufficient amount of metal originally present in
the purified protein. This would suggest that the protein loses a metal ion during the course of sample preparation (purification), which is probably due to the weak metal binding. By supplying excess zinc in the buffer it is evident from the increased activity that the MPH active site becomes fully occupied which explains the substantial increase in the \( k_{cat} \).

The inactivity of His234Glu and His234Gln mutants highlights the importance of the histidine in this position as a metal ligating residue. The activity of these proteins does not improve with the presence of excess metal. It is thought that altering this histidine residue disrupts the coordination in the \( \beta \) site and subsequently affects the activity of the enzyme. The activity of these residues is for the worst part down by better than 3 orders of magnitude from the native activity (\( k_{cat} \)). For the purposes of further discussion this will be taken as background level.

In many ways the activity measurements contrast with the stability measurements described in the previous section. For example, changes to residue 151 cause a drop in stability, that is significantly less than the drop caused by changes to residues 152 and 302 yet the 151 mutants have barely discernable activity while the 152 and 302 mutants give clear (albeit weak) activity. Similarly, when mutated the histidine residues coordinating the \( \alpha \) metal give very poor stability, but measurable activity while those coordinating the \( \beta \) metal exhibit the opposite character – relatively good stability but background activity. It appears that the \( \alpha \) metal has a greater impact on the stability of the protein while the \( \beta \) metal is clearly more crucial for activity. Changes to residues 150 that links coordinating residues 149 and 151 causes a discernable drop in activity. One would anticipate this effect since slight alterations to the active site can give rise to significant changes in activity. However, one would also expect that removal of the linking residue (Asp255) would inactivate the protein. Clearly, this is not
observed. The activities of Asp255Ala and Asp255Asn are down by single digit factors and not orders of magnitude. This is probably due to a water molecule taking up the role played by Asp255. Although mutants of 2 of 3 secondary coordination sphere residues have caused a significant drop in stability, there is little effect on the kinetic parameters – they are very similar to those of the native protein.

4.2.5. Isothermal titration calorimetry (ITC)

It is proposed that the incorporation of Zn$^{2+}$ into the binuclear metal centre of each MPH subunit is a two-stage process consisting of (i) binding of the first metal into the site with strong binding affinity and (ii) binding of the second metal ion into the site with weaker affinity. The stepwise binding process is evident from observation of the ITC data where two distinct binding affinities (one strong and one weak) and two different $\Delta H^\circ$ are observed. The binding of Zn$^{2+}$ to the first site is approximately 1000-fold stronger than to the second site.

In defining the affinity of the metal binding sites and the occupancy of MPH, the crystal structure published by Dong, et al., (2005) has been examined (5). The study revealed that one of the MPH subunit has a hybrid Cd$^{2+}$/Zn$^{2+}$ centre. However, the metal binding centre was spectroscopically found to be Zn$^{2+}$. The presence of Cd$^{2+}$ is suspected to come from the crystallisation buffer where MPH was crystallised in the presence of Cd$^{2+}$.

Indeed, it is reasonable for a less tightly bound ion to be replaced by the Cd$^{2+}$, which proposed the occurrence of the mixed hybrid binuclear centre. The site with the tighter binding affinity on the other hand, is anticipated to retain the native metal in its position. According to the crystal structure, the Cd$^{2+}$ is found substituting the $\beta$ metal, a site that is more solvent exposed compared to the $\alpha$ metal site, which is more buried. It is therefore assume that the $\alpha$ metal has a
tighter binding affinity that is anticipated to bind first to the binuclear centre then followed by \( \beta \) metal in regards of having weaker binding constant. The results from the ITC and structural analysis are in agreement in this regard.

It should be noted that the observed binding constants sit at either end of the range of values measurable to ITC that is \( \sim 10^3 \) to \( \sim 10^9 \) \((6,7)\). Although binding constants can be measured for mutant proteins, it is not clear whether the active site structure has been preserved. It is unfortunate that MPH could not be crystallized in a form suitable for structure analysis.

Mutating active site residues of MPH causes changes in zinc binding to the protein. In a number of cases there is an increase in affinity due principally to a change in \( \Delta H \) where it becomes more negative. However, in most cases there is also a decrease in \( \Delta S \) that would have the effect of reducing the binding affinity.

It was not possible to monitor two binding events for all the mutants. One could interpret this observation to mean that only 1 metal bound to the protein, His152Gln would be a good example. However, this mutant exhibits similar levels of activity to the His152Glu mutant for which two binding events were identified. This latter observation is consistent with the idea the His152Gln binds 2 metals but only 1 was detected in the experiment described herein. Similarly, mutation made to other sites led to stability readings that were consistent with 2 metal binding events despite the fact that only 1 was detected with ITC. The His152Glu and His302Glu residues both coordinates the \( \alpha \) metal and both gave significantly tighter binding for the first event as would be consistent with the replacement of a neutral residue with a negatively charged residue.
Altering the secondary residues has not completely damaged the protein-metal coordination, however such alterations have slightly affected the binding constants. The metal binding affinity for MPH variants with mutations to secondary coordinating residues are changed due to changes they cause in hydrogen bonding and metal coordination involving residues in the first coordination sphere within the active site. The exact effects are dictated by the location of the secondary residue in question and the interaction between its connecting primary ligand. In summary, this site directed mutagenesis study has provided insight into how the coordinating residues, primary and secondary have tuned the strength of the metal ion binding.

