Studies on the
inhibitor selectivity and
inhibitory signal transfer of
$\alpha$-Isopropylmalate synthase

A thesis submitted for the fulfilment of the requirements
for the Degree of
Master of Philosophy in Biochemistry
in the University of Canterbury
by
Tyler Brooke Clarke

Department of Chemistry
University of Canterbury
September 2013
Abstract

α-Isopropylmalate synthase (α-IPMS) is responsible for catalysing the first committed step in leucine biosynthesis. This pathway is found in plants and microorganisms, including pathogenic bacteria such as *Mycobacterium tuberculosis* and *Neisseria meningitidis*. α-IPMS catalyses a Claisen condensation reaction between α-ketoisovalerate (KIV) and acetyl coenzyme A (AcCoA) to form the product α-isopropylmalate (IPM). This enzyme undergoes feedback inhibition by the end product of the pathway, leucine. This regulation allows the control of the rate leucine biosynthesis.

This project focuses on the α-IPMS enzymes from *M. tuberculosis* and *N. meningitidis* (MtuIPMS and NmeIPMS). These α-IPMS enzymes are homodimeric in structure. Each monomer consists of a catalytic domain which comprises of a (β/α)$_8$ barrel fold, two subdomains and a regulatory domain, to which the allosteric binding of the natural inhibitor leucine occurs. The mechanism by which the allosteric binding of leucine leads to a decrease in enzymatic activity is not yet fully understood.

Citramalate synthase (CMS) is responsible for catalysing the first committed step of threonine-independent isoleucine biosynthesis. This enzyme is extremely similar to α-IPMS in both the reaction which it catalyses and the catalytic and regulatory domain structure. CMS catalyses a Claisen condensation reaction between pyruvate and AcCoA to produce citramalate (CM). CMS is also feedback inhibited by the end product of its pathway, isoleucine.

The similarity between α-IPMS and CMS enzymes resulted in and examination of the inhibitor selectivity of MtuIPMS. Amino acids in the leucine binding site were altered to their counterparts in the isoleucine binding site of the CMS enzyme to see if the selectivity of the leucine binding site could be interchanged.
Results from this study show that it is possible to change inhibitor selectivity with a single amino acid substitution. However, changing the selectivity from leucine to isoleucine was unsuccessful. Instead, one of the \textit{Mtu}IPMS variants displayed significantly increased sensitivity to an alternative amino acid, norvaline. The \textit{Mtu}IPMS variants were expressed and purified using immobilised metal affinity chromatography and size-exclusion chromatography. These variants were then kinetically characterised and displayed similar binding affinities and turnover rates for the natural substrates to the wild-type enzyme. As expected changes to the leucine binding pocket had drastic effects on the sensitivity of the enzyme to its natural inhibitor. This work is described in Chapter 2 of this thesis.

The mechanism by which the regulatory signal is transferred from the allosteric leucine binding site to the catalytic site in \(\alpha\)-IPMS is not fully understood. \textit{Nmel}IPMS variants were created based on preliminary molecular dynamic simulations which indicated that significant changes in residue contacts were associated with leucine binding. Chapter 3 describes studies that explore the effect of single amino acid substitutions of \textit{Nmel}IPMS. The \textit{Nmel}IPMS variants were expressed and purified similarly to \textit{Mtu}IPMS, using immobilised metal affinity chromatography and size-exclusion chromatography. Variants were subsequently characterised via mass spectrometry, differential scanning fluorimetry and kinetic assays. It was found that each variant generated retained sensitivity to leucine but displayed significant differences in the catalytic efficiencies with AcCoA. One of the generated variants also displayed a significant increase in thermal stability.

Results are drawn together in Chapter 4 along with future directions of this research. This chapter details knowledge gained into protein structure and allosteric mechanisms in this thesis.
Acknowledgments

Firstly, I would like to thank Emily Parker (the closest thing to super woman in the real world) for her supervision and guidance. Without her input this thesis would not have been possible. I would like to thank her for always helping me out when I had a problem (even if she was in a different time-zone).

I would like to specially thank Michael Hunter and Frances Huisman for getting me up and running in the laboratory and answering all of my questions.

Thanks to everyone in the Parker group for their support and friendship, with a special thanks to Penel Cross, Mohamad Othman, Ryu Toyama, Vicky Zhang, Michael (2.0) Weusten, Dmitri Joseph, Tammie Cookson, Gerd (one part of G²) Mittlestaedt and Nicky Blackmore who were always there to bounce ideas off and to chat.

A special thanks to Dr. Wanting Jiao and Chloe Thompson for molecular dynamics modelling and all the technical staff in the UC Chemistry and Biology departments for their help.

Thanks to all my family, especially mum and dad, for all their encouragement and support and lastly thanks to Helen for all of her support and for keeping up my morale when the chips were low.
Contents

Abstract iii
Acknowledgments v
List of Figures xiii
List of Tables xvi
List of Abbreviations xvii

Chapter 1: Introduction

1.1 Branched chain amino acid biosynthesis .................................................. 1
1.2 The isoleucine and valine biosynthetic pathway ........................................ 2
1.3 Valine and isoleucine synthesis ................................................................. 2
1.4 The leucine biosynthetic pathway ............................................................. 4
1.5 The citramalate pathway ............................................................................ 5
1.6 α-isopropylmalate synthase ....................................................................... 6
  1.6.1 Enzyme activity .................................................................................. 6
  1.6.2 α-IPMS catalysed reaction and alternate substrates .............................. 7
  1.6.3 Alternate inhibitors ............................................................................. 8
  1.6.4 Metal dependency ............................................................................... 8
  1.6.5 Proposed reaction mechanism ............................................................. 8
  1.6.6 Structure ............................................................................................ 9
  1.6.7 Feedback inhibition .......................................................................... 12
1.7 Ligand binding in Mycobacterium tuberculosis .......................................... 13
  1.7.1 α-KIV binding .................................................................................. 13
  1.7.2 Leucine binding .................................................................................. 14
Chapter 2: Effects on regulatory domain substitutions on inhibitor selectivity of α-isopropylmalate synthase from *Mycobacterium tuberculosis*

2.1 Overview .............................................................................................................. 28
2.2 Design of *Mtu*IPMS mutants ........................................................................... 29
2.3 Justification of choice of variants ..................................................................... 31
   2.3.1 *Mtu*IPMS A558I and A558V ................................................................. 31
   2.3.2 *Mtu*IPMS V551L ................................................................................... 32
   2.3.3 *Mtu*IPMS A567V ................................................................................... 32
   2.3.4 *Mtu*IPMS I627A ................................................................................... 32
2.4 *Mtu*IPMS mutant generation using PCR ......................................................... 33
2.5 Expression of protein ......................................................................................... 35
2.6 Purification ........................................................................................................... 36
2.6.1 Immobilised metal affinity chromatography ........................................ 37
2.6.2 Desalting .............................................................................................. 37
2.6.3 TEV protease cleavage ........................................................................ 37
2.6.4 Size-exclusion chromatography .......................................................... 38

2.7 Mass spectrometry .................................................................................. 39

2.8 MtuIPMS wild-type .................................................................................. 40
2.8.1 Physical characterisation ...................................................................... 40
2.8.2 Secondary structure analysis ............................................................... 40
2.8.3 Thermal stability .................................................................................. 41
2.8.4 Kinetic characterisation ....................................................................... 43
2.8.5 Michaelis-Menten kinetics .................................................................. 43
2.8.6 Allosteric inhibition ............................................................................. 45

2.9 Demonstration of slow-onset inhibition .................................................. 46
2.9.1 MtuIPMS wild-type observed slow-onset inhibition ............................. 47

2.10 Variant structure analysis ...................................................................... 48

2.11 Thermal stability of variants .................................................................. 49

2.12 Kinetic properties of variants ................................................................. 51

2.13 Allosteric inhibition of variants .............................................................. 52
2.13.1 Leucine inhibition .............................................................................. 52
2.13.2 Isoleucine inhibition .......................................................................... 53
2.13.3 Norvaline inhibition .......................................................................... 54

2.14 Observed slow-onset inhibition data ..................................................... 55
2.14.1 MtuIPMS V551L observed slow-onset inhibition ............................... 55
2.14.2 MtuIPMS A567V observed slow-onset inhibition ............................. 56

2.15 Summary of findings ............................................................................. 57
Chapter 4: Summary of thesis and future directions

4.1 Changes to the regulatory pocket of MtuIPMS can have a large effect on the inhibitor selectivity and sensitivity of the enzyme ................................................................. 93
4.2 Disruption of hydrogen bonding contacts can have large consequences on enzyme activity, regulation and stability ................................................................. 94
4.3 Future Experiments ............................................................................................................. 97
   4.3.1 What is the effect upon inhibitor selectivity of double and triple amino acid substituted variants of MtuIPMS? ......................................................... 97
   4.3.2 Does removing multiple possible regulatory signal transfer pathways reduce enzyme regulation in NmeIPMS? .............................................. 98
4.4 Concluding Remarks ........................................................................................................ 99

Chapter 5: Materials and Methods

5.1 General methods .................................................................................................................. 101
   5.1.1 Water .......................................................................................................................... 101
   5.1.2 Determination of pH ................................................................................................ 101
   5.1.3 Protein structure viewing and images ....................................................................... 101
   5.1.4 Sequence alignments ................................................................................................. 101
5.2 Site-directed mutagenesis ................................................................................................ 102
   5.2.1 Primers ......................................................................................................................... 102
5.2.2 PCR equipment ................................................................. 103
5.2.3 Mutagenesis of MtuIPMS variants........................................... 103
5.2.4 Mutagenesis of NmeIPMS variants........................................... 103
5.2.5 Agarose gel electrophoresis .................................................. 103
5.2.6 Chemical transformation ..................................................... 104
5.2.7 Plasmid preparation and purification ....................................... 104
5.2.8 DNA sequencing ............................................................... 105

5.3 Cell cultures ............................................................................. 105
5.3.1 Escherichia coli cell lines ....................................................... 105
5.3.2 Glycerol stocks ..................................................................... 105
5.3.3 Antibiotics ............................................................................ 106
5.3.4 LB media .............................................................................. 106
5.3.5 Protein expression ............................................................... 106
5.3.6 Cell harvesting ..................................................................... 106

5.4 Purification ................................................................................ 107
5.4.1 Cell lysis .............................................................................. 107
5.4.2 Chromatography equipment ................................................. 107
5.4.3 Immobilised metal affinity chromatography ............................. 107
5.4.4 TEV protease treatment ....................................................... 108
5.4.5 Size-exclusion chromatography .......................................... 108
5.4.6 Concentration of protein samples ....................................... 109
5.4.7 Determination of protein concentration ................................. 109
5.4.8 Buffer exchange .................................................................. 109
5.4.9 Protein storage ..................................................................... 109

5.5 Protein characterisation ............................................................ 110
5.5.1 Protein parameters ............................................................. 110
5.5.2 Polyacrylamide gel electrophoresis ...................................... 110
5.5.3 Circular dichroism spectrometry ......................................... 110
5.5.4 Mass spectrometry .............................................................. 111
5.5.5 Differential scanning fluorimetry .......................................................... 111

5.6 Activity assays ........................................................................................ 111
  5.6.1 Kinetic assay equipment ................................................................... 111
  5.6.2 4-4’-Dithiodipyridine-coupled assays at 324 nm ......................... 111
  5.6.3 Substrate concentration determination ........................................ 112
  5.6.4 Michaelis-Menten kinetics ............................................................... 112
  5.6.5 MtulPMS wild-type and variant Michaelis-Menten conditions .... 112
  5.6.6 NmelPMS wild-type and variant Michaelis-Menten conditions ...... 113
  5.6.7 Inhibition assays ............................................................................ 114

Appendix A: Sequence alignments .............................................................. 116

Appendix B: Michaelis-Menten plots ......................................................... 122

Bibliography ............................................................................................... 127
List of figures

1.1 Chemical structures of the three branched-chain amino acids.......................... 1
1.2 The branched-chain amino acid biosynthetic pathway................................. 3
1.3 The leucine biosynthesis pathway.................................................................... 4
1.4 The citramalate biosynthesis pathway.............................................................. 5
1.5 Reaction catalysed by α-IPMS........................................................................... 7
1.6 Proposed reaction mechanism for α-IPMS and CMS........................................ 9
1.7 Crystal structure of MtuIPMS ............................................................................ 10
1.8 Differences in conformation between MtuIPMS monomers............................. 11
1.9 Active site of MtuIPMS..................................................................................... 13
1.10 Leucine binding.............................................................................................. 14
1.11 Crystal structure of truncated MtuIPMS........................................................ 15
1.12 Crystal structure of truncated NmeIPMS........................................................ 16
1.13 Reaction catalysed by CMS............................................................................ 19
1.14 Crystal structure of the catalytic domain of LiCMS........................................ 20
1.15 Crystal structure of the regulatory domain of LiCMS..................................... 21
1.16 Active site of LiCMS...................................................................................... 22
1.17 Similarities in the active site of MtuIPMS an LiCMS....................................... 23
1.18 Regulatory site of LiCMS................................................................................ 24
1.19 Flexible regulatory loop in MtuIPMS and LiCMS............................................ 25

2.1 Superposition of the MtuIPMS and LiCMS regulatory domain ...................... 29
2.2 Residues contributing to the regulatory site in MtuIPMS and LiCMS............... 30
2.3 Agarose gel showing purified MtuIPMS gene.................................................. 33
2.4 Agarose gel showing MtuIPMS PCR products after Dpn1.............................. 34
2.5 SDS-PAGE gel showing MtulPMS expression trial ............................................. 35
2.6 MtulPMS purification procedure ........................................................................ 36
2.7 Sequence of TEV cleavage site in the MtulPMS gene ........................................ 37
2.8 SDS-PAGE gel showing MtulPMS IMAC purification ........................................ 38
2.9 SDS-PAGE gel showing purified MtulPMS enzymes .......................................... 39
2.10 CD spectrum of wild-type MtulPMS .................................................................. 40
2.11 DSF curve for wild-typeMtulPMS with no inhibitor ......................................... 41
2.12 Effects on denaturation temperature of wild-type MtulPMS with leucine, isoleucine and norvaline ................................................................. 42
2.13 Wild-type MtulPMS Michaelis-Menten plots ...................................................... 44
2.14 Wild-type MtulPMS activity with leucine, isoleucine and norvaline ................. 45
2.15 Energy profile of slow-onset inhibition ............................................................. 46
2.16 Observed slow-onset inhibition of wild-type MtulPMS .................................... 47
2.17 CD spectrum showing MtulPMS variants vs. wild-type ..................................... 48
2.18 DSF data of MtulPMS variant denaturation temperatures ............................... 49
2.19 Leucine inhibition of MtulPMS variants based on initial rates .......................... 52
2.20 Isoleucine inhibition of MtulPMS variants based on initial rates ...................... 53
2.21 Norvaline inhibition of MtulPMS variants based on initial rates ...................... 54
2.22 Slow-onset inhibition of MtulPMS V551L ....................................................... 55
2.23 Slow-onset inhibition of MtulPMS A567V ....................................................... 56
2.24 MtulPMS with norvaline modelled into the leucine binding site ....................... 58
2.25 MtulPMS with isoleucine modelled into the leucine binding site ..................... 58
2.26 Location of mutated residues in the MtulPMS structure ................................... 59
2.27 Regulatory site of MtulPMS A567V ................................................................. 61