4.3. Discussion by individual residue

4.3.1. Primary coordination sphere residues

4.3.1.1. Primary coordination residues (a centre)

i) Asp151

Asp151 is a key residue in coordinating the binuclear metal centre of MPH. It is part of a sequence motif (HXHDXD) that is found in numerous other enzymes. It makes a coordinate link to the α metal and a hydrogen bond to the bridging hydroxide and in doing so modulates the properties of that ion. It is one of only two acid residues that coordinate the active site metals. Asp151 is located such that it can coordinate the α but not the β metal while Asp255 can coordinate either the α or β metal. It is the close proximity of Asp151 to the α metal that suggests that metals bind to the α site much more tightly than to the β site.

The objective of the present set of experiments was to mutate Asp151 and monitor the effects on metal binding and catalytic activity. Removing the charged group of Asp151 as in the Asp151Ala mutant had surprisingly little effect on metal binding (Figure 4.2). The first metal
bound with an affinity very similar to that of the native protein while the second metal bound with a slightly lower affinity. It is probable that a water molecule would bind in place of the side chain of Asp151 and the mutation would not prohibit the bridging hydroxide from binding. However, this mutation could affect the structure of the metal centre and the position of the bridging hydroxide as well as the charge on this ion – both of these factors may be the cause of the reduced binding affinity at the β site. A shift in the protein of the bridging hydroxide and a change in its charge are likely the causes of the low of activity that are exhibited by the mutant protein. The dependence of activity on metals suggests that two metals are required for activity. Little activity is observed at sub-micromolar concentrations of substrate (see Figure 3.18) and activity increases as binding occurs at the second site.

The Asp151Asn mutation still allows Asp151 to make a coordinate link with the protein as well as a hydrogen bond to the bridging hydroxide, but both interactions will be quite different to those of the native protein. However, the binding affinities are very close to those of the native protein. The first binding event is tighter than that of the native protein, but is subject to a large error that makes it hard to distinguish from the native. The second binding event is clearly indistinguishable from the native protein when errors are taken into account.

The Asp151Asn mutant is a proton donor in the hydrogen bond donor to the bridging hydroxide, even if the position of the bridging hydroxide is the same in the native and Asp151Asn mutant, the nucleophilicity and basicity will be dramatically different to that of the native protein and it is not surprising that it has very little activity.
The similarity of the pH profiles for the activities of the native and the Asp151Asn proteins is surprising – one possibility is that the activity of Asp151Asn mutant is due to native protein produce (at very low level) by translational mis-incorporation.

The present study demonstrates that Asp151 can be easily replaced with little effect on the ability of the protein to bind metals, but it is essential for the catalytic activity of the protein. It must ensure that the bridging hydroxide is appropriately activated for catalysis and that it is in the correct position with respect to the substrate. The stability of the Asp151Asn and Asp151Ala mutants suggest that structures of these proteins differ from that of the native protein and that it is not the ionisation state of the bridging hydroxide alone that causes the dramatic drop in activity.
Figure 4.2. Comparison of D151 variant results. A. Structure of MPH active site locating Asp151; B. Asp151Asn pH profile comparison with native pH profile; C. Comparison of Asp151 variants metal binding, kinetic and stability results.
Both His residues coordinating the α site were converted to Gln and Glu. Three of four mutants exhibited easily detected activity that was well above background for example, His152Glu had a $k_{cat}$ value that was about 12% that of the native protein (Figure 4.3). However, ITC only gave two metal binding affinities for His152Glu and His302Glu. All four mutants were only a little more stable than the apo-protein, and suggesting that their structures had been altered by the mutations and that this was probably the cause of the drop in affinities.

The His152Glu and His302Glu mutants both exhibit higher binding constants for the first event, consistent with the fact that the negatively charged acid residues would bind the α ion more tightly. The second binding constant for His152Glu is down almost an order of magnitude, but the mutant retains some level of activity. Conversely, the second binding event for His302Glu is very similar to that of the native protein, but it has barely detectable activity.

The above results are consistent with the idea that His152 and His302 have little direct influence on the activity of MPH, but are necessary to bind the metals in the position that is optimal for activity. It would also appear that structural alterations suggested by the poor stabilities of these mutants do not cause the activity to drop to background levels, as was found with the Asp151 mutants. In other words, the activity of the protein is more tolerant of changes in residues His152 and His302 while the overall stability of the protein is very sensitive to the same changes.
Figure 4.3. Comparison of α variant results. A. Structure of MPH active site locating Asp151, Asp152 and His302; B. Comparison of α variants metal binding, kinetic and stability results.
4.3.1.2. Primary coordination residues of the β site

i) His147, His149 and His234

As in previous section the His residues of the β site were converted to Glu and Gln. All have stabilities that are similar to those of Asp151Ala and Asp151Asn (Figure 4.4). That is stabilities that are about mid-way between that of the apo-protein and the native protein with two metals bound. These stabilities are much higher than mutants of the His residue coordinating the α site. This observation is consistent with the mutant proteins binding 2 metals. However, none of these mutant proteins has activity that is significantly above background. The ITC experiments exhibited two binding sites for only the His149Gln and His234Gln mutants. In both cases the binding constants for the α site ($K_1$) are similar to that of the native protein while affinities for the β site are down compared to that of the native protein.

It appears that activity is very sensitive to changes at the β site, but these same changes have a far less dramatic effect on the overall stability of the protein.
Figure 4.4. Comparison of β variant results. A. Structure of MPH active site locating His147, His149 and His234; B. Comparison of β variants metal binding, kinetic and stability results.
4.3.2. Bridging residue

i) Asp255

Asp255 was mutated to an Ala and an Asn. The first mutation removes the acid group of the residue, but allows a water molecule in to take up its coordination site. The second mutation replaces the acid residue with an amide group that could still link the two metals. Both mutants exhibit detectable activity that is well above background (Figure 4.5). The Asp255Ala mutant has a $k_{cat}$ value that is close to 30% of the native protein and has a $K_m$ value that is a little higher than that of the native protein. The fact that these two mutants have reasonable level of activity suggests that both bind two metals. However, only the Asp255Ala mutant gave rise to an ITC trace that was consistent with two metal binding metals. This mutant bound the first metal with higher affinity than the native protein. The second metal bound with only slightly lower affinity than the native protein. The stabilities of these two mutants is above the stabilities for the His mutations at the $\alpha$ site and less than that of the His at the $\beta$ site. The drop in stability due to mutations of Asp255 is consistent with a structural change that does not obliterate activity. In other words, Asp255 is used primarily to position the metals for optimal activity.
Figure 4.5. Comparison of bridging ligand variant results. A. Structure of MPH active site locating Asp255; B. Comparison of Asp255Ala and Asp255Asn metal binding, kinetic and stability results.
4.3.3. **Link residue**

i) **Pro150**

Residue Pro150 is connected to both metals in the active site through the engagement with Asp151 that coordinates the α metal and His149 that coordinates the β metal. Altering Pro to an Ala is predicted to allow both primary ligands more freedom of movement, as these ligands will no longer be held by such a rigid segment of peptide backbone as provided by the proline. Therefore, the substitution was expected to cause some modifications on the active site of this protein. According to the binding study, Pro150Ala has weakened the second metal binding by an order of magnitude and retained the first metal binding slightly down on that of the wild-type (Figure 4.6). The change has altered the substrate binding site by decreasing the $k_{cat}$ by three-fold. The structure rearrangement does not greatly affect the protein stability.

Changing the rigid proline residue to Gly, an even more flexible residue than Ala, was predicted to distort both binding sites where the flexibility may allow Asp151 and His149 a greater degree than the Ala mutation. The change however, has caused a subtle modification on the α metal binding. The change has increased the α and β binding compared to that of wild-type MPH. This binding modification considerably affected the catalytic activity by reducing the $k_{cat}$ by 40-fold. In summary, alteration of the binding site did not modify the whole protein structure suggesting that Pro150, which is not a conserved residue, has not provided any importance in the protein structure. It does however, play a role in metal coordination and changes can significantly affect catalysis, via slight changes in metal coordination.
Figure 4.6. Comparison of link residue variant results. A. Structure of MPH active site locating Pro150; B. Comparison of Pro150Ala and Pro150Gly metal binding, kinetic and stability results.
4.3.4. Secondary coordination sphere residues

Three secondary coordination sphere residues were identified that made links with residues coordinating metals. Each of these residues was converted to Ala residues. Activity and ITC results were consistent with two metals being taken up by the mutant protein.

The activities of the three mutants were similar to that of the native protein with the Asp112Ala mutant exhibiting higher $K_m$ and $k_{cat}$ compared to the native protein (Figure 4.7). This mutant gave a higher value for the first binding event, but the error in this determination was also high.

The second binding event had an affinity similar to that of the native protein and higher than the other two mutants. This was consistent with the $K_d$ values attain in the metal titration (Table 3.5). The stability of this mutant was only slightly higher than that of the apo-protein. This drop in stability was similar to that induced by mutating the His residues to the $\alpha$ metal. This mutant also was more quickly converted to the apo-enzyme that was the native protein (Figure 3.10).

The stability of the protein and metal lability appear to be very sensitive to changes in the secondary coordination sphere.

The mutation of Thr146 caused little change in the kinetic properties of MPH but it did cause a significant drop in the stability of the protein (Figure 4.7) with little effect on the ease of forming the apo protein (Figure 3.10).

The Asp274Ala mutation was different to the other two secondary coordination sphere mutants in that it did not cause a significant drop in stability. Like the other two mutants it exhibited activity similar to that of the native protein. As can be seen in Figure 3.10, this mutation also gave rise to increase ease in the formation of the apo-protein.
In summary, the effects of mutating secondary coordination sphere residues were varied. None seemed to have a dramatic effect on the activity of the protein and ease of forming the apo-enzyme were varied.

Figure 4.7. Comparison of secondary coordination residue variant results. A. Structure of MPH active site locating Asp112, Thr146 and Asp274; B. Comparison of secondary coordination residues metal binding, kinetic and stability results.
4.4 Concluding remarks

During the course of this research it became apparent that experimental plan had not been adequately throughout or that the choice of experimental techniques limited the conclusions that could be drawn. The scholarship supporting this research had a 3.5 year tenure and this placed limitations on what could be done.

There is one aspect of the research that was poorly throughout. Residue 149 coordinates the metal through its δ N rather than the ε N that is the case for the other His ligands. In this case it should have been mutated to Asp and Asn. His149Glu and His149Gln mutations are very likely to be too bulky to allow the metal to bind in the β site. The His149Gln mutant did give rise to two binding sites; $K_1$ was similar to that of the native protein while $K_2$ was down by a factor of 4 on the native protein. This observation suggests that there is some flexibility in metal coordination. However, this mutant showed little activity compared to the native protein like the other β site mutants. The coordination of His149 was not noticed till late in the research and alternative mutants could not be made or characterized. In addition, given the results obtained with mutants of His147 and His234, it was not thought that pursuit of mutants of His149 would be a good use of time.