3.1 Homology model of NmelPMS .......................................................................... 65
3.2 Average ligand-free and leucine-bound NmelPMS models ................................. 67
3.3 Hydrogen bond interaction between Ser352 and Glu466 ..................................... 69
3.4 Hydrogen bond interactions between Glu353, Ser468 and Arg470 ..................... 70
3.5 Hydrogen bond interactions between Arg310, Glue314 and Glu319 ................. 71
3.6 Agarose gel of the isolated plasmid containing the NmelPMS gene .......... 72
3.7 Sequence of TEV cleavage site in the NmelPMS gene .................................. 75
3.8 SDS-PAGE gel showing purified NmelPMS enzymes ..................................... 76
3.9 CD spectra of wild-type NmelPMS .................................................................. 77
3.10 DSF curve for wild-type NmelPMS with 5 mM leucine .............................. 78
3.11 Effects of leucine concentration on denaturation temperature ........ 79
3.12 Wild-type NmelPMS Michaelis-Menten plots ........................................... 80
3.13 Inhibition of wild-type NmelPMS by leucine ............................................. 81
3.14 CD spectrums of NmelPMS variants ......................................................... 82
3.15 Denaturation temperatures of NmelPMS variants and wild-type ........ 83
3.16 Inhibition of NmelPMS R310A by leucine .................................................. 86
3.17 Inhibition of NmelPMS E353A by leucine .................................................. 87
3.18 Inhibition of NmelPMS E466A by leucine .................................................. 88
3.19 NmelPMS homology model showing theoretical AcCoA binding .......... 90

4.1 MtuIPMS regulatory site with I627A and V551E substitutions ............... 98
List of Tables

1.1 Reported kinetic data for α-IPMS enzymes ................................................................. 7
1.2 Reported kinetic data for CMS enzymes ................................................................. 18
2.1 Distance of chosen residues from bound leucine in the MtuIPMS regulatory site ......................................................................................................................... 30
2.2 Conservation of chosen MtuIPMS residues and corresponding LiCMS residues ................................................................................................................................. 31
2.3 Theoretical and experimental masses of MtuIPMS variants ................................... 39
2.4 MtuIPMS wild-type and variant denaturation temperatures ................................... 50
2.5 Michaelis-Menten parameters of MtuIPMS enzymes ........................................... 51
3.1 Theoretical and experimental masses of NmelIPMS variants ................................. 76
3.2 NmelIPMS wild-type and variant denaturation temperatures ............................... 84
3.3 Michaelis-Menten parameters of NmelIPMS enzymes ......................................... 84
3.4 Catalytic efficiencies of NmelIPMS enzymes ....................................................... 85
5.1 Primers used to generate MtuIPMS and NmelIPMS variants ................................. 102
5.2 Primers used for DNA sequencing .......................................................................... 105
List of Abbreviations

α-AL $\alpha$-acetolactate
α-IPMS $\alpha$-isopropylmalate synthase
α-KB $\alpha$-ketobutyrate
α-KIC $\alpha$-ketoisocaproate
α-KIV $\alpha$-ketoisovalerate
AcCoA acetyl coenzyme A
AHAR acetohydroxyacid synthase
AHAS acetohydroxyacid synthase
BCAT branched-chain amino transferase
BTP 1,3-bis{(tris(hydroxymethyl)methylamino)propane
CD circular dichroism
CMS citramalate synthase
CoA coenzymeA
DHAD dihydroxyacid dehydratase
DNA deoxyribonucleic acid
DSF differential scanning fluorimetry
DTP 4,4′-dithiodipyridine
DTT dithiothreitol
EDTA ethylenediaminetetraacetic acid
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Ile isoleucine
IMAC immobilised metal affinity chromatography
IPMD $\beta$-isopropylmalate dehydrogenase
IPMI isopropylmalate isomerase
TD threonine deaminase
IPM α-isopropylmalate
IPTG isopropyl β-D-1-thiogalactopyranoside
LB lysogeny broth
Leu leucine

LiCMS *Leptospira interrogans* CMS
MMD 3-methylmalate dehydrogenase
MMH 2-methylmalate hydrolyase
MS mass spectrometry
MtuIPMS *Mycobacterium tuberculosis* α-IPMS
NmIPMS *Neisseria meningitidis* α-IPMS
Nor norvaline

PCR polymerase chain reaction
PDB Protein Data Bank
RFU relative fluorescence unit
SDS-PAGE sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
SEC size-exclusion chromatography
TEV tobacco etch virus
Chapter 1

Introduction

1.1 Branched chain amino acid biosynthesis

All organisms require a vast range of different key compounds and energy in order to function. These key molecules can be obtained by diet or by metabolic biosynthesis. Therefore each organism makes use of a complicated network of chemical reactions which are known as metabolic pathways. Humans are able to synthesise ten of the twenty common α-amino acids, which are used to build proteins, via key metabolic pathways.\textsuperscript{1,2} These ten amino acids which can be produced by humans are known as the non-essential amino acids. The other ten naturally occurring amino acids are known as the essential amino acids. Among the essential amino acids are the branched chain amino acids leucine, isoleucine and valine. Humans and other mammalian cells lack the metabolic machinery to produce the essential amino acids and need to obtain them from the environment, such as by ingesting and digesting proteins in food. While humans and other mammalian cells need to obtain the branched amino acids from the environment, bacteria\textsuperscript{3}, fungi\textsuperscript{4} and plants\textsuperscript{5} are able to manufacture their own supply of these compounds using enzymes that function in metabolic pathways that are not present in mammalian cells.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.pdf}
\caption{Chemical structures of the three branched chain amino acids.}
\end{figure}
As these essential amino acids are able to be manufactured in pathogenic bacteria such as *Mycobacterium tuberculosis* but not in human and other mammalian cells, this makes the metabolic pathways responsible for the production of these amino acids a viable target for drug design. If a key enzyme in a metabolic pathway is inhibited the enzyme is prevented from functioning and the end product can no longer be produced in the necessary quantity or obtained from the environment, the organism may die or undergo growth attenuation.\(^6\)

### 1.2 The isoleucine and valine biosynthetic pathway

The initial starting compound for the biosynthesis of all three of the branched-chain amino acids is pyruvate. However, for the majority of bacteria and plants, the production of isoleucine also utilizes α-ketobutyrate (α-KB), which is derived from threonine.\(^7\) The enzyme which catalyses the formation of α-KB from threonine is known as threonine deaminase (TD). α-KB can also be formed via a threonine independent route in some bacteria; this is achieved via the citramalate pathway which will be discussed in detail.\(^8\) Isoleucine and valine are synthesised in similar ways and share a number of enzymes in their pathway (Figure 1.2) whereas the synthesis of leucine branches off from α-ketoisovalerate (α-KIV), which is the precursor for valine.\(^9,10\) The first committed steps in these pathways are all highly regulated by the branched-chain amino acids being formed to ensure minimal metabolite waste.

### 1.3 Valine and isoleucine synthesis

The pathway for valine biosynthesis begins with the condensation of two molecules of pyruvate to form α-acetolactate (α-AL) via the enzyme acetohydroxy acid synthase (AHAS). AHAS is also responsible for the condensation of α-KB and pyruvate in the first committed step of the isoleucine biosynthesis pathway and is regulated by all three branched-chain amino acids.\(^7,11\) In some organisms a synergistic regulatory system exists where combinations of these three amino acids lead to differing levels of inhibition.\(^7,11\) The formation of valine and isoleucine run
in parallel from this point on, using the same enzymes to catalyse each transformation. The next enzyme involved in these pathways is responsible for the isomerisation and reduction of $\alpha$-acetolactate and $\alpha$-acetohydroxybutyrate and is known as acetohydroxyacid isomeroreductase (AHAIR). These products are then dehydrated via dihydroxyacid dehydratase (DHAD) and then transaminated by branched-chain aminotransferase (BCAT) which forms the amino acid products. Both AHAIR and DHAD are also inhibited by valine and leucine in some organisms.

Figure 1.2: The branched-chain amino acid biosynthetic pathway where TD = threonine deaminase; AHAS = acetohydroxyacid synthase; AHAIR = acetohydroxyacid isomeroreductase; DHAD = dihydroxyacid dehydratase; BCAT = branched-chain aminotransferase.
1.4 The leucine biosynthetic pathway

The first committed step in the biosynthesis of leucine is the condensation of α-KIV, which is one of the products near the end of the valine biosynthesis pathway, and acetyl coenzyme A (AcCoA) to form α-isopropylmalate (IPM) catalysed by the enzyme α-isopropylmalate synthase (α-IPMS). IPMS is feedback inhibited by the end product of the pathway, leucine. The next step is the transfer of a hydroxyl group between adjacent carbons on IPM via the enzyme isopropylmalate isomerase (IPMI) to form β-isopropylmalate. The new hydroxyl group is then oxidised to give the keto functionality while the other carboxylate group is removed to form α-ketoisocaproate (α-KIC). Both these steps are catalysed by the enzyme β-isopropylmalate dehydrogenase (IPMD). The final step in the pathway is the transamination of α-KIC to form leucine. This reaction is performed by the BCAT enzyme responsible for the formation of isoleucine and valine.

\[ \text{AcCoA} + \text{H}_2\text{O} \xrightarrow{\alpha-\text{IPMS}} \alpha-\text{isopropylmalate} \xrightarrow{\text{IPMI}} \beta-\text{isopropylmalate} \xrightarrow{\text{IPMD}} \alpha-\text{ketoisocaproate} \xrightarrow{\text{BCAT}} \text{leucine} \]

Figure 1.3: The leucine biosynthesis pathway where \(\alpha-\text{IPMS}\) = α-isopropylmalate synthase; \(\text{IPMI}\) = α-isopropylmalate isomerase; \(\text{IPMD}\) = β-isopropylmalate dehydrogenase; \(\text{BCAT}\) = branched-chain aminotransferase.
1.5 The citramalate pathway

As previously mentioned, the production of isoleucine can also be achieved via a threonine independent pathway in some organisms such as *Leptospira interrogans*\(^{13}\). This route is known as the citramalate synthase pathway. This pathway begins with the condensation of pyruvate and AcCoA which is catalysed by citramalate synthase (CMS) to form citramalate (CM). CMS is feedback regulated by isoleucine to control the flux through the pathway. The next step is a dehydration reaction via the enzyme 2-methylmalate hydrolase (MMH) to form citraconate. MMH then hydroxylates citraconate to form 3-methylmalate which is further oxidized by 3-methylmalate dehydrogenase (MMD) to form α-KB, the starting product seen in the threonine-dependent pathway.\(^{14}\)

![Figure 1.4: The citramalate pathway. Where CMS = citramalate synthase; MMH = 2-methylmalate hydrolase; MMD = 3-methylmalate dehydrogenase; TD = Threonine deaminase.](image-url)
1.6 α-Isopropylmalate synthase

Overview

α-IPMS (EC 2.3.3.13) catalyses the first committed step in the leucine biosynthesis pathway. The gene which encodes α-IPMS is known as *leuA* which in the case of *Mycobacterium tuberculosis* (*Mtu*) is polymorphic due to an insertion of a 57-base pair repeating unit which creates a variable number of tandem repeats.\(^\text{15}\) α-IPMS belongs to a family of enzymes known as Claisen-condensing enzymes. Claisen-condensing enzymes are responsible for the condensation reaction of AcCoA with α-keto acids. Other members of this family include citramalate synthase, which is introduced below, malate synthase, homocitrate synthase, re- and si-citrate synthases and methylthioalkylmalate synthase.\(^\text{16-19}\) All known α-IPMS enzymes require a divalent metal ion to polarize the carbonyl group of the α-keto acid, activating it for nucleophilic attack.

A large range of α-IPMS enzymes have been studied from plants, fungi and bacteria. The most detailed studies on α-IPMS have been carried out on MtuIPMS as it is a viable drug target to slow or stop the growth of this pathogen.\(^\text{20}\) *M. tuberculosis* was also of interest as the *leuA* gene exhibits polymorphism, where a 57-base pair tandem repeating unit occurs between 2 and 21 times. The number of tandem repeats does not affect the enzyme activity or expression and deletion of the repeat has no observed effect.\(^\text{21,22}\) The MtuIPMS used in this thesis was obtained from the H37Ra strain of *M. tuberculosis*, which contains two tandem repeats.

1.6.1 Enzyme activity

α-IPMS is responsible for the condensation of substrates AcCoA and α-KIV. Reported *K*\(_m\) values for the natural substrates for α-IPMS from most organisms are typically between 5 and 600 µM with turnover numbers between 2 and 15 s\(^{-1}\) at pH levels between 7.0 and 8.0 (Table 1.1).\(^\text{23-28}\) Slightly basic pH levels have been found to be most activating for this enzyme which may be due to deprotonation of AcCoA being a required step for catalysis as shown in the proposed mechanism (Figure 1.6).
Table 1.1: Kinetic data reported for α-IPMSs from different organisms where NR = not reported.

<table>
<thead>
<tr>
<th>Organism</th>
<th>α-KIV (K_m) (µM)</th>
<th>AcCoA (K_m) (µM)</th>
<th>(k_{cat}) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em>(^{23})</td>
<td>12</td>
<td>136</td>
<td>3.5</td>
</tr>
<tr>
<td><em>N. meningitidis</em>(^{24})</td>
<td>30</td>
<td>35</td>
<td>13</td>
</tr>
<tr>
<td><em>N. crassa</em>(^{25})</td>
<td>10</td>
<td>25</td>
<td>NR</td>
</tr>
<tr>
<td><em>S. cerevisiae</em>(^{26})</td>
<td>16</td>
<td>9</td>
<td>NR</td>
</tr>
<tr>
<td><em>S. typhimurium</em>(^{27})</td>
<td>60</td>
<td>200</td>
<td>NR</td>
</tr>
<tr>
<td><em>C. maltosa</em>(^{28})</td>
<td>570</td>
<td>64</td>
<td>NR</td>
</tr>
</tbody>
</table>

1.6.2 α-IPMS catalysed reaction and alternate substrates

\[ \alpha-KIV + \text{CoA} \rightarrow \alpha-\text{IPM} + \text{HCoA} \]

\[ \text{CoA} = \text{S-} - \text{S-} - \text{N-} - \text{N-} - \text{O-} - \text{P-} - \text{P-} \]

*Figure 1.5:* Reaction catalysed by α-IPMS.