The technique that would have provided a great deal of useful information was x-ray crystallography, although there was a structure available, attempts to grow crystal suitable for structure determination were unsuccessful. This work was carried out by other members of the laboratory and involved trying to use published crystallization conditions as well as using commercial screens to identify new crystallization conditions. Structures of the mutant proteins
would have been invaluable in determining the extent of structural change caused by mutations.

Metal analysis would have also provided confirmation of the extent metal binding in mutant proteins. However, the amount of time required to prepare protein required for these experiments would have meant that other experiments could not have been done. The binding constant determined by ITC implied that the “excess” zinc added to the mutant proteins should have saturated both sites.

Despite any limitations on the experimental design or methods, a great deal has been learnt. It is clear that nature has related active site residues to give good activity and stability. The stability of MPH is very dependent upon the active site metals. This could be due to the two active site acid residues. These are both negatively charged and would repel one another in the absence of positively charged metals – similar suggestions have been made previously, based on genetic evidence (8). In this case direct observations of changes in stability have been made. It is also clear that changes to active site residues can have quite different effects on stability.

Throughout the discussion, it has frequently assumed that changes in stability would be caused by or associated with changes in structure. There is no experimental evidence to support this and it should be clear that large changes in stabilities do not necessarily imply large changes in structure. However, it seems reasonable to assume that stability and structure will be related.
One disappointment aspect of this work is the inability to say much about catalytic mechanism. There are two prevailing theories as to how binuclear metal centres function. One uses the bridging hydroxide as a nucleophile, while the other uses it as a base to abstract a proton from terminal water in the β metal. The data collected so far do not allow a clear distinction to be made between these two mechanisms. The inactivity of the Asp151Asn mutant would tend to favour the bridging hydroxide as the nucleophile, while the abolition of activity by mutating the β metal ligands would tend to favour the terminal water as the nucleophile. However, these interpretations could be better labelled as speculations.

A summary of the results and suggestions for further work are to be found in the final chapter.
4.5 References


The binuclear metalloenzymes have a multitude of functions. Because of their importance a great deal is known about their functions, their structures and even their catalytic mechanism. However little has been published about the metal ion coordination that forms the heart of these important catalyses. This study set out to probe the metal coordination of one binuclear metalloenzyme. The aim was to determine the binding constants for the two metals, the role of selected amino acids (the first and second coordination sphere) in binding metals and whether activity could be obtained with a single metal in the active site. It was hoped that this study would give some insight into how binuclear metal centers form and also into the catalytic mechanism of the protein being studied.

As can be seen in the foregoing chapters a great deal of work is required in accomplishing the objective of the proposed study. This has limited the study to a single protein, that is MPH. Other organophosphate degrading enzymes had been studied in the lab, but they were not considered appropriate for the proposed work. The OPDA protein coordinated metals through a carbamylated lysine that would have complicated experiments (it would have meant that
dissolved CO₂ would have had to be controlled for example). As it stands this work with MPH has been limited by the inability to produce useful crystals of the native and mutant proteins. However, this deficiency is to some extend tied up with the relationship between metal content and protein stability.

The observation that the stability of MPH depends upon metal content is intriguing. It has first been reported by Fersht (1) that enzyme active site evolve and optimise catalysis and that this can result in a low or reduction in the overall stability of the protein. In the case of MPH, it appears as if the protein structure has been designed to accommodate the two metals and that the overall stability of the protein depends critically upon their presence whether this is the case in other binuclear metalloenzymes remains to be seen.

A cursory examination of the metal coordination site of MPH, reveals two distinct coordination sites. The α site has two acid groups (one of which is shared with the β site) and appears to be the one more likely to bind incoming metals with higher affinity. All of the amino acids could be mutated in such a way as to allow two metals to bind and with the exceptions of the Asp151 mutants, low levels of activity were observed. Similarly, the results of the previous chapter suggest that there is good evidence to support the idea that each of the β site residues could be mutated in such a way as to allow both metals to bind.

It is remarkable that mutating each of the coordinating residues, one at a time, did not abolish metal binding. However, at this stage it is not clear how each of the mutations affected the metal centre. Mutations to Asp151 abolished activity, yet the overall stability of the protein was retained. This residue is clearly very important for catalytic activity. Mutations to residues
His152 and His302 give rise to mutants which retain low levels of activity, while giving rise to significant reductions in overall stability. Conversely, mutations made to ligands at the β site resulted in background levels of activity, with levels of stability similar to that of the Asp151Asn mutant. This result was quite surprising. Clearly, the coordination of the β site is crucial for the activity of the protein. Whereas, mutations to the α site as well as the bridging ligand, give rise to variants with low levels of activity, no much activity was obtained for His147, His149 or His234. Clearly, changes to the metal ligands affect the activity in a variety of ways that were unanticipated at the start of this work.

As noted in the previous chapter, most of the goals of this study have been achieved. As in the case of any study of the enzyme, more can be done to gain still more information. In the present case, it is clear that more effort needs to be made to gain structural information for the mutant forms of MPH or some other binuclear metalloenzymes. The Asp151Asn mutant deserves special mention in this regard. It is clear that this mutant binds two metals, retains reasonable overall stability yet has very little activity. If obtained in crystallize form, it may be possible to obtain the low temperature (frozen) structure of the protein with substrate bound. This may shed some light on the mechanism of the protein. Does the mutant lose activity because of structural changes or is the low of activity due to protonation of the bridging hydroxide?

The fact that all of the ligands can be replaced by other amino acids leads to the question of whether an amino acid ligand is required in all cases. By replacing the acid groups (Asp151 and Asp255) with Ala, water has been allowed to coordinate in some cases without total loss of activity. Similar experiments should have been carried out with the His ligands. These mutants
will clearly bind metals differently to the wild-type protein and the Glu and Gln mutants and they should therefore provide additional information.

Some arguments could be made for making multiple mutations to a single protein. Could the mutant with Asp151Ala and Asp255Ala mutation bind 2 metals and if so with what affinity studies like this could address questions as to whether amino acid ligands are required to bind metal tightly or rather to place metals in locations appropriate for catalysis.

Much of the time devoted to the present study has been taken up with the preparation of materials for physical characterisation. This has to a great extend limited the number of experiments that have been done. Technical advances have will mean that much of this type of work will be done much more quickly in the future. This technical advances combined with experience gained in the present study will mean that future attempts at understanding binuclear metal centres will be much more productive. It may be possible in future studies to engineer metal centres. This may be to better accommodate different metals for the purpose of binding toxic metals. Alternatively it may be possible to alter or simply enhance catalytic activity.

5.2 References

Appendix A: Suppliers of equipments, consumables and chemicals

Equipment and Consumable Suppliers

<table>
<thead>
<tr>
<th>Equipments and consumables</th>
<th>Manufacturers and suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>5415 Microcentrifuge</td>
<td>Eppendorf</td>
</tr>
<tr>
<td>5804 Centrifuge</td>
<td>Eppendorf</td>
</tr>
<tr>
<td>AKTA FPLC System</td>
<td>G.E. Healthcare</td>
</tr>
<tr>
<td>Amicon Ultra-15 Centrifugal Filter Unit</td>
<td>Millipore</td>
</tr>
<tr>
<td>ASB270BT Autoclave</td>
<td>Astell Scientific</td>
</tr>
<tr>
<td>C1000 Thermal Cycler</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>CO8000 Cell Density Meter</td>
<td>WPA Biowave</td>
</tr>
<tr>
<td>French Pressure Cell Press</td>
<td>SLM Instruments</td>
</tr>
<tr>
<td>GeneAmp PCR System 9700</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>HisTrap QFF Column</td>
<td>G.E. Healthcare</td>
</tr>
<tr>
<td>HiTrap Desalting Column</td>
<td>G.E. Healthcare</td>
</tr>
<tr>
<td>HiTrap SP FF Column</td>
<td>G.E. Healthcare</td>
</tr>
<tr>
<td>I-Cycler</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Perkin-Elmer</td>
</tr>
<tr>
<td>MicroPulser Electroporator</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>MJ Mini Personal Thermal Cycler</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>NanoDrop® ND-1000 Spectrophotometer</td>
<td>NanoDrop</td>
</tr>
<tr>
<td>Orion ROSS Combination pH Electrode</td>
<td>Orlon Pacific Pty Ltd</td>
</tr>
<tr>
<td>Q Sepharose Affinity Column</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>QIAprep Spin Miniprep kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>QIAquick PCR Purification Kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>R20A2 and R9A Rotors for VX22G</td>
<td>Hitachi</td>
</tr>
<tr>
<td>Rotorfix 32 Centrifuge</td>
<td>Hettich</td>
</tr>
<tr>
<td>SE250 Mighty Small II Mini Vertical Electrophoresis Unit</td>
<td>Hoefer</td>
</tr>
<tr>
<td>SpectraMax® M2/M2e Microplate Reader</td>
<td>Molecular Devices</td>
</tr>
<tr>
<td>Superdex 200 Size Exclusion Column</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>UV transilluminator 312 nm with UVItec camera</td>
<td>Hanimax Statesman</td>
</tr>
<tr>
<td>Varian Cary UV/Vis Spectrophotometer</td>
<td>Varian</td>
</tr>
<tr>
<td>Veriti 96-Well Thermal Cycler</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>VP-ITC</td>
<td>Microcal</td>
</tr>
<tr>
<td>VX22G High Speed Centrifuge</td>
<td>VWR</td>
</tr>
<tr>
<td>Wide Mini Sub Cell Electrophoresis Tank</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Wizard® SV Gel and PCR Clean-Up System</td>
<td>Promega</td>
</tr>
</tbody>
</table>
## Chemical Suppliers

**Chemicals**
- 1 kb DNA Marker
- 1,10-phenanthroline
- 2-propanol
- 2,6-pyridine dicarboxylate
- 3-methyl-1-butanol
- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- 8-hydroxyquionoline-5-sulfonic acid
- Acetone
- Acetonitrile
- Acryl/Bis 37.5:1, 40% (w/v)
- Agar
- Agarose
- Ammonium Persulfate
- Ampicilin
- ASEL and Buffer
- β-mercaptoethanol
- BigDye® Terminator and Buffer
- BioTaq™ Polymerase
- bis-p-(nitrophenyl) Phosphate
- Bromophenol Blue
- BugBuster® Protein Extraction Reagent
- Calf Intestinal Alkaline Phosphatase and Buffer
- Chelex
- Dimethyl Sulfoxide
- dNTPs
- DpnI and Buffer
- Dual Color Precision Plus Protein Standards
- EcoRI and Buffer New England
- EDTA
- Ethanol
- Ethidium Bromide
- Ethyl Parathion
- Gel Filtration Markers Kit 12,000 - 200,000 Da
- Glycerol
- Glycine
- HCl
- HNO₃
- Imidazole
- Isopropyl β-D-1-thiogalactopyranoside
- K₂HPO₄
- KH₂PO₄
- Low Range SDS-PAGE Marker
- Methanol