α-IPMS converts the substrates α-KIV and AcCoA into α-IPM and HCoA. α-IPMS has been observed functioning with a number of other α-keto acids such as pyruvate\(^{23}\), α-
ketobutyrate\textsuperscript{23,25}, \(\alpha\)-ketovalerate\textsuperscript{23}, \(\alpha\)-ketoisocaproate and \(\alpha\)-ketocaproate.\textsuperscript{29} Despite the promiscuity with \(\alpha\)-keto acids strict adherence to AcCoA as the second substrate is observed.

1.6.3 Alternate inhibitors

As well as being able to catalyse reactions with other \(\alpha\)-keto acids than the natural substrate, \(\alpha\)-IPMS is also inhibited by molecules other than the natural inhibitor, leucine. Some known alternate inhibitors for \(\alpha\)-IPMS are: \(\alpha\)-ketovalerate\textsuperscript{29}, \(\alpha\)-ketoisocaproate\textsuperscript{23}, (\(S\)-\(\alpha\)-hydroxyisovalerate\textsuperscript{23}, valine\textsuperscript{25} and leucine halides.\textsuperscript{25}

1.6.4 Metal dependency

All studied \(\alpha\)-IPMS enzymes require a divalent metal ion for activity. The most common metal ion for obtaining the maximal enzyme activity is \(\text{Mg}^{2+}\).\textsuperscript{30,31} Effects on activity have been studied with various other divalent metal ions and it was discovered that metal ions which may activate the \(\alpha\)-IPMS from one organism may have no effect or be inhibitory for the \(\alpha\)-IPMS from other organisms.\textsuperscript{29-31} For example \(\text{K}^+\) ions are inhibitory for the \(\alpha\)-IPMS from \(C.\ maltosa\textsuperscript{32}. \(\alpha\)-IPMS from some organisms such as \(Mtu\textsuperscript{IPMS}\) also require a monovalent cation, with the preference being for \(\text{K}^+\), although others such as \(\text{Na}^+\) are also activating. The monovalent cations have been shown not to directly affect the enzyme activity in \(Mtu\textsuperscript{IPMS}\), but may instead act to recruit the divalent metal ions to the active site.\textsuperscript{30}

1.6.5 Proposed reaction mechanism

The proposed mechanism of the condensation of \(\alpha\)-KIV and AcCoA in \(Mtu\textsuperscript{IPMS}\) is shown in figure 1.6.\textsuperscript{23} The mechanism shows a divalent metal ion coordinating to the carbonyl groups of \(\alpha\)-KIV withdrawing electron density from the carbonyl carbon. This makes the nucleophilic attack of AcCoA more favourable. Intermediate 2 is then nucleophilically attacked by water and subsequently loses the CoA group forming IPM.
1.6.6 Structure

The only full length structure which has been elucidated at this time is from *M. tuberculosis* (PDB codes 3HPS, 3HPZ, 3HQ1, 3FIG and 1SR9). Molecules which have been found bound in the active site of the enzyme in the crystals are α-KIV, α-KIC, bromopyruvate and citrate. At the regulatory domain only leucine has been shown bound. A variety of metal ions have also been found at the active site in the crystal structures, such as Zn$^{2+}$, Mn$^{2+}$, Ni$^{2+}$ and Mg$^{2+}$. There have been no full length structures solved without ligands bound yet suggesting the ligands may play a role in stabilising the enzyme leading to a more favourable conformation for crystallisation.
Figure 1.7: Crystal structure of α-IPMS from *M. tuberculosis* (PDB code 1SR9). One monomer is show in light grey while the other monomer is colour coded by domains and subdomains. The catalytic domain is shown in purple, subdomain I is in yellow, subdomain II is in red and the regulatory domain is blue. A-KIV (cyan) and Zn$^{2+}$ (black) are shown in the active site.

The crystal structure of *Mtul*IPMS (Figure 1.7) is known to form a dimer both in solution and when crystallised. The monomeric weight of *Mtul*IPMS is 70 kDa. α-IPMS from other organisms have been reported to exist as trimer and tetramers.$^{25,29}$

Each *Mtul*IPMS monomer is further divided into two domains, the catalytic and regulatory domains, and two subdomains, subdomain I and subdomain II. Between the two subdomains on each monomer is a flexible linker region which was not elucidated due to lack of electron density. The catalytic domain consists of a ($\beta/\alpha)_8$-barrel and contains the active site where the substrate and metal binding sites are located at the C-terminal of the barrel. Subdomain I consists of an $\alpha$-helix and 2 beta sheets which cap the active site of the adjacent monomer and
may contribute residues involved in the binding of AcCoA. Subdomain I is then connected to subdomain II by the short unresolved linker region. The subdomain II is larger than subdomain I and consists of three α-helices. Finally the regulatory domain is comprised of a (βββα)₂ fold which interlinks with the other monomer forming a sandwich in which two leucine molecules are able to bind at the interface between the monomers and allosterically inhibit the enzyme. The regulatory domain also contains two tandem repeats at the C-terminus corresponding to residues 575-612 which are flexible and undefined in the structure.¹⁵

Interestingly, the monomers in MtuIPMS are not aligned symmetrically to one another, instead the catalytic domain and subunit I are rotated relative to each other (Figure 1.8).

![Figure 1.8: Crystal structure of MtuIPMS (PDB code 1SR9) showing the different conformations of the two monomers. The catalytic domain is shown in purple, subdomain I in yellow, subdomain II in red and the regulatory domain is shown in blue.](Image)
1.6.7 Feedback inhibition

α-IPMS is feedback inhibited by leucine, the end product of the pathway.\textsuperscript{25} Since this is the enzyme responsible for catalysing the first committed step in the biosynthesis of leucine having feedback regulation allows for control of metabolite flux through the pathway in line with cellular requirements for leucine.

Leucine binds to α-IPMS in the regulatory domain (Figure 1.10).\textsuperscript{33} Currently, it is unknown how the signal is carried through the enzyme and this is one of the aspects that is being examined in this thesis. In MtuIPMS, leucine inhibits similarly well in both substrate bound and unbound enzymes with a reported $K_i$ value of 8 ± 1 µM and $K_i'$ value of 22 ± 2 µM.\textsuperscript{34}

MtuIPMS also displays mixed inhibition which is slow-onset.\textsuperscript{35} The overall mechanism of slow-onset inhibition for allosterically inhibited enzymes is still unclear. It is thought that the slow-onset nature of inhibition in MtuIPMS is a consequence of a flexible loop which is involved in the binding of leucine.\textsuperscript{36}

As mentioned, it is unknown how the binding of leucine leads to inhibition of α-IPMS. Crystal structures show little difference between leucine-bound and unbound forms suggesting the enzyme is controlled by enzyme molecular dynamics. In order to examine possible differences in solvent accessibility between the leucine-bound and unbound forms solution-phase amide hydrogen/deuterium exchange was performed on MtuIPMS. This technique measures the incorporation of deuterium into the backbone amide hydrogens of proteolysed peptide fragments.\textsuperscript{37} Comparison of changes in deuterium uptake can be used to view dynamic changes which may be hidden or suppressed in crystal structures.

Upon binding leucine the majority of difference in deuterium incorporation was found in the regulatory domain and in the region of subdomain II which interacts with the regulatory domain. It was found that the deuterium incorporation decreased upon leucine binding which corresponds to a conformational shift towards increased protection from solvent.\textsuperscript{38}
Only residues 78-87 in the catalytic domain showed a change in deuterium exchange. This part of \textit{Mtu}IPMS contains Arg80 which binds to $\alpha$-KIV and Asp81 which is involved in metal ion coordination. Examination of the Y410F variant of \textit{Mtu}IPMS also exhibits a decrease in deuterium exchange of these residues.\textsuperscript{38} The Y410F variant maintains the ability to bind leucine with a $K_0$ of 21 $\pm$ 1 $\mu$M but has a catalytic rate similar to maximally inhibited wild-type \textit{Mtu}IPMS.\textsuperscript{39}

These experiments indicate important regions involved in the regulatory signal transfer, however it still remains unclear exactly how regulation is achieved.

1.7 Ligand binding in \textit{Mycobacterium tuberculosis}

1.7.1 $\alpha$-KIV binding

\textbf{Figure 1.9}: Active site of \textit{Mtu}IPMS (PDB code 1SR9) with $\alpha$-KIV (cyan) shown interacting with Zn\textsuperscript{2+} (black). Residues involved in binding $\alpha$-KIV and Zn\textsuperscript{2+} are also shown in purple. Water is shown as a sphere in yellow.
The active site of MtulPMS (viewed as PDB code 1SR9, Figure 1.9) consists of a metal ion coordinating to α-KIV. The metal ion, in this case Zn$^{2+}$, is held in place by coordination to a water molecule and the residues Asp81, His285 and His287. The divalent metal ion polarizes the α-KIV making it more susceptible to nucleophilic attack as shown in the proposed reaction mechanism (Figure 1.6). Monovalent metal ions have not been observed in the crystal structure suggesting that they do not play a role in the physical binding of the substrates. As well as coordination to the divalent metal ion, α-KIV also interacts with the residues R80 and T284. The active sites of α-IPMSs are highly conserved. Asp81, His285, Arg80 and Thr254 are all conserved across the range of α-IPMSs. A hydrophobic pocket is also present around α-KIV which is thought to dictate the enzyme’s specificity for the α-keto acid. The residues involved in the formation of the hydrophobic pocket are Leu143, His167, Ser126, Asn250 and pro252, all of which are highly conserved.

1.7.2 Leucine binding

*Figure 1.10*: Interface between monomers of the regulatory domain of MtulPMS (PDB code 3FIG). Shows residues from each monomer (orange and cyan) binding to leucine (yellow).
α-IPMS is allosterically inhibited by a leucine molecule in each monomer, which are found bound at the regulatory domain. The regulatory binding site of *Mtu*IPMS (PDB code 3FIG) (figure 1.10) consists of interactions between Asn532 and Leu535 from one monomer and Ala565 and Ile627 from the other monomer. A hydrophobic binding pocket around the hydrophobic end of leucine is formed by residues Val551, Leu535 and Tyr554.

Residues in the regulatory site of α-IPMS are well conserved. The motif G-x-G-P-[VIL] is known where x can be any residue. The motif corresponds to residues 531-535 in *Mtu*IPMS.

### 1.8 *Mtu*IPMS truncation

[Figure 1.11](#): Crystal structure of the truncated *Mtu*IPMS (PDB code 3U6W) showing the catalytic domain. Each domain is shown as different colours.

A truncated variant of *Mtu*IPMS was also created in which the catalytic domain was isolated and characterised. The protein is comprised of residues 1-425 of the wild-type enzyme that corresponds to the catalytic domain and subdomain I therefore excluding the regulatory
domain and subdomain II. The protein had a molecular mass of 47.5 kDa as compared to the wild type mass of 70 kDa. It was determined that \textit{Mtu}IPMS existed as a homodimer in solution using size-exclusion chromatography and the protein was found to be a homodimer in the crystalline form. A slight loss of thermal stability was observed using differential scanning fluorimetry (DSF) where the melting point of the truncated variant was decreased from the wild-type value of 42.5 ± 0.1 °C to 40.8 ± 0.1 °C. Kinetic studies showed no catalytic activity for this truncated variant.

1.9 \textit{Nme}IPMS truncation

\textbf{Figure 1.12}: Crystal structure of truncated \textit{Nme}IPMS (PDB code 3RMJ) showing the catalytic domain. The two monomers are shown in different colours.

A truncated variant of the \textit{α}-IPMS from \textit{Neisseria meningitidis} (\textit{Nme}) was created in which the catalytic domain was isolated and characterised. There is currently no full length structure available for this variant. The protein is made up of residues 1-365 of the \textit{Nme}IPMS which is
comprised of the catalytic domain, subdomain I and two α-helices of subdomain II and had a molecular mass of 40 kDa. The full length protein has a mass of 56 kDa. Size-exclusion chromatography indicated that the truncated variant was homodimeric despite the significant loss of potential contacting residues due to the truncation. It was shown using DSF that the truncated variant had the same melting point as the wild-type enzyme of 44.5 ± 0.1 °C suggesting the regulatory domain does not add to the stability of the enzyme. The enzyme displayed AcCoA hydrolysis in the absence of α-KIV, but was unable to catalyse the Claisen-condensation between α-KIV and AcCoA. AcCoA hydrolysis was not affected by leucine concentrations up to 10 mM as was expected with a lack of the regulatory domain.
1.10 Citramalate synthase

1.10.1 Overview

Citramalate synthase (CMS), an enzyme found in the citramalate pathway, is one of the key enzymes responsible for the creation of isoleucine via the threonine-independent pathway found in archaea and some bacteria such as Leptospira interrogans (Li), which is the causative agent for Zoonotic disease. This enzyme also catalyses a Claisen-condensation reaction similar to α-IPMS.41

Only a small number of CMS enzymes have been studied as this pathway is relatively rare due to most organisms using the threonine-dependent pathway for isoleucine synthesis. CMS has been studied in L. interrogans⁴¹, G. sulfurreducens⁴², M. sannaschii⁴³, R. rubrum⁴⁴ and Thermoanaerobacter sp.⁴⁵

1.10.2 Enzyme activity

CMS is responsible for the condensation reaction of AcCoA and pyruvate to form citramalate (Figure 1.13). Reported $K_m$ values for the natural substrates vary significantly (Table 1.2). Slightly basic pH levels have been shown to lead to the greatest enzyme activities with most assays being performed between pH 7.0 and 8.0.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Pyruvate $K_m$ (µM)</th>
<th>AcCoA $K_m$ (µM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. interrogans⁴¹</td>
<td>60</td>
<td>1118</td>
<td>10.3</td>
</tr>
<tr>
<td>R. rubrum⁴⁴</td>
<td>NR</td>
<td>3200</td>
<td>NR</td>
</tr>
<tr>
<td>M. jannaschii⁴³</td>
<td>184</td>
<td>303</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Table 1.2: Kinetic data for CMS from different organisms. NR = not reported.
LiCMS is able to use glyoxalate and α-KB as alternate α-keto acid substrates but exhibits no observable activity with α-KIV, the substrate for α-IPMS.41

1.10.3 Catalysed reaction

\[
\text{pyruvate} + \text{AcCoA} \xrightarrow{H_2O} \text{citramalate} + \text{HCoA}
\]

Figure 1.13: Reaction catalysed by CMS

1.10.4 Metal dependency

Like α-IPMS, LiCMS requires a divalent metal ion for catalysis. Unlike MtuIPMS monovalent cations are not essential for activity, however, when K\(^+\) ions are added a ~50 % increase in activity is observed.41 It is thought the K\(^+\) ions stimulate the divalent metal ions in the active site.

1.10.5 Proposed mechanism

The mechanism proposed for the condensation of AcCoA and pyruvate by LiCMS is analogous to the mechanism proposed for IPMS catalysis (Figure 1.6).
1.10.6 Feedback inhibition

CMS is allosterically feedback regulated by the end product of its pathway, isoleucine. This allows for control of the metabolic flux throughout the pathway ensuring over-production of isoleucine does not occur. Isoleucine binds and inhibits the enzymes at an allosteric position in the regulatory domain. LiCMS has a reported $K_i$ of 19 ± 5 µM for isoleucine and no inhibition observed with leucine. Two LiCMS mutants have been created which show some inhibition by leucine. These are Y454A and V468A which have respective $K_i$ values of 1302 ± 406 µM and 75 ± 24 µM.\textsuperscript{13}

1.11 Structure

There are currently no full length crystal structures solved of CMS from any source. Only five crystal structures of parts of the protein have been solved so far (PDB codes 3F6G, 3F6H, 3BLF, 3BLE and 3BLI) and all of these are from \textit{L. interrogans}. Two of these structures are for the regulatory domain complexed with isoleucine at the regulatory site, and one of these structures also contains sulfate. The other three structures show the catalytic domain complexed with malonate, pyruvate or pyruvate and AcCoA with Zn$^{2+}$ as the metal ion.

\textbf{Figure 1.14:} Catalytic domain of \textit{LiCMS} (PDB code 3BLI) with α-KIV (yellow) and AcCoA (orange) bound. Zn$^{2+}$ (black) is shown as the divalent metal ion.
The full length LiCMS is comprised of 516 amino acids with a molecular mass of 56 kDa for each monomer. The crystal structure of the regulatory domain shows LiCMS as a homodimer. LiCMS appears to be comprised of an N-terminal catalytic domain (residues 1-330) and a C-terminal regulatory domain (residues 390-516) which based on homology models with MtuIPMS are thought to be connected via a flexible linker region (residues 331-389). The catalytic domain is composed of a (β/α)_8-barrel as with MtuIPMS. The regulatory domain adopts a (βαβ) sandwich structure made of two anti-parallel β-sheets and two α-helices. The inhibitor binding pocket is located in the same place as it is on MtuIPMS, at the dimer interface.

**Figure 1.15**: Regulatory domain of LiCMS (PDB code 3F6G) showing isoleucine (cyan) bound. One monomer is shown in red and the other in grey.
1.11.1 Pyruvate binding

![Diagram]

**Figure 1.16**: Catalytic domain of LiCMS (PDB code 3BLI) showing the active site with pyruvate (yellow) interacting with Zn$^{2+}$ (black) and important active site residues (cyan). The water molecule is shown as a yellow sphere.

Pyruvate binds at the active site of LiCMS near the N-terminus. A Zn$^{2+}$ ion interacts with the carbonyl and carboxylate functional groups withdrawing electron density which allows for a more favourable nucleophilic attack from AcCoA. The Zn$^{2+}$ ion is held in place by three residues - Asp17, His207 and His209. Asp16 is also able to hydrogen bond pyruvate which may help to stabilise pyruvate binding.
1.11.2 AcCoA binding

No structure with AcCoA bound to α-IPMS has been solved. The catalytic domain of LiCMS does however show both pyruvate and AcCoA bound in the active site. Figure 1.17 shows the overlay of MtulPMS with LiCMS illustrating residues which play a similar role in substrate binding and suggest a location in MtulPMS where AcCoA is likely to bind. There are many similarities in the active sites of these two enzymes. The LiCMS residues Arg16, Asp17, His207, His209 and Thr179 are also found in almost identical places in MtulPMS suggesting these residues are extremely important in the condensation of α-keto acids and AcCoA. Glu146 is thought to act as a base which enolises AcCoA while Arg16 stabilises the enol form.¹³ These residues are highly conserved for both enzymes.

![Figure 1.17: Overlay of the active site of LiCMS (blue)(PDB code 3BLI) and MtulPMS (purple)(PDB code 1SR9). AcCoA is shown in orange, α-KIV in cyan, pyruvate in yellow. Zn²⁺ in LiCMS is shown in black and in MtulPMS in grey. CMS hydrogen bonding is shown.](image-url)
1.11.3 Isoleucine binding

**Figure 1.18:** LiCMS (PDB code 3F6G) regulatory site showing isoleucine (cyan) binding at the dimer interface. One monomer is shown in grey and the other in red.

LiCMS forms hydrogen bonds with residues Asp431, Thr464, Ala466 and Gln495. The selectivity towards isoleucine over other ligands is also influenced by hydrophobic interactions which is the work described in chapter 2. The residues Thr464 and Ala466 are located on a flexible β-strand which is thought to play a role in ‘capping’ the regulatory pocket when isoleucine is bound. The same ‘capping’ strand is found in the *MtuIPMS* structure (Figure 1.19).[36]
Figure 1.19: Regulatory domains of *MtulPMS* (blue) (PDB code 3FIG) and *LiCMS* (red) (PDB code 3F6G) overlaid illustrating the flexible ‘capping’ β-strand in yellow (*MtulPMS*) and green (*LiCMS*). Leucine is shown in cyan and isoleucine in orange.
1.12 Objectives of this thesis

The main objective of this thesis was to investigate the determinants of the inhibitor selectivity in MtulIPMS from leucine and gain an understanding of the role the residues in the regulatory binding pocket play in inhibitor selectivity. A change in inhibitor selectivity from leucine to isoleucine was attempted based upon the regulatory site of LiCMS. This work was carried out using α-IPMS from *M. tuberculosis*.

The secondary objective in this thesis was to investigate three residues which were thought to be involved in the transmission of the regulatory signal from the regulatory domain through the enzyme to the catalytic domain. This part of the thesis was carried out using α-IPMS from *N. meningitidis*.

Research goals:

- Gain a greater understanding of how inhibitor selectivity is determined
- Increase the knowledge of α-IPMS enzymes and the roles they play
- Gain an understanding of how the regulatory signal may be transferred throughout the protein with minimal conformational change

α-IPMS from *M. tuberculosis* and *N. meningitidis* were chosen due to the pathogenic nature of the organisms they originate from and their availability. A range of amino acid substituted variants were created to investigate inhibitor selectivity and regulatory signal transfer.
Chapter 2

Effects of regulatory domain variants on inhibitor selectivity of α-isopropylmalate synthase from *Mycobacterium tuberculosis*

2.1 Overview

The goal of this research was to make single amino-acid changes to the allosteric inhibitory site of *Mtu*IPMS in order to see if inhibitor selectivity could be tuned towards isoleucine over the natural inhibitor leucine.

These variants were characterised via enzyme kinetic studies, protein mass spectrometry, differential scanning fluorimetry and circular dichroism. Enzyme kinetic studies included obtaining Michaelis-Menten constants and determining the relative inhibition data for each variant generated. Protein mass spectrometry was implemented to ensure the purified protein was of the correct mass. Differential scanning fluorimetry was used to determine the melting temperatures of the wild-type and each variant in the presence of different inhibitors and in the absence of inhibitor. Finally circular dichroism was used to ensure the chosen variants did not significantly alter the secondary structure of the enzyme via comparison to the wild-type enzyme.
2.2 Design of MtuIPMS variants

In order to change the MtuIPMS inhibitor selectivity from leucine to isoleucine, the isoleucine-sensitive enzyme that is responsible for catalysing a similar reaction to α-IPMS, CMS, was used as a template. The crystal structure of the regulatory domain of the only currently described CMS structure, LiCMS, was overlaid with the MtuIPMS regulatory domain crystal structure to make structural comparisons.\textsuperscript{13,33}

\textbf{Figure 2.1:} Superposition of the regulatory domains from MtuIPMS (blue, PDB code 3FIG) and LiCMS (red, PDB code 3F6G) showing natural inhibitors leucine (cyan) and isoleucine (orange) bound.

This overlay shows a high similarity in tertiary structure and subunit interaction between IPMS and CMS enzymes and illustrates the similar binding poses and positions of the inhibitors. A closer analysis of the binding sites in LiCMS was carried out to reveal differences and similarities in key residues involved in the hydrophobicity of the regulatory pocket (Figure 2.2).
Figure 2.2: Residues contributing to the regulatory pockets of MtuIPMS (PDB code 3FIG) and LiCMS (PDB code 3F6G). Leucine is shown in cyan, and isoleucine in orange. Residues for LiCMS are shown in yellow for one monomer and orange for the opposing monomer. MtuIPMS residues are shown in cyan for one monomer and blue for the other. Residue labels for LiCMS are coloured black and grey for MtuIPMS.

By comparing the residues involved in binding isoleucine in LiCMS with the residues involved in binding leucine in MtuIPMS, the following residues in MtuIPMS were selected as potential residues for mutation for further analysis: V551, A558, A567 and I627.

Table 2.1: Distance of chosen residues from the bound leucine in the regulatory domain of MtuIPMS (PDB code 3FIG).

<table>
<thead>
<tr>
<th>MtuIPMS residue</th>
<th>Nearest distance from bound leucine (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val551</td>
<td>4.3</td>
</tr>
<tr>
<td>Ala558</td>
<td>4.1</td>
</tr>
<tr>
<td>Ala567</td>
<td>4.4</td>
</tr>
<tr>
<td>Ile627</td>
<td>3.9</td>
</tr>
</tbody>
</table>

30
The chosen residues were then analysed further by inspecting a multiple sequence alignment (Appendix A) of α-IPMS sequences in order to ensure the residues chosen are well conserved across the α-IPMS enzymes (Table 2.2), suggesting they play an important role in the binding of leucine. A comparable multiple sequence alignment of CMS enzymes was not carried out as it was not clear which of the sequences annotated as CMS are in fact CMS enzymes.

**Table 2.2:** Residues in LiCMS that correspond to the chosen residues in MtuIPMS and conservation of these residues across α-IPMS enzymes.

<table>
<thead>
<tr>
<th>MtuIPMS residue</th>
<th>LiCMS residue</th>
<th>Conserved in α-IPMS (Yes/No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val551</td>
<td>Leu451</td>
<td>Yes</td>
</tr>
<tr>
<td>Ala558</td>
<td>Ile458</td>
<td>Yes</td>
</tr>
<tr>
<td>Ala567</td>
<td>Val468</td>
<td>Yes</td>
</tr>
<tr>
<td>Ile627</td>
<td>Gln495</td>
<td>Yes (Ile or Thr)</td>
</tr>
</tbody>
</table>

### 2.3 Justification of choice of variants

#### 2.3.1 MtuIPMS A558I and A558V

The Ala558 residue of MtuIPMS is well conserved across α-IPMS enzymes in the multiple sequence alignment. All but one α-IPMS enzyme in the multiple sequence alignment (Appendix A) have an alanine at this position. The other residue observed at this position is threonine. The mutation to an isoleucine was performed to mimic the residue in the same position in LiCMS. Ala558 was also changed to valine in case the introduction of an isoleucine at this position led to an unstable regulatory site due to steric factors possibly preventing inhibitors being able to bind to the regulatory site or due to interactions with the side chains of neighbouring residues.
2.3.2 \textit{Mtu}\textsubscript{IPMS} V551L

The Val511 residue of \textit{Mtu}\textsubscript{IPMS} is well conserved. There are two enzymes in the multiple sequence alignment which contain an isoleucine and a leucine at this position. The corresponding residue at this position in \textit{LiCMS} is a leucine. The mutation from valine to leucine was carried out to mimic the \textit{LiCMS} regulatory site.

2.3.3 \textit{Mtu}\textsubscript{IPMS} A567V

The Ala567 residue of \textit{Mtu}\textsubscript{IPMS} is conserved across α-IPMS enzymes in the multiple sequence alignment. Ala567 corresponds to a valine in \textit{LiCMS}, and was mutated into a valine to mimic \textit{LiCMS}.

2.3.4 \textit{Mtu}\textsubscript{IPMS} I627A

The Ile627 residue of \textit{Mtu}\textsubscript{IPMS} is conserved as an isoleucine or threonine across α-IPMS enzymes. Based upon the crystal structure it was substituted for an alanine to determine the effect of shortening the side chain at this position and potentially to make room for the methyl group at the C3 position of isoleucine. This residue was not exchanged for glutamine (the equivalent residue in \textit{LiCMS}) due to the high possibility of steric classes with other residues within the regulatory pocket resulting from increased chain length.
2.4 *MtulPMS* variant generation using PCR

*MtulPMS* variants were created via the mutation of the wild-type *MtulPMS* gene using a polymerase chain reaction (PCR) method. The *MtulPMS* wild-type gene was located in pPROEX-HTa plasmid. This plasmid also contains coding for an N-terminal His-tag to allow for easy purification by immobilised metal affinity chromatography, a TEV protease cleavage sequence located between the *MtulPMS* gene and the His-tag sequence to allow for tag removal after purification, a T7 promoter region binding a lac operon to enable over-expression of the protein of interest after addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) and an ampicillin resistance gene to allow for selection of cells containing the plasmid during growth on media containing ampicillin. A plasmid extraction was carried out to obtain the plasmid containing the wild-type *MtulPMS* gene. The purified plasmid was then examined on an agarose gel to ensure the obtained plasmid was the expected size of approximately 4700 bp.

![Figure 2.3: Agarose gel showing purified pPROEX-HTa plasmid which contains the wild-type *MtulPMS* gene. Marker weights shown are in base pairs (bp).](image)
Once the wild-type template DNA was obtained PCR was performed using primers which were designed to introduce the mutations of choice into the wild-type gene. The amplified PCR products were subjected to the addition of Dpn1 which digests the methylated wild-type template DNA leaving only the amplified PCR product which containing the mutation.

The PCR products were then analysed using agarose gel electrophoresis to ensure a band corresponding to the theoretical calculated size of the PCR product had been obtained.

**Figure 2.4**: Agarose gel showing PCR products post Dpn1 treatment. Lane 1, A558V; lane 2, V551L; lane 3, ladder of which marker weights are shown in base pairs; lane 4, A567V and lane 5, I627A. Marker weights above 5000 are not shown for viewing clarity. Each marker band above 5000 is another 1000 bp where the largest band corresponds to 12000 bp.
Once the PCR product had been viewed as the correct size on agarose gel transformation into *E. coli* One Shot TOP10 cells was performed. The plasmids were then isolated from the cells and sequenced. The sequences were compared to the wild-type gene sequence to ensure that the PCR had been successful and the correct mutation had been introduced into the gene. The plasmids which contained the mutations of interest were then transformed into BL21(DE3)Star cells for protein expression.