**Manufacturers and suppliers**
- New England Biolabs
- Sigma Corporation
- Merck
- Sigma Corporation
- Sigma Corporation
- Amresco
- Sigma
- Merck
- Sigma Corporation
- Amresco
- Difco
- Sigma Corporation
- Sigma Corporation
- Sigma Corporation
- Amresco
- New England Biolabs
- Sigma Corporation
- BRF, JCSMR
- Bioline
- Sigma Corporation
- Sigma Corporation
- Novagen
- New England Biolabs
- Bio-Rad
- Sigma Corporation
- Roche
- New England Biolabs
- Bio-Rad
- Biolas
- Ajax Chemicals
- Merck
- Sigma Corporation
- Chemservice
- Sigma Corporation
- Merck
- Amresco
- Ajax Chemicals
- Merck
- Sigma Corporation
- Astral
- Ajax Chemicals
- Ajax Chemicals
- Bio-Rad
- Merck
Methyl Parathion
MgCl₂
MnCl₂
N,N,N',N'-Tetramethylethylenediamine
NaCl
NaOH
Native Pfu Polymerase and Buffer
Nde I and Buffer
Nutrient Broth
p-nitrophenol
Phenylmethylsulfonyl fluoride
Phusion Polymerase
Piperazine
Polyethylene Glycol 8000
Propylene Glycol
Pst I and Buffer
RedSafe™ DNA Stain
rLysozyme™
Sodium Acetate
Sodium Dodecyl Sulfate
T4 DNA Ligase

Taq Polymerase
Tris Base
Tryptone
Tween 20
Yeast Extract

Chemservice
Sigma Corporation
Ajax Chemicals
Sigma Corporation
Chem Supply
Ajax Chemicals
Thermo Scientific
New England Biolabs
Difco
Sigma Corporation
Sigma Corporation
Finnzyme; New England Biolabs
Sigma Corporation
Fluka
Fluka
New England Biolabs
Chembio
Novagen
ICN Biomedical
Amresco
Thermo Scientific; Fermentas
New England Biolabs
Roche
Amresco
Difco
Sigma Corporation
Difco
Appendix B: Recipes

**Bromophenol Blue Loading Dye (for DNA agarose electrophoresis)**

This solution contained 30% (v/v) glycerol and 0.25% bromophenol blue in dH$_2$O.

**LB (Luria-Bertani)**

This media contained 10 g of tryptone (Difco), 5 g of Bacto yeast extract (Difco) and 10 g of NaCl (for 1000 ml of water). This media was sterilised by autoclaving. A final concentration of 100 µg/mL of ampicillin (Amresco) was added into the media once had cooled to approximately 60 °C.

**Organophosphate Substrate Stock**

As much as 18.99 mM of methyl parathion was dissolved in 100% methanol.

**SB Buffer (for DNA agarose electrophoresis)**

20x stock solution contained 8 g of NaOH was prepared in 1 L dH$_2$O. Final pH was adjusted to pH 8.0.

**SDS Running Buffer (for SDS-PAGE)**

10x stock solution contained 144 g glycine, 30 g Tris base and 10 g SDS was prepared in 1 L of dH$_2$O.

**SDS-PAGE stock Sample Buffer**

The solution contained 120 µl of 0.1% (w/v) bromophenol blue, 1.12 ml of 80% (v/v) of
glycerol, 800 μl of 2 M Tris buffer at pH 6.8, 2 ml of 10 % (w/v) SDS and 0.31 g of dithiothreitol. Water (dH2O) was added to a final volume of 10 ml and as much as 10 μl of β-mercaptoethanol was added to every 190 μl of sample buffer.

**Terrific Broth**

A mixture of 900 ml of Tryptone (12 g), Yeast Extract (24 g) and Glycerol (4 ml) was prepared. A separate mixture 100 ml of KH2PO4 and K2HPO4 was also prepared prior autoclave- sterilizing. Both solutions were allowed to cool to room temperature before mixing them together to make the media into 1 L.