### 2.5 Expression of protein

Expression of the wild-type *MtulPMS* and variants was carried out as described in the materials and methods section (Chapter 5). Following cell growth and protein expression, cell pellets were stored at -80°C until required. Proteolysis can occur in the cell lysate during purification steps. To minimize this, immobilised metal affinity chromatography (IMAC) steps were performed immediately after lysis and protease inhibitor was added to the lysate.

![Figure 2.5: SDS-PAGE gel of wild-type MtulPMS over-expression after lysis using BugBuster® showing the supernatant (SN) in lane 1 and the cell pellet (CP) in lane 2 with ladder shown in lane 3. Marker weights indicated are in kDa.](image-url)
Cell lysis was performed using sonication for full scale cell growths or using detergent based disruption (Bugbuster®) for small scale growths such as expression trials. A large proportion of the over-expressed protein is found in the supernatant at the expected molecular mass of 77 kDa which also includes the 4 kDa His-tag. Due to the presence of many other proteins observed in the cell pellet it appears that lysis is incomplete.

2.6 Purification

The wild-type \textit{Mtu}IPMS and mutants were purified using the method shown in figure 2.6.\textsuperscript{40}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{flow_diagram.png}
\caption{Flow diagram of the procedure used for purification of \textit{Mtu}IPMS.}
\end{figure}

This purification procedure produced protein of high purity for all characterisation assays. Dithiothreitol (DTT) was added prior to addition of TEV protease in order to reduce disulfide bonds which may form between the proteins allowing for better access to the TEV cleavage site. The dilution with SEC buffer following the IMAC step was performed to reduce imidazole concentration in preparation for TEV protease cleavage of the His-tag.

This purification procedure usually yielded 10-15 mg of purified protein for a 1 L cell culture grown in lysogeny broth (LB).
2.6.1 Immobilised metal affinity chromatography

The first step in the purification procedure of *MtuPMS* is IMAC. A HisTrap charged with Ni$^{2+}$ ions was used to separate *MtuPMS* from background *E. coli* proteins via the N-terminal His-tag. Protein which bound to the column was eluted with imidazole. The bound and unbound fractions typically obtained from this procedure can be viewed in lanes 1 and 2 of figure 2.8.

2.6.2 Desalting

Protein was eluted from the IMAC column in a buffer which contained a high imidazole concentration. For this reason, prior to the addition of TEV protease, the pooled fractions were diluted in SEC buffer containing no imidazole to ensure the imidazole did not interfere with protein stability or TEV protease efficiency.

2.6.3 TEV protease cleavage

Once the desalting step had been completed the protein was digested with TEV protease overnight at 4°C to remove the N-terminal His-tag. Removal of the His-tag was carried out to minimise the chances of the tag interfering with the characterisation of *MtuPMS*.

The His-tag added 25 residues to the N-terminus of *MtuPMS* including the six histidine residues used to bind the protein to the IMAC column and the TEV protease recognition sequence. After digestion two residues (glycine and alanine) are left attached to the N-terminus of *MtuPMS* (Figure 2.7).

![Sequence of the pPROEX-HTa plasmid polyhistidine tag showing the polyhistidine tag (red), the TEV recognition site (green) the extra residues after digestion (blue) and the beginning of the MtuPMS sequence (pink).](image)

**Figure 2.7:** Sequence of the pPROEX-HTa plasmid polyhistidine tag showing the polyhistidine tag (red), the TEV recognition site (green) the extra residues after digestion (blue) and the beginning of the *MtuPMS* sequence (pink).
After digestion was complete the solution was once again run through the HisTrap column, which allowed the His-tag cleaved protein to travel through the column without binding. The TEV protease contains a His-tag so that it binds to the column separating it from MtuIPMS. An example of the bound and unbound fractions from this step are shown in figure 2.8.

![Figure 2.8: SDS-PAGE gel showing fractions after each IMAC step for MtuIPMS I627A. Lane 1, shows the flowthrough from the first IMAC run; lane 2, shows the bound fraction from the first IMAC run; lane 3, shows the marker ladder; lane 4 and 5, show the flowthrough of the second IMAC run and lane 6, shows the bound fraction of the second IMAC run. Indicated marker weights are in kDa.](image)

2.6.4 Size-exclusion chromatography

The last step in the purification protocol was size-exclusion chromatography (SEC), which elutes MtuIPMS in one peak and aggregated proteins in a separate peak which has a smaller retention volume. The mass of the purified protein was that of the expected molecular mass of 70 kDa (Figure 2.9).
**Figure 2.9:** SDS-PAGE gel of final collected fractions after SEC of wild-type MtuIPMS and variants. Lane 1, shows the marker ladder; lane 2, shows the wild-type; lane 3, shows A558I; lane 4, shows A558V; lane 5, shows V551L; lane 6, shows A567V and lane 7, shows I627A. Marker weights are shown in kDa.

### 2.7 Mass spectrometry

The masses of all the MtuIPMS enzymes were measured using electrospray mass spectrometry (MS) to ensure all enzymes were of the expected mass calculated from amino acid sequence using ProtParam. A summary of the wild-type and variant masses is shown in table 2.3.

**Table 2.3:** Expected masses and masses calculated via MS of MtuIPMS enzymes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Expected mass (Da)</th>
<th>Calculated mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtuIPMS WT</td>
<td>70211.7</td>
<td>70212.6</td>
</tr>
<tr>
<td>MtuIPMS A558I</td>
<td>70253.8</td>
<td>70252.7</td>
</tr>
<tr>
<td>MtuIPMS A558V</td>
<td>70239.7</td>
<td>70237.2</td>
</tr>
<tr>
<td>MtuIPMS V551L</td>
<td>70225.7</td>
<td>70227.3</td>
</tr>
<tr>
<td>MtuIPMS A567V</td>
<td>70239.7</td>
<td>70237.8</td>
</tr>
<tr>
<td>MtuIPMS I627A</td>
<td>70169.6</td>
<td>70169.9</td>
</tr>
</tbody>
</table>
2.8 *Mtu*IPMS wild-type

2.8.1 Physical characterisation

This section details the examination of the wild-type enzyme. This work took place for later comparison with the *Mtu*IPMS variants.

2.8.2 Secondary structure analysis

A circular dichroism (CD) spectrum (Figure 2.10) was obtained for *Mtu*IPMS wild-type enzyme to ensure the protein was folded correctly and to use as a comparison for the *Mtu*IPMS variants to ensure no major structural change had taken place.

![Circular dichroism spectrum](image)

*Figure 2.10:* Circular dichroism spectrum of *Mtu*IPMS wild-type. Spectrum was obtained using 0.2 mg/mL protein in distilled H$_2$O at pH 7.0.
The shape of the obtained spectrum is consistent with the shape of the properly folded protein. This presence of secondary structure suggests it is suitable for further characterisation.

2.8.3 Thermal stability

Differential scanning fluorimetry (DSF) was used to examine the thermal stability of the wild-type enzyme and the effects which occurred to the denaturation temperature in the presence of the inhibitors leucine, isoleucine and norvaline. DSF uses a dye which binds to hydrophobic regions of the protein and fluoresces. As the protein is subjected to steady heating it reaches a point where it denatures revealing previously buried hydrophobic regions leading to a steep rise in the fluorescence intensity. This allows the denaturation point of the enzyme to be calculated by plotting the differentiated function and looking for the lowest point on the graph (Figure 2.11).

![Figure 2.11: Example of a DSF curve for the wild-type MtuIPMS enzyme with no ligand present where in green is the relative fluorescence unit (RFU) and shown in purple is the negative derivative of the RFU. Red shows the negative control which contains no enzyme. The melting temperature is expressed as the steepest part of the RFU trace and the minimum on the negative derivative. The experiment was carried out in BTP buffer at pH 8.0.](image-url)
It was found using DFS that wild-type MtuIPMS has a denaturation temperature of 47.6 ± 0.1 °C which is similar to the denaturation temperature ($T_m$) obtained for the IPMS from *N. meningitidis* (Figure 3.9).24

DSF can be used as evidence for ligand binding as when a ligand binds to the protein an overall increase in the stability is sometimes observed.46 Responses to the thermal stability of the wild-type enzyme when in the presence of 5 mM leucine, isoleucine and norvaline are displayed in figure 2.12.

![Figure 2.12: Effects on $T_m$ of MtuIPMS in the presence of 5 mM leucine, isoleucine and norvaline in 25 mM BTP buffer (pH 8.0).](image)

From this data a small but significant increase in the thermal stability of the protein was observed in the presence of all three ligands, suggesting that the enzyme is stabilised by all of the tested ligands. Leucine was expected to provide the greatest increase in stability due to its role as the natural inhibitor of MtuIPMS and this was observed, however, norvaline was found to stabilise the enzyme to almost the same extent. Isoleucine did stabilise the enzyme but to a lesser degree. The tested concentrations of tested ligands were very high compared to the *in vivo* concentrations, and this may have led to fully occupied regulatory binding sites with the
tested ligands despite having a low binding affinity, leading to a lack of discrimination between different ligands at this concentration.

2.8.4 Kinetic characterisation

The activity of *Mtul*PMS was studied first by finding the apparent Michaelis-Menten constants for the two substrates (α-KIV and AcCoA). Once this data had been elucidated, assays involving leucine, isoleucine and norvaline were performed.

All assays were carried out using a 4,4'-dithiodipyridine (DTP) coupled assay which was monitored at 324 nm. This measures the formation of a thio-pyridine as DTP reacts with the free thiol group of the enzyme reaction product CoA.\(^{34}\)

2.8.5 Michaelis-Menten kinetics

Apparent \(K_m\) and turnover values were measured at 25 °C and pH 8.0. Apparent AcCoA \(K_m\) values were found by holding the α-KIV concentration at 400 μM while the AcCoA concentration was varied from low to high concentrations. The apparent α-KIV \(K_m\) values were determined by holding the AcCoA concentration at 300 μM while the α-KIV was varied from low to high concentration. \(K_m\) values of 41 ± 2 μM and 8.4 ± 0.9 μM were calculated for AcCoA and α-KIV respectively. A \(k_{cat}\) value of 3.6 ± 0.1 s\(^{-1}\) was also obtained for the wild-type enzyme (Figure 2.13).
Figure 2.13: Michaelis-Menten plots obtained for wild-type MtuIPMS showing the apparent AcCoA (left) and α-KIV (right) $K_m$. Standard assay conditions were as follows: 500 µM DTP, 3 mM MgCl$_2$ and 20 mM KCl. A $k_{cat}$ value of $3.6 \pm 0.1$ s$^{-1}$ was obtained.
2.8.6 Allosteric inhibition based upon initial rates

Figure 2.14: Remaining activity (%) of MtuIPMS wild-type in the presence of differing concentrations of leucine, isoleucine and norvaline when initial rates are measured. Experimental conditions as follows: 500 µM AcCoA, 400 µM α-KIV, 500 µM DTP, 20 mM KCl and 3 mM MgCl₂ in 50 mM Tris-HCl buffer at pH 8.0.

The activity of MtuIPMS in the presence of leucine at 500 µM is maximally inhibited and the activity drops to approximately 15% of the uninhibited activity and remains at this level as the leucine concentration is increased. This high level of inhibition was expected as leucine is the natural inhibitor for the wild-type enzyme. Little effect on the activity of the enzyme at 1 mM isoleucine was observed. However, at 5 mM a decrease in activity to approximately 38% was observed. The relative insensitivity of the enzyme to the lower leucine concentration suggests that isoleucine inhibition may not be physiologically relevant \textit{in vivo}; otherwise leucine production would be highly dependent upon intracellular isoleucine levels. MtuIPMS shows a similar sensitivity towards norvaline where at 1 mM a slight decrease in activity was observed and when the concentration was increased to 5 mM the enzyme activity dropped to approximately 44%.
2.9 Demonstration of slow-onset inhibition

*Mtu*IPMS displays a rare type of inhibition in which a time-dependent effect on rate is observed, this is known as allosteric slow-onset inhibition. It is thought that this is due to *Mtu*IPMS acting through a two-step mechanism where equilibrium exists between two conformations of the enzyme-inhibitor complex. These two conformations display different kinetic properties and are separated by an energy barrier.\(^{36,46}\)

![Energy Profile Diagram](image)

**Figure 2.15**: Diagram showing energy profile for allosteric slow-onset inhibition where E = Unbound enzyme; EI = High energy enzyme-inhibitor complex transition state; EI* = Low energy enzyme-inhibitor complex.

Data to examine the slow onset inhibition was obtained for the wild-type *Mtu*IPMS (Figure 2.16). Allosteric slow-onset inhibition was observed at all tested leucine concentrations. Reaction rates measured after two minutes were slightly lower than the initial rates. This behaviour was not a result of decreasing substrate concentrations as the reaction progressed, as this change in rate was not seen with the uninhibited enzyme over this time course or reaction extent. Interestingly slow-onset kinetics was observed with all tested ligands which disagrees with results published recently by Casey *et al.*\(^{36}\) where show onset inhibition was only observed with leucine. This effect may arise from the relatively high ligand concentrations used in the assays.
2.9.1 *MtulPMS* wild-type observed slow-onset inhibition

The data obtained by measuring the initial rates and the rates after two minutes shows that the wild-type *MtulPMS* enzyme displays slow-onset inhibition with all three tested ligands. Similar decreases in the remaining activity percentages are seen between the initial and two minute measurements for all leucine concentrations and isoleucine at 5 mM. Norvaline showed a much larger difference in remaining activity percentage, dropping from 45% to under 30% in two minutes.
2.10 Variant structure analysis

To ensure no major structural changes had taken place due to the introduced amino acid substitutions, CD spectra were obtained for MtuIPMS variants and these were compared to the spectrum obtained for the wild-type enzyme. The spectra show a high level of similarity suggesting the overall fold of the protein has not been significantly altered by the amino acid substitutions.

![CD spectrum graph]

**Figure 2.17**: CD spectrum of all MtuIPMS mutants using 0.2 mg/mL enzyme exchanged into double-distilled water at pH 7.0. Wild-type = blue; A558I = red; A558V = green; V551L = orange; A567V = purple; I627A = yellow.
2.11 Thermal stability of variants

The thermal stabilities of the *Mtu*PMS variants were examined to establish whether any major differences in stability in the presence of leucine, isoleucine and norvaline have occurred when compared to the wild-type enzyme under the same conditions.

![Figure 2.18: DSF data showing denaturation temperatures of *Mtu*PMS wild-type and variants in the presence of 5 mM leucine, isoleucine and norvaline. The wild-type is shown in blue, A558I in red, A558V in green, V551L in purple, A567V in yellow and I627A in orange. Experiment was carried out with 20mM KCl, 3mM MgCl$_2$ in 25 mM BTP buffer at pH 8.0.]