**YENB (Yeast Extract Nutrient Broth)**

As much as 7.5 g of Bacto yeast extract (Difco) and 8 g Bacto Nutrient Broth (Difco) were dissolved in 1000 mL water and sterilized with autoclaving.
### Appendix C: Oligonucleotide primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mph D151N(F)</td>
<td>Forward D151N mutagenic</td>
<td>5′-ATG CAT CCC AAT CAT GTT GGT GGT CTG-3′</td>
</tr>
<tr>
<td>mph D151N(R)</td>
<td>Reverse D151N mutagenic</td>
<td>5′-CAG ACC ACC AAC ATG ATG GGT CTT GGT CTG-3′</td>
</tr>
<tr>
<td>mph D151A(F)</td>
<td>Forward D151A mutagenic</td>
<td>5′-ATG CAT CCC GCT CAT GTT GGT GGT CTG-3′</td>
</tr>
<tr>
<td>mph D151A(R)</td>
<td>Reverse D151A mutagenic</td>
<td>5′-CAG ACC ACC ATG AGG ATG GGT CAT-3′</td>
</tr>
<tr>
<td>mph H152E(F)</td>
<td>Forward H152E mutagenic</td>
<td>5′-ATG CAT CCC GAT GAA GGT GGT CTG ATG-3′</td>
</tr>
<tr>
<td>mph H152E(R)</td>
<td>Reverse H152E mutagenic</td>
<td>5′-CAT CAG ACC ACC AAC TCT ATG CGG ATG CAT-3′</td>
</tr>
<tr>
<td>mph H152Q(F)</td>
<td>Forward H152Q mutagenic</td>
<td>5′-ATG CAT CCC GAT CAA GTT GGT GGT CTG ATG-3′</td>
</tr>
<tr>
<td>mph H152Q(R)</td>
<td>Reverse H152Q mutagenic</td>
<td>5′-CAT CAG ACC ACC TTG GCT ATG CAT-3′</td>
</tr>
<tr>
<td>mph H302E(F)</td>
<td>Forward H302E mutagenic</td>
<td>5′-ATG CAT CCC GAT CAA CTG AGC TTT CCG GGT-3′</td>
</tr>
<tr>
<td>mph H302E(R)</td>
<td>Reverse H302E mutagenic</td>
<td>5′-ACC CGG AAA GCA TCC GCT TGC TGC AAT-3′</td>
</tr>
<tr>
<td>mph H302Q(F)</td>
<td>Forward H151 mutagenic</td>
<td>5′-ATG CAT CCC GAA CTG AGC TTT CCG GGT-3′</td>
</tr>
<tr>
<td>mph H302Q(R)</td>
<td>Reverse H151 mutagenic</td>
<td>5′-ACC CGG AAA GCA TCG GCT TGC TGC AAT-3′</td>
</tr>
<tr>
<td>mph H147E(F)</td>
<td>Forward D151N mutagenic</td>
<td>5′-ATG CAT CCC GAT CAA CTG AGC TTT CCG GGT-3′</td>
</tr>
<tr>
<td>mph H147E(R)</td>
<td>Reverse D151N mutagenic</td>
<td>5′-ACC CGG AAA GCA TCG GCT TGC TGC AAT-3′</td>
</tr>
<tr>
<td>mph H147Q(F)</td>
<td>Forward D151N mutagenic</td>
<td>5′-ATG CAT CCC GAT CAA CTG AGC TTT CCG GGT-3′</td>
</tr>
<tr>
<td>mph H147Q(R)</td>
<td>Reverse D151N mutagenic</td>
<td>5′-ACC CGG AAA GCA TCG GCT TGC TGC AAT-3′</td>
</tr>
<tr>
<td>mph H149Q(F)</td>
<td>Forward D151N mutagenic</td>
<td>5′-ATG CAT CCC GAT CAA CTG AGC TTT CCG GGT-3′</td>
</tr>
<tr>
<td>mph H149Q(R)</td>
<td>Reverse D151N mutagenic</td>
<td>5′-ACC AAC ATG ATC CCG GGT CAT-3′</td>
</tr>
<tr>
<td>mph H149E(F)</td>
<td>Forward D151N mutagenic</td>
<td>5′-ATG CAT CCC GAT CAA CTG AGC TTT CCG GGT-3′</td>
</tr>
<tr>
<td>mph H149E(R)</td>
<td>Reverse D151N mutagenic</td>
<td>5′-ACC AAC ATG ATC CCG GGT CAT-3′</td>
</tr>
<tr>
<td>mph H234E(F)</td>
<td>Forward D151N mutagenic</td>
<td>5′-ATG CAT CCC GAT CAA CTG AGC TTT CCG GGT-3′</td>
</tr>
<tr>
<td>mph H234E(R)</td>
<td>Reverse D151N mutagenic</td>
<td>5′-ACC AAC ATG ATC CCG GGT CAT-3′</td>
</tr>
<tr>
<td>mph H234Q(F)</td>
<td>Forward D151N mutagenic</td>
<td>5′-ATG CAT CCC GAT CAA CTG AGC TTT CCG GGT-3′</td>
</tr>
<tr>
<td>mph H234Q(R)</td>
<td>Reverse D151N mutagenic</td>
<td>5′-ACC AAC ATG ATC CCG GGT CAT-3′</td>
</tr>
<tr>
<td>mph P150A(F)</td>
<td>Forward D151N mutagenic</td>
<td>5′-ATG CAT CCC GAT CAA CTG AGC TTT CCG GGT-3′</td>
</tr>
<tr>
<td>mph P150A(R)</td>
<td>Reverse D151N mutagenic</td>
<td>5′-ACC AAC ATG ATC CCG GGT CAT-3′</td>
</tr>
<tr>
<td>mph P150G(F)</td>
<td>Forward D151N mutagenic</td>
<td>5′-ATG CAT CCC GAT CAA CTG AGC TTT CCG GGT-3′</td>
</tr>
<tr>
<td>mph P150G(R)</td>
<td>Reverse D151N mutagenic</td>
<td>5′-ACC AAC ATG ATC CCG GGT CAT-3′</td>
</tr>
<tr>
<td>mph D255A(F)</td>
<td>Forward D151N mutagenic</td>
<td>5′-ATG CAT CCC GAT CAA CTG AGC TTT CCG GGT-3′</td>
</tr>
<tr>
<td>mph D255A(R)</td>
<td>Reverse D151N mutagenic</td>
<td>5′-ACC AAC ATG ATC CCG GGT CAT-3′</td>
</tr>
<tr>
<td>mph D255N(F)</td>
<td>Forward D151N mutagenic</td>
<td>5′-ATG CAT CCC GAT CAA CTG AGC TTT CCG GGT-3′</td>
</tr>
<tr>
<td>mph D255N(R)</td>
<td>Reverse D151N mutagenic</td>
<td>5′-ACC AAC ATG ATC CCG GGT CAT-3′</td>
</tr>
<tr>
<td>mph D274A(F)</td>
<td>Forward D151N mutagenic</td>
<td>5′-ATG CAT CCC GAT CAA CTG AGC TTT CCG GGT-3′</td>
</tr>
<tr>
<td>mph D274A(R)</td>
<td>Reverse D151N mutagenic</td>
<td>5′-ACC AAC ATG ATC CCG GGT CAT-3′</td>
</tr>
<tr>
<td>F_SSM_FlankMIT</td>
<td>Forward flanking primer for MPh with signal in pETMCS1 and pCY76. Contains Asel site</td>
<td>5′-GCT TCT ATT AAT GGC AGC CCA GGT T-3′</td>
</tr>
<tr>
<td>R_SSM_FlankMIT</td>
<td>Reverse flanking primer for MPh without signal in pETMCS1 and pCY76. Contains EcoRI site</td>
<td>5′-TGC CAT GAA TTC TTA TTA TTA TTT CGG ATT CAC-3′</td>
</tr>
</tbody>
</table>