The *Mtu*PMS A558I variant was destabilised under all experimental conditions compared to the wild-type enzyme and all other variants. This protein also did not show any additional stabilisation in the presence of the tested ligands suggesting the ability of the A558I variant to bind these ligands has been severely attenuated.
The *MtulPMS* A558V variant showed the same denaturation temperature (*T*<sub>m</sub>) as the wild-type enzyme when no ligands were present showing this amino acid substitution has not led to a significant change in stability of the unbound enzyme. However, this variant shows a similar stabilisation trend to the wild-type enzyme in the presence of all three tested ligands but to a lesser degree.

*MtulPMS* V551L also showed the same *T*<sub>m</sub> as the wild-type enzyme with no ligand present, however, in the presence of both leucine and isoleucine an increase in *T*<sub>m</sub> was observed compared to the wild-type enzyme with these ligands, suggesting increased enzyme stability under these conditions. In the presence of 5 mM norvaline the same *T*<sub>m</sub> was observed as the wild-type enzyme suggesting the amino acid substitution had not affected the stability of the protein in the presence of norvaline.

*MtulPMS* A567V showed increased stability across all tested conditions suggesting this amino acid substitution had an overall stabilising effect on the enzyme. Similar increases in *T*<sub>m</sub> in response to the three amino acid ligands were observed as for the wild-type enzyme.

The *MtulPMS* I627A variant showed a slightly higher *T*<sub>m</sub> than the wild-type enzyme, however, in the presence of leucine and isoleucine the same absolute level of enzyme stability was observed as for the wild-type enzyme, so overall less stability was gained by addition of these ligands than observed for the wild-type enzyme. Interestingly, no effect on enzyme stability was observed in the presence of norvaline.

Table 2.4: Denaturation temperature for *MtulPMS* wild-type and variants without ligand and in the presence of 5 mM leucine, isoleucine and norvaline. Results were obtained in 25 mM BTP buffer at pH 8.0.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>No ligand</th>
<th>Leucine</th>
<th>Isoleucine</th>
<th>Norvaline</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MtulPMS</em> WT</td>
<td>47.6 ± 0.1</td>
<td>51.6 ± 0.1</td>
<td>49.6 ± 0.1</td>
<td>51.6 ± 0.1</td>
</tr>
<tr>
<td><em>MtulPMS</em> A558I</td>
<td>45.3 ± 0.1</td>
<td>45.4 ± 0.1</td>
<td>44.9 ± 0.1</td>
<td>45.3 ± 0.1</td>
</tr>
<tr>
<td><em>MtulPMS</em> A558V</td>
<td>47.6 ± 0.1</td>
<td>49.6 ± 0.1</td>
<td>47.7 ± 0.1</td>
<td>49.6 ± 0.1</td>
</tr>
<tr>
<td><em>MtulPMS</em> V551L</td>
<td>47.6 ± 0.1</td>
<td>52.5 ± 0.1</td>
<td>51.4 ± 0.1</td>
<td>51.6 ± 0.1</td>
</tr>
<tr>
<td><em>MtulPMS</em> A567V</td>
<td>49.6 ± 0.1</td>
<td>52.5 ± 0.1</td>
<td>51.5 ± 0.1</td>
<td>52.6 ± 0.1</td>
</tr>
<tr>
<td><em>MtulPMS</em> I627A</td>
<td>48.6 ± 0.1</td>
<td>51.5 ± 0.1</td>
<td>49.5 ± 0.1</td>
<td>48.8 ± 0.1</td>
</tr>
</tbody>
</table>
2.12 Kinetic properties of variants

Kinetic data was obtained for each variant to ensure no significant change in the catalytic activity of the variants had taken place due to the amino acid substitutions (Table 2.5). No significant differences in the kinetic parameters were observed between the variants and the wild-type enzyme. $K_m$ values obtained for $\alpha$-KIV were between 4 and 10 µM. $K_m$ values obtained for AcCoA were between 40 and 72 µM. Turnover rates of between 3 and 4 s$^{-1}$ were found for the wild-type and all variants.

**Table 2.5:** Michaelis-Menten data obtained for the wild-type (WT) MtulIPMS and each variant. Conditions for obtaining $\alpha$-KIV data: 300 µM AcCoA, 500 µM DTP, 3 mM MgCl$_2$, 20 mM KCl. Conditions for obtaining AcCoA data: 400 µM $\alpha$-KIV, 500 µM DTP, 3 mM MgCl$_2$, 20 mM KCl. All data was obtained at pH 8.0 in 50 mM Tris-HCl buffer. Michaelis-Menten plots are shown in Appendix B.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$\alpha$-KIV $K_m$ (µM)</th>
<th>AcCoA $K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtulIPMS WT</td>
<td>8.4 ± 0.8</td>
<td>41 ± 3</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>MtulIPMS A558I</td>
<td>9.7 ± 1.0</td>
<td>72 ± 5</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>MtulIPMS I627A</td>
<td>4.2 ± 0.4</td>
<td>40 ± 3</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>MtulIPMS A567V</td>
<td>5.0 ± 0.4</td>
<td>56 ± 3</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>MtulIPMS V551L</td>
<td>9.1 ± 0.8</td>
<td>46 ± 3</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>MtulIPMS A558V</td>
<td>6.7 ± 0.6</td>
<td>53 ± 3</td>
<td>4.0 ± 0.1</td>
</tr>
</tbody>
</table>
2.13 Allosteric inhibition of variants

Initial rate data

Inhibition data with leucine, isoleucine and norvaline was obtained for each variant. Data shown is taken from the initial measured rate however, each variant also displayed allosteric slow-onset inhibition with all three tested inhibitors (Figures 2.22 and 2.23).

2.13.1 Leucine inhibition

![Graph showing leucine inhibition](image)

**Figure 2.19**: Response of wild-type and variant *MtulPMS* to 0.5 mM (blue), 1 mM (red) and 5 mM (green) of leucine. Experimental conditions: 500 µM AcCoA, 400 µM α-KIV, 500 µM DTP, 20 mM KCl and 3 mM MgCl₂ in Tris-HCl buffer at pH 8.0.

As noted previously the wild-type *MtulPMS* displays maximal inhibition at 500 µM of leucine, and maintains the same level of inhibition when the leucine concentration is increased to 5 mM. The *MtulPMS* A558I variant displays a significant loss in sensitivity towards leucine; a concentration of 5 mM leucine was only able to reduce the remaining activity to 67 %. This decrease in sensitivity towards leucine was also observed for the *MtulPMS* A558V variant suggesting this residue plays a very important role in the inhibition of the enzyme by leucine.
The *Mtu*PMS A558V variant only reaches approximately 86% remaining activity at 5 mM of leucine making this the least sensitive variant to leucine created in this study. No significant differences between the wild-type enzyme and *Mtu*PMS V551L were observed at all leucine concentrations. The inhibition data of the *Mtu*PMS A567V variant was also similar to the data determined for the wild-type enzyme, except that at 500 µM leucine less inhibition was observed. However, interestingly, at 5 mM leucine greater inhibition was observed compared to the wild-type enzyme suggesting a different means of inhibition may be occurring such as ligand binding at another region of the protein. The *Mtu*PMS I627A variant displays very similar inhibition to the *Mtu*PMS A558I variant.

### 2.13.2 Isoleucine inhibition

![Graph showing inhibition of wild-type and variant *Mtu*PMS to 1 mM (blue) and 5 mM (red) of isoleucine.](image)

**Figure 2.20**: Response of wild-type and variant *Mtu*PMS to 1 mM (blue) and 5 mM (red) of isoleucine. Experimental conditions: 500 µM AcCoA, 400 µM α-KIV, 500 µM DTP, 20 mM KCl and 3 mM MgCl₂ in Tris-HCl buffer at pH 8.0.

There was no significant difference in inhibition observed between the wild-type *Mtu*PMS and any of the variants when assayed with 1 and 5 mM isoleucine.
2.13.3 Norvaline inhibition

Wild-type \textit{MtulPMS} displays little inhibition at 1 mM of norvaline and is inhibited to approximately 45\% of the full enzyme activity at 5 mM norvaline. \textit{MtulPMS} A558I and A558V both display a similar lack of inhibition even at 5 mM norvaline. The \textit{MtulPMS} V551L variant displays similar inhibition to the wild-type enzyme but is slightly more inhibited than the wild-type enzyme at both tested concentrations of norvaline. Interestingly the \textit{MtulPMS} A567V variant displays a huge increase in sensitivity towards norvaline suggesting it is possible to tune the amino acid selectivity from one ligand to another using only a single amino acid substitution. The A567V variant is inhibited to approximately 10\% of the uninhibited activity at 1 mM norvaline and is further inhibited to approximately 6\% activity at 5 mM norvaline. The \textit{MtulPMS} I627A variant displays a lack of sensitivity towards norvaline and behaves similarly to the A558I and A558V variants.
2.14 Observed slow-onset inhibition data

In order to investigate the slow-onset allosteric inhibition characteristics of MtuIPMS, reaction rates were measured upon initiation of the reaction and two minutes after reaction initiation with 5 mM concentrations of each of the tested ligands. No slow-onset inhibition data was obtained with the MtuIPMS variants A558I, A558V or I627A as their lack of sensitivity towards the tested inhibitors meant that the available substrate was used too quickly to view if any slow-onset behaviour was occurring.

2.14.1 MtuIPMS V551L observed slow-onset inhibition

Figure 2.22: Observed slow-onset allosteric inhibition of MtuIPMS V551L with differing concentrations of inhibitors. Shown in blue are remaining activity percentages based upon the measurement of initial rates. Shown in red are the remaining activity percentages of rates measured 2 minutes after reaction initiation. Experimental conditions: 500 µM AcCoA, 400 µM α-KIV, 500 µM DTP, 20 mM KCl and 3 mM MgCl₂ in Tris-HCl buffer at pH 8.0.

The MtuIPMS V551L variant shows similar slow-onset nature to the wild-type enzyme except the difference in the remaining activity percentage with norvaline is not as great between the initial and steady state values.
2.14.2 MtulPMS A567V observed slow-onset inhibition

Figure 2.23: Observed slow-onset allosteric inhibition of MtulPMS A567V with differing concentrations with tested inhibitors. Shown in blue are remaining activity percentages based upon initial rates measured. Shown in red are the remaining activity percentages of rates measured 2 minutes after reaction initiation. Experimental conditions: 500 µM AcCoA, 400 µM α-KIV, 500 µM DTP, 20 mM KCl and 3 mM MgCl$_2$ in Tris-HCl buffer at pH 8.0.

The MtulPMS A567V variant showed similar slow-onset characteristics to the other variants where 5 mM concentrations of leucine and norvaline produced almost identical inhibition data.
2.15 Summary of findings

*Mtu*IPMS variants were successfully created, expressed and purified by metal-affinity and size-exclusion chromatography. Mass spectra showed each variant was of the expected size. CD spectra showed the variants had a similar folding profile to the wild-type suggesting there were no major structural changes due to the amino acid substitutions.

Kinetic assays revealed the Michaelis-Menten kinetics of the wild-type enzyme and each generated variant. The enzyme activity of the variants in the absence of the tested inhibitors seemed unchanged with AcCoA $K_m$ values between 40 and 72 µM, $\alpha$-KIV $K_m$ values between 5 and 10 µM and turnover rates between 3 and 4 s$^{-1}$. This kinetic data is similar to the kinetic data found for other $\alpha$-IPMS enzymes.$^{30,40}$

It was found that the residues in the regulatory site play a vital role in the regulation of the enzyme where even one amino acid change can lead to drastic changes in the inhibition behaviour of the enzyme. No change in regulatory sensitivity towards isoleucine was observed for any of the variants, however the I627A, A558I and A558V variants displayed a greater relative sensitivity towards isoleucine than leucine due to the loss in leucine sensitivity. The A567V variant showed increased sensitivity to norvaline while maintaining similar sensitivity to leucine at the tested concentrations.

Molecular modelling was carried out to examine the structure of *Mtu*IPMS with norvaline and isoleucine-bound instead of leucine. The regulatory sites of *Mtu*IPMS illustrating the positions of norvaline and isoleucine binding with respect to leucine can be used to help analyse the effects that the substitutions have had on the response of the inhibitors (Figures 2.24 and 2.25).
Figure 2.24: *MtulPMS* with norvaline modelled in the leucine binding site. Norvaline is shown in orange, leucine is shown in yellow and surrounding residues are shown in blue. Residues substituted in this study are labeled in red.

Figure 2.25: *MtulPMS* with isoleucine modelled in the leucine binding site. Norvaline is shown in orange, leucine is shown in yellow and surrounding residues are shown in blue. Residues substituted in this study are labelled in red.
The inhibition results from this study suggest the Ala558 and Ile627 residues in MtuIPMS play an important role in the binding of the natural inhibitor, leucine, and norvaline, as alteration of these residues to different amino acids with the same properties (non-polar amino acids) leads to insensitivity to inhibition. Interestingly, all changes made to these residues had no effect on the inhibition observed with isoleucine, introducing the possibility that the inhibition which is seen with isoleucine may be due to the ligand binding elsewhere on the protein scaffold inducing an inhibitory effect, although this is unlikely, and the modelling studies suggest that isoleucine can be accommodated at the leucine binding site. The lack in sensitivity of the Ala558 variants may be due to the increase in side chain length disrupting the binding of the inhibitors due to a decrease in available space within the regulatory pocket. Another possibility
of the lack in inhibition is the location of Ala558 in the protein. Ala558 is located at the terminal of the flexible β-strand which is thought to form a lid over the regulatory site (Figure 2.26). The increase in side chain length may force the afore-mentioned β-strand into a position where it blocks the tested inhibitors entering the regulatory site leading to a lack of inhibition.

The lack of sensitivity of the I627A variant to all tested inhibitors may be associated with the shortening of the chain length increasing the available space within the regulatory pocket which may lead to a lack in hydrophobic interaction between the tested ligands and the regulatory residues. It is possible the increase in the size of the regulatory pocket may also allow water to enter the site making it unfavourable for binding of the inhibitors.

Substitution of Val551 to leucine showed little change in the inhibition compared to the wild-type enzyme with any of the tested inhibitors suggesting either this residue plays a minimal role in the formation of the regulatory pocket and inhibitor binding or that the slightly shorter leucine residue plays a similar role at this position as both of these residues are non-polar (Figures 2.24 – 2.26).

The final residue examined, Ala567, displayed some interesting inhibitory characteristics. While the inhibition with isoleucine was comparable to the wild-type enzyme, a decrease in sensitivity was displayed towards leucine at lower concentrations. As the leucine concentration increased the maximal inhibition percentage of the enzyme was at a lower level than the wild-type enzyme. The most unexpected result was observed with norvaline where a high level of sensitivity was observed even at 1 mM norvaline showing that the substitution of one amino acid residue can tune the inhibitor selectivity towards another ligand. The effects of this substitution could be due to the side chain of norvaline being flexible. This flexibility may allow it to adopt a stable conformation within the regulatory pocket despite the increase in side chain length due to the substitution of the Ala567 residue leading to possible occlusion of one side of the regulatory pocket (Figure 2.27).
Figure 2.27: Regulatory site of MtulIPMS showing the Ala567 residue exchanged to valine (green) with norvaline (blue) overlapped with leucine (cyan). Val551 is shown in orange, Ala558 in purple and Ile627 in yellow.

The wild-type MtulIPMS enzyme and the V551L and A567V variants displayed slow-onset inhibition with each of the tested inhibitors suggesting these residues do not play an important role in the slow-onset mechanism for the MtulIPMS enzyme.

DSF revealed that the denaturation temperature of the wild-type and variants increased in the presence of the tested ligands leucine, isoleucine and norvaline at concentrations of 5 mM except for the A558I variant in which similar results for the denaturation temperature were found both in the presence and absence of ligand. The overall trend of increasing denaturation temperature in the presence of ligands suggests that upon binding these inhibitors the enzyme complex created is more stable than the unbound enzyme alone.

Some comparisons can be drawn between the DSF data obtained and the inhibition data. The MtulIPMS A558I, which experienced little change in stability in the presence and absence of inhibitor, showed little inhibition with any of the tested inhibitors as would be expected. The
A558V variant however, shows little inhibition by the tested ligands but still displays a substantial increase in denaturation temperature with both leucine and isoleucine (though not as much as for the wild-type enzyme) and shows no stabilisation by isoleucine despite being inhibited by this ligand. The V551L variant displays similar responses to the wild-type enzyme for both the inhibition and DSF data once again illustrating the unlikelihood for this residue playing a major role in the inhibition of the enzyme. The A567V variant shows an overall increase in absolute denaturation temperature both in the presence and absence of ligands; however, the relative denaturation temperatures in the presence of the inhibitors are less than for the wild-type enzyme. The inhibition data suggests significantly increased norvaline affinity, so it was expected that an increase in stabilisation by norvaline relative to the wild-type enzyme might be observed. These discrepancies were perhaps due to the use of 5 mM ligand concentrations in the DSF assays whereas 1 mM may have been more discriminatory and show more about the nature of the stabilisation of each ligand. The I627A variant displayed an increase in denaturation temperature in the absence of ligands. The same absolute denaturation temperature as the wild-type enzyme was obtained with leucine and isoleucine meaning the relative increase in temperature due to the addition of ligands was not as great. No stabilisation effect was observed in the presence of norvaline.
Chapter 3

Effects of amino acid substitutions made to the \( \alpha \)-IPMS from \textit{Neisseria meningitidis} on the regulatory signal transfer throughout the enzyme

3.1 Overview

The goal of this research was to see if single amino acid changes could be made to the \( \alpha \)-IPMS from \textit{N. meningitidis} (NmeIPMS) at positions located away from the regulatory and active sites of the enzyme which would lead to an alteration in the inhibition by leucine due to the disruption in the signal transfer from the regulatory domain to the catalytic domain.

These NmeIPMS variants were characterised via enzyme kinetic studies, protein mass spectrometry, differential scanning fluorimetry and circular dichroism. Enzyme kinetic studies included obtaining Michaelis-Menten constants and inhibition data with leucine for each variant generated. Protein mass spectrometry was implemented to ensure the purified protein was of the correct mass. Differential scanning fluorimetry was used to determine the melting temperatures of the wild-type and each variant in the presence of different inhibitors and in the absence of inhibitor. Finally circular dichroism was used to ensure the chosen variants did not significantly alter the secondary structure of the enzyme via comparison to the wild-type protein.
3.2 Design of the *Nme*IPMS variants

The *Nme*IPMS variants were made based upon molecular dynamic simulations performed by Dr. Wanting Jiao and Chloe Thompson on the BlueFern supercomputer at the University of Canterbury.

3.3 *Nme*IPMS variant justification

As there is no current full length crystal structure solved for *Nme*IPMS, analysis of the enzyme was done using a homology model generated by Dr. Wanting Jiao based on the crystal structure of *Mtu*IPMS (PDB code 3FIG).

3.3.1 *Nme*IPMS homology model

![Homology model of *Nme*IPMS](image)

**Figure 3.1**: Homology model of *Nme*IPMS showing one monomer in grey. The other monomer is separated into the catalytic domain (purple), subdomain I (red), subdomain II (yellow) and the regulatory domain (blue). Leucine is shown in cyan.

65
The *NmeIPMS* homology model (Figure 3.1) was created based upon sequence alignment with *MtuIPMS* and the structural alignment with the truncated *NmeIPMS* structure.\textsuperscript{24} As for *MtuIPMS*, the model is homodimeric and comprised of a catalytic and regulatory domain separated by subdomains I and II. Structurally the *NmeIPMS* enzyme is very similar to *MtuIPMS* despite being 127 amino acid residues shorter. This extra length of *MtuIPMS* was in part, the reason why the *NmeIPMS* homology model was built. A significant proportion of the structure of *MtuIPMS* is unresolved in the crystal structures, indicating that these regions show significant conformational flexibility. Sequence alignments of α-IPMS enzymes show that these regions are not present in α-IPMS proteins from other species, therefore the *NmeIPMS* model was built as a more representative structure of the majority of α-IPMS enzymes.

Molecular dynamic studies were then carried out comparing the dynamic trajectories and movement of residues between leucine-bound and ligand-free forms of the enzyme to predict residues that may play an important role in allosteric function. Residues located in the catalytic domain of *NmeIPMS* showed little movement between bound and unbound forms, the subdomains I and II however, which are thought to play a significant role in the signal transfer through the protein, display the greatest flexibility.

Interestingly, the leucine-bound and ligand-free forms of the enzyme display a clear difference in orientation and motion in subdomain I, II and the regulatory domain. In the ligand free system, subdomain I is disordered and oriented into the opening of the catalytic barrel. When leucine is bound, subdomain I displays significantly less movement due to hydrogen bonding between key residues (Figure 3.3-3.5) and other important interactions within the protein scaffold. The increase in rigidity of the structure holds subdomain I over the top of the catalytic barrel for the majority of the trajectory. Subdomain II adopts significantly different orientations between the leucine-bound and ligand-free forms. When no leucine is bound, the α-helices adopt a conformation in which subunit II is parallel to the β-sheets of the regulatory domain. When leucine binds, the orientation of the α-helices changes propping up the regulatory domain (Figure 3.2). The regulatory domain undergoes a large shift from the leucine-bound to
ligand-free form, which likely disrupts bonds involved in the interface connection between the regulatory domain and subdomain II preventing regulation of the enzyme.

Preliminary results from molecular dynamics simulations (112.6 ns for the ligand free system and 119 ns for the leucine-bound system) suggest that a number of the residues which are involved in structurally important hydrogen bonds and salt bridges display a conformational shift between leucine-bound and ligand-free forms of the enzyme suggesting they may play a vital role in the inhibitory signal transfer from the leucine binding site to the catalytic site. Detailed below are some of these important residues which have been altered to investigate the inhibitory signal transfer mechanism.

Figure 3.2: Homology model of the NmelPMS average ligand-free system (left) and average leucine-bound system (right) displaying significant differences in positions of the subunits. Catalytic domain (purple), subdomain I (red), subdomain II (yellow) and the regulatory domain (blue). Leucine is shown in cyan.
The importance of asymmetry between the two monomers in the structure (as modelled from the MtuIPMS structure)\textsuperscript{33} was displayed when looking at which residues on each monomer that were forming important hydrogen bonds, as without this asymmetry these residues would be located in a position which they would not be able to interact. It is possible that for MtuIPMS and other variants that this asymmetry is an important part of the regulatory system.
3.3.2 *NmelPMS E466A*

In the leucine-bound form of *NmelPMS*, Glu466 located on the β-sheet of the regulatory domain forms a hydrogen bond with Ser352 located on the flexible loop of subdomain II below the regulatory site (Figure 3.3). This interaction keeps subdomain II stacked in an upright position (Figure 3.2) below the regulatory domain. The average trajectory for the ligand-free form shows a hydrogen bond occupancy of 1.45% which increases to 63.4% for the average trajectory in the leucine-bound form. This implies the interaction may play an important role in communication of leucine binding to subdomain II. Upon binding leucine, the dynamics simulations indicate that subdomain II also loses its upright orientation. In the ligand-free model a slight rotation of subdomain II is permitted after the loss of this anchoring (as the S353-E466 hydrogen bond is disrupted) and subdomain II becomes more parallel to the β-sheets of the regulatory domain (Figure 3.2).

![Figure 3.3](image)

*Figure 3.3:* Demonstration of the change in distance between residues E466 and S352 in the average ligand-free (left) and leucine-bound (right) form of *NmelPMS*. In the ligand-free model a hydrogen bond distance of 9.6 Å was observed compared to a distance of 4.1 Å in the leucine-bound model.
3.3.3 *Nmel*PMS E353A

Glu353 interacts strongly with both Ser468 and Arg470 via hydrogen bonding in the leucine-bound form (Figure 3.4). Differences in hydrogen bond occupancy for the Glu352-Ser468 interaction were determined as 21.9 % in the leucine-bound form of *Nmel*PMS and 2.6 % in the ligand-free form calculated over the entire simulation. The Glu353-Arg470 occupancy decreases from 40.2 % in the leucine-bound form to 0.1 % in the ligand-free form. Both Ser468 and Arg470 are located on the lower β-sheets of the regulatory domain which are able to interact with Glu353 which is located on the loop of subdomain I.

**Figure 3.4:** Demonstration of interactions between E353, S468 and R470 in the average ligand-free (left) and leucine-bound (right) forms of *Nmel*PMS. Hydrogen bond distances in ligand-free form: E353-S468 = 7.4 Å, E353-R470 = 6.6 Å. Hydrogen bond distances in leucine-bound form: E353-S468 = 3.8 Å, E353-R470 = 3.0 Å.
3.3.4 NmelPMS R310A

Arg310 is located in subdomain I on a flexible linker region thought to form a ‘capped lid’ over the catalytic domain that prevents substrate entering the active site (Figure 3.5). This interaction between subdomains is not observed in the leucine unbound structure. This is supported by hydrogen bond occupancy between Arg310 and Glu319 decreasing from 43.8 % in the leucine-bound form to 5.0 % in the ligand-free form. Arg310-Glu314 hydrogen bond occupancy also drops from 51.2 % in the leucine-bound form to 12.2 % in the ligand-free form. Hydrogen bond occupancies were calculated over the entire simulation of the leucine-bound and ligand-free form.

Figure 3.5: Demonstration of interactions between R310, E314 and E319 in the ligand-free form (left) and leucine-bound form (right) of NmelPMS. Hydrogen bond distances in ligand-free form: R310-E314 = 11.3 Å, R310-E319 = 14.0 Å. Hydrogen bond distances in leucine-bound form: R310-E314 = 2.6 Å, R310-E319 = 2.9 Å.
3.4 *Nmel*IPMS variant generation using PCR

*Nmel*IPMS variants were created via mutation of the wild-type *Nmel*IPMS gene using PCR. The *Nmel*IPMS gene was found inserted in a pET-151 plasmid. The wild-type gene contained coding for a TEV protease cleavage site attached to the 5’ end of the gene. The plasmid also included coding for an N-terminal polyhistidine tag to make the protein easier to purify after expression, a T7 promoter region binding a lac operon to enable over-expression of the protein of interest after addition of IPTG and an ampicillin resistance gene to allow for growth selection of cells which contain the gene of interest. The TEV protease cleavage site was included so that the His-tag could be removed after IMAC had been performed. The plasmid was located in *E. coli* One Shot TOP10 cells and was obtained using plasmid isolation. The purified plasmid was then run on an agarose gel to ensure the obtained plasmid was the expected size of 5000 bp.

![Figure 3.6: Agarose gel showing isolated plasmid containing the wild-type *Nmel*IPMS gene. Marker sizes are shown in base pairs. Marker weights between 3000 and 12000 bp increase 100 bp per band.](image)
After the plasmid bearing the wild-type gene was isolated, PCR was used to introduce the mutations of choice into the wild-type NmelIPMS gene. The PCR products were then subjected to Dpn1 in order to digest the parental methylated DNA leaving the copies of the gene which contain the mutation intact. The PCR products were then analysed on an agarose gel, however no bands were observed. Based upon the site-directed mutagenesis kit guide, the PCR products were transformed into *E. coli* One Shot TOP10 cells which were grown on LB/agar plates containing ampicillin as a selection marker. The plasmids were then obtained from the cells and sequenced. The sequences were then compared to the wild-type sequence to ensure the correct mutations had been made in the gene. Plasmids which contained the correct gene mutation were then transformed into *E. coli* BL21(DE3)Star cells for protein expression.

## 3.5 Expression of protein

The expression of the NmelIPMS wild-type enzymes and variants was carried out as described in the materials and methods section (Chapter 5). After expression cell pellets were stored at -80°C until required. Proteolysis can occur in the cell lysate during purification steps. To minimise this lysis to immobilised metal affinity chromatography (IMAC) steps were performed in close succession and protease inhibitor was added to the lysate.

Cell lysis was performed using sonication. A large proportion of the over-expressed protein was found in the supernatant at the expected molecular mass of 60 kDa for the His-tagged protein.
3.6 Purification

The wild-type NmelPMS and variants were purified in the same manner as MtulPMS (described in Chapter 2) except instead of using a Ni$^{2+}$ containing IMAC column, a Talon® column containing Co$^{2+}$ was used. This procedure involved IMAC chromatography of the crude protein after which TEV protease was added to remove the His-tag. The protein sample was then run back through the Talon® column to separate the clean untagged protein from the tag and uncleaved protein. SEC was used as a final cleaning step to ensure protein was of high-purity.

This purification procedure resulted in protein of high purity for all characterisation assays. The addition of DTT was performed prior to addition of TEV protease in order to reduce disulfide bonds that may form between the proteins allowing for better access to the TEV cleavage site. The dilution with SEC buffer is performed to reduce imidazole concentration in preparation for TEV protease activity.

This purification procedure usually led to 5-10 mg of protein for a 1 L cell culture in lysogeny broth (LB).

3.6.1 Immobilised metal affinity chromatography

The first step used in the purification procedure of NmelPMS was IMAC. A Talon® column charged with Co$^{2+}$ ions was used to separate NmelPMS from background E. coli proteins via the N-terminal His-tag. Protein which bound to the column was eluted with 150 mM imidazole.