154
Appendix D: Calorimetric titration curves (ITC)

1. Wild-type

2. Asp151Asn
3. Asp151Ala

4. His152Glu
5. His152Gln

6. His302Glu
7. His302Gln

![Graph showing His302Gln's analysis.](image)

8. His147Glu

![Graph showing His147Glu's analysis.](image)
9. His147Gln

10. His149Gln
11. His149Glu

12. His234Glu
13. His234Gln

14. Pro150Ala
15. Pro150Gly

16. Asp255Ala
17. Asp255Asn

18. Asp122Ala
19. Thr126Ala

20. Asp274Ala
Appendix E: Apo protein reactivation curves

Reactivation of apo-native with varying amounts of Zn(II). Apo-enzyme (100 nM) was incubated independently overnight at different zinc ion concentrations. Fitted with one site-specific binding with Hill slope. $V_{max}: 115.1 \pm 4.7; K_d: 2.8 \pm 0.1; R^2: 0.9745$.

Asp151Asn reactivation plot. Apo-enzyme (100 nM) was incubated independently overnight at different zinc ion concentrations (nM). Fitted with one site-specific binding with Hill slope. $V_{max}: 94.4 \pm 1.5; K_d: 0.9 \pm 0.1; R^2: 0.985$. 
Pro150Ala reactivation plot. Apo-enzyme (100 nM) was incubated independently overnight at different zinc ion concentrations (nM). Fitted with one site-specific binding with Hill slope. \(V_{\text{max}}: 97 \pm 3; K_d: 3 \pm 1; R^2: 0.9616\)

Pro150Gly reactivation plot. Apo-enzyme (100 nM) was incubated independently at different zinc ion concentration (nM) for overnight. Fitted with one site-specific binding with Hill slope. \(V_{\text{max}}: 100 \pm 3; K_d: 4 \pm 1; R^2: 0.9649\).
Titration curves for the reactivation of apo-Asp112Ala. [Asp112Ala]: 100 nM; $V_{\text{max}}$: 92.99 ± 2.5; $K_d$: 2.35 ± 0.05; $R^2$: 0.9659.

Titration curves for the reactivation of apo-Thr146Ala. [Thr146Ala]: 100 nM; $V_{\text{max}}$: 96.8 ± 2.9; $K_d$: 2.92 ± 0.08; $R^2$: 0.9747.
Titration curves for the reactivation of apo-MPH native. Sample was incubated independently overnight at different cobalt ion concentrations. The curve was fitted with one site-specific binding with Hill slope. [protein]: 5 μM; $V_{\text{max}}$: 97 ± 3; $K_d$: 10 ± 1; $R^2$: 0.9725.

Pro150Ala reactivation plot. Apo-enzyme (5 μM) was incubated independently overnight at different cobalt ion concentrations (μM). Fitted with one site-specific binding with Hill slope. $V_{\text{max}}$: 100 ± 4; $K_d$: 23 ± 1; $R^2$: 0.9797.
I S O "100"

IC (uM)

Prol50Gly reactivation plot. Apo-enzyme (5 µM) was incubated independently at different cobalt ion concentration (µM) for overnight. Fitted with one site-specific binding with Hill slope. $V_{\text{max}}$: 98 ± 1; $K_C$: 12 ± 1; $R^2$: 0.9983.

Pro150Gly reactivation plot. Apo-enzyme (5 µM) was incubated independently at different cobalt ion concentration (µM) for overnight. Fitted with one site-specific binding with Hill slope. $V_{\text{max}}$: 98 ± 1; $K_C$: 12 ± 1; $R^2$: 0.9983.

Titration curves for the reactivation of apo-Asp112Ala. [protein]: 5 µM; $V_{\text{max}}$: 96 ± 2; $K_C$: 9 ± 1; $R^2$: 0.9897
Titration curves for the reactivation of apo-Thr146Ala. [protein]: 5 µM; \( V_{\text{max}} \): 108 ± 2; \( K_d \): 13 ± 1; \( R^2 \): 0.9910.

Titration curves for the reactivation of apo-Asp274Ala. [protein]: 5 µM; \( V_{\text{max}} \): 97 ± 2; \( K_d \): 5 ± 1; \( R^2 \): 0.9896.