3.6.2 Desalting

Protein is eluted from the IMAC column in a buffer containing a high concentration of imidazole. For this reason prior to the addition of TEV protease the pooled fractions were diluted with SEC buffer to ensure the imidazole did not interfere with protein stability or TEV protease efficiency.
3.6.3 TEV protease cleavage

Once the desalting step had been completed the protein was digested with TEV protease overnight at 4°C to remove the N-terminal His-tag. Removal of the His-tag was carried out to minimize the chances of the tag interfering with the characterisation of *NmelPMS*.

The His-tag added 33 amino acid residues to the N-terminus of *NmelPMS* including the 6 histidine residues used to bind to the IMAC column and the TEV protease recognition sequence. After digestion, 6 residues (GIDPFT) are left attached to the N-terminus of *NmelPMS*.

![Figure 3.7: Residues located at the N-terminal of NmelPMS. Red shows the His-tag, green shows the TEV protease recognition sequence and cyan shows the extra residues introduced to the N-terminal of the NmelPMS after TEV protease activity.](image)

After the incubation with TEV was complete the solution was run back through the Talon® Co²⁺ column. The untagged protein did not bind to the column and was eluted in the flow-through, while the cleaved tag and background *E. coli* protein that bound to the column in the last step bound to the column.

3.6.4 Size-exclusion chromatography

The last step in the purification protocol is SEC, which elutes *MtuIPMS* in one peak and aggregated proteins in an earlier peak. The mass of the purified protein is of the expected molecular mass of 56 kDa. The gel of the wild-type and variants is shown in figure 3.8.
Figure 3.8: SDS-PAGE showing purified *Nmel*IPMS wild-type and variants. Lane 1, wild-type; lane 2, R310A variant; lane 3, E353A variant; lane 4, E466A variant and lane 5 shows the marker ladder. Marker weights are shown in kDa.

3.7 Mass spectrometry

All *Nmel*IPMS enzymes were measured using MS to ensure all enzymes were of the expected mass calculated from amino acid sequence using ProtParam (Table 3.1).  

Table 3.1: Expected and calculated masses of each *Nmel*IPMS variant. Calculated masses obtained using mass spectrometry.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Expected mass (Da)</th>
<th>Calculated mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nmel</em>PMS WT</td>
<td>56027.4</td>
<td>56026.7</td>
</tr>
<tr>
<td><em>Nmel</em>PMS R310A</td>
<td>55942.3</td>
<td>55940.8</td>
</tr>
<tr>
<td><em>Nmel</em>PMS E535A</td>
<td>55969.4</td>
<td>55967.7</td>
</tr>
<tr>
<td><em>Nmel</em>PMS E466A</td>
<td>55969.4</td>
<td>55969.3</td>
</tr>
</tbody>
</table>
3.8 *NmIPMS* wild-type

3.8.1 Physical characterisation

This section details the examination of the wild-type enzyme. This work took place for later comparison with the *NmIPMS* variants.

3.8.2 Secondary structure analysis

A CD spectrum was obtained for *NmIPMS* wild-type enzyme to ensure the protein was folded properly and to use as a comparison for the *NmIPMS* variants to ensure no major structural change had taken place.

![CD spectrum](image)

**Figure 3.9**: CD spectra of the wild-type *NmIPMS* in double-distilled water at pH 7.0. The protein concentration was 0.2 mg/mL.
The shape of the CD spectrum of *Nme*IPMS wild-type suggests proper folding of the protein. This presence of secondary structure suggests it is suitable for further characterisation.

### 3.8.3 Thermal stability

The wild-type *Nme*IPMS was subjected to DSF to investigate its thermal denaturation temperatures in the absence of ligand and in the presence of 1 and 5 mM leucine.

![DSF curve example](image)

**Figure 3.10:** Example of a DSF curve for the wild-type *Nme*IPMS enzyme with 5 mM leucine present where in blue is the relative fluorescence unit (RFU) and shown in red is the negative derivative of the RFU. The melting temperature is recorded as the steepest part of the RFU trace and the minimum on the negative derivative. The experiment was carried out in 25 mM BTP buffer supplemented with 20 mM MgCl\(_2\) and 20 mM KCl at pH 7.5.
The DSF results showed the wild-type NmelPMS (Figure 3.11) had a denaturation temperature of \(43.6 \pm 0.1 \, ^\circ C\) with no leucine present. When leucine was added at concentration of 1 mM the denaturation temperature increased to \(45.2 \pm 0.1 \, ^\circ C\) which is a small but significant difference in the thermal stability of the enzyme. Upon addition of 5 mM leucine the denaturation temperature increased further to \(46.5 \pm 0.1 \, ^\circ C\). These results suggest that leucine binding to the wild-type enzyme results in a more thermally stable protein.

![Figure 3.11](image.png)

**Figure 3.11**: Effects on \(T_m\) of NmelPMS wild-type in the presence 1 and 5 mM leucine in 25 mM BTP buffer (pH 7.5).

### 3.8.4 Kinetic characterisation

The activity of NmelPMS was studied first by finding the apparent Michaelis-Menten constants for the two substrates (\(\alpha\)-KIV and AcCoA). Once these results had been obtained inhibition studies were performed to examine the response of the wild-type NmelPMS and variants to varying leucine concentrations.

All assays were carried out using a DTP coupled assay which was monitored at 324 nm.
3.8.5 Michaelis-Menten kinetics

Apparent $K_m$ and turnover values were found for the wild-type NmeIPMS at 25 °C and pH 7.5 (Table 3.3). While obtaining the AcCoA $K_m$ the $\alpha$-KIV concentration was held at 500 µM. While obtaining the $\alpha$-KIV $K_m$ the AcCoA concentration was held at 500 µM. A $K_m$ value of 37.3 ± 3.4 µM was obtained for AcCoA and a $K_m$ of 31.9 ± 1.5 µM for $\alpha$-KIV. A $k_{cat}$ value of 8.9 ± 0.1 s$^{-1}$ was obtained.

Michaelis-Menten curves obtained are shown in figure 3.12. Michaelis-Menten data can be viewed in table 3.3.

![Figure 3.12](image)

**Figure 3.12:** Michaelis-Menten plots obtained for wild-type NmeIPMS showing the apparent AcCoA (left) and $\alpha$-KIV (right) $K_m$. A $K_m$ of 37.3 ± 3.4 µM was obtained for AcCoA and a $K_m$ of 31.9 ± 1.5 µM was obtained for $\alpha$-KIV. A $k_{cat}$ value of $8.9 \pm 0.1$ s$^{-1}$ was obtained. Each assay contained: 20 mM MgCl$_2$, 20 mM KCl and 500 µM DTP in 50 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH 7.5.
3.8.6 Allosteric inhibition by leucine

The inhibition of wild-type *NmelPMS* by leucine (Figure 3.13) was analysed by varying the concentration of leucine present in the cuvette while holding the substrate and metal ion concentrations at the same conditions.

![Inhibition of wild-type NmelPMS with varying leucine concentrations.](image)

**Figure 3.13:** Inhibition of wild-type *NmelPMS* with varying leucine concentrations. Inhibition data was determined with 500 µM AcCoA, 550 µM α-KIV, 20 mM MgCl₂, 20 mM KCl and 500 µM DTP in 50 mM HEPES buffer at pH 7.5. Amount of enzyme used in each assay was 1.6 µg.

The inhibition data of wild-type *NmelPMS* at different leucine concentrations showed the enzyme was maximally inhibited at a level of 22 ± 4 % activity. An approximate half-maximal effective concentration (EC50) value of 5 µM leucine was obtained showing the natural inhibitor of *NmelPMS*, leucine, is a potent inhibitor as expected.
3.9 Analysis of *Nme*IPMS variants

3.9.1 Structure analysis

CD spectra of *Nme*IPMS variants were overlaid with the wild-type spectrum to ensure the variants were folded properly and no major perturbations had occurred due to the amino acid substitutions.

![Figure 3.14](image-url): CD spectrums of *Nme*IPMS wild-type and variants overlaid. The wild-type is shown in black, R310A in red, E353A in blue and E466A in purple.

The high degree of similarity between the *Nme*IPMS enzymes spectra suggests no large differences in the structure of variants had occurred due to the changes in sequence.
3.9.2 Thermal stability with leucine

The denaturation temperature of each of the variants was determined with no leucine, 1 mM leucine and 5 mM leucine for comparison with the wild-type NmelPMS enzyme (Figure 3.15).

![Figure 3.15: Denaturation temperature of wild-type NmelPMS and variants obtained using DSF. Blue shows denaturation temperatures with no leucine, red with 1 mM leucine and green with 5 mM leucine. Experiments were carried out in the presence of 20 mM MgCl₂ and KCl in 25 mM BTP buffer at pH 7.5.](image)

Results from DSF studies show an overall trend in all of the enzymes where in the presence of leucine an increase in denaturation temperature occurs (Table 3.2), suggesting the binding of leucine leads to a more thermally stable enzyme-inhibitor complex. The R310A variant displays a slightly higher denaturation temperature than the wild-type enzyme with no leucine present suggesting that this amino acid mutation has led to an increase in thermal stability of the protein. The increase in the thermal stability of this variant with 1 mM leucine is comparable to the rise observed in the wild-type enzyme, however, no further increase in denaturation temperature was observed from 1 to 5 mM. The E353A variant showed a similar response to leucine as the wild-type enzyme except all denaturation temperatures were approximately 2 °C.
lower than for the wild-type enzyme. The E466A variant showed some interesting results. The denaturation temperature with no leucine present increased dramatically from 43.6 to 50.1 °C. This suggests that this amino acid substitution provides thermal stability to the enzyme. Substantial increases in denaturation temperature were observed in the presence of both 1 and 5 mM leucine further demonstrating the change in thermal stability this substitution has made, and with 5 mM leucine a denaturation temperature of 65.1 °C is observed.

**Table 3.2:** Denaturation temperature for *NmelPMS* wild-type and variants without ligand and in the presence of 1 and 5 mM leucine. Results were obtained with 20 mM MgCl$_2$ and 20 mM KCl in 25 mM BTP buffer at pH 7.5.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>No ligand</th>
<th>1 mM leucine</th>
<th>5 mM leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>NmelPMS</em> WT</td>
<td>43.6 ± 0.1</td>
<td>45.2 ± 0.1</td>
<td>46.5 ± 0.1</td>
</tr>
<tr>
<td><em>NmelPMS</em> R310A</td>
<td>45.3 ± 0.1</td>
<td>46.0 ± 0.1</td>
<td>45.9 ± 0.1</td>
</tr>
<tr>
<td><em>NmelPMS</em> E353A</td>
<td>41.6 ± 0.1</td>
<td>43.2 ± 0.1</td>
<td>43.9 ± 0.1</td>
</tr>
<tr>
<td><em>NmelPMS</em> E466A</td>
<td>50.1 ± 0.1</td>
<td>61.9 ± 0.5</td>
<td>65.1 ± 0.5</td>
</tr>
</tbody>
</table>

3.9.3 Kinetic properties

Michaelis-Menten constants were obtained for each variant to observe if any significant changes in the catalytic activity of the variants had taken place due to the amino acid substitutions (Table 3.3). Michaelis-Menten values were found using different co-substrate concentrations for each variant due to the differences in their kinetic behaviour.

**Table 3.3:** Michaelis-Menten constants for wild-type *NmelPMS* and variants.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>α-KIV $K_m$ (µM)</th>
<th>AcCoA $K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>NmelPMS</em> WT</td>
<td>32 ± 2</td>
<td>37 ± 3</td>
<td>13 ± 1</td>
</tr>
<tr>
<td><em>NmelPMS</em> R310A</td>
<td>ND*</td>
<td>750 ± 260</td>
<td>5.1 ± 0.7</td>
</tr>
<tr>
<td><em>NmelPMS</em> E353A</td>
<td>29 ± 2</td>
<td>19 ± 1</td>
<td>11.6 ±0.1</td>
</tr>
<tr>
<td><em>NmelPMS</em> E466A</td>
<td>29 ± 3</td>
<td>79 ± 3</td>
<td>9.3 ± 0.1</td>
</tr>
</tbody>
</table>

* ND means not determined.
**Details of experimental conditions are provided in chapter 5.
Table 3.4: $k_{cat}/K_m$ values for wild-type NmeIPMS and variants where ND means not determined.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>α-KIV $k_{cat}/K_m$ (µM$^{-1}$s$^{-1}$)</th>
<th>AcCoA $k_{cat}/K_m$ (µM$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NmeIPMS WT</td>
<td>0.41 ± 0.06</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>NmeIPMS R310A</td>
<td>ND*</td>
<td>0.007 ± 0.003</td>
</tr>
<tr>
<td>NmeIPMS E353A</td>
<td>0.40 ± 0.03</td>
<td>0.61 ± 0.04</td>
</tr>
<tr>
<td>NmeIPMS E466A</td>
<td>0.32 ± 0.04</td>
<td>0.12 ± 0.01</td>
</tr>
</tbody>
</table>

* ND means not determined.

The complete Michaelis-Menten data set for the NmeIPMS variant R310A was not obtained due to the need for high concentrations of AcCoA to determine the apparent α-KIV $K_m$. Initial results did reveal a much higher AcCoA $K_m$ value than the wild-type enzyme of around 750 µM and a low $k_{cat}/K_m$ value of 0.007 µM$^{-1}$s$^{-1}$.

The other NmeIPMS variants display similar $K_m$ values for α-KIV suggesting these amino acid substitutions do not play an important role in the binding of α-KIV. Similarly the AcCoA $K_m$ values obtained for the variants are similar to the wild-type enzyme with the E353A variant displaying a slightly lower $K_m$ value of 19 ± 1 µM and the R310A variant showing a higher $K_m$ than the wild-type with a value of 79 ± 3 µM. Each of the variants displays a small decrease in $k_{cat}$ showing these amino acid substitutions have had a detrimental effect on the rate of substrate turnover.

The $k_{cat}/K_m$ values for α-KIV show that little effect on the enzymes’ catalytic performances results from these amino acid substitutions. More significant differences are observed when looking at the $k_{cat}/K_m$ values for AcCoA. The NmeIPMS E353A variant displays a significant increase in catalytic efficiency of 0.61 ± 0.04 µM$^{-1}$s$^{-1}$ compared with the wild-type value of 0.35 ± 0.06 µM$^{-1}$s$^{-1}$. Alternatively a decrease in the catalytic efficiency is observed with the NmeIPMS E466A variant where a decrease in the $k_{cat}/K_m$ value from 0.35 ± 0.06 µM$^{-1}$s$^{-1}$ to 0.12 ± 0.01 µM$^{-1}$s$^{-1}$ was observed.
3.10 Inhibition by leucine of \textit{NmelPMS} variants

3.10.1 \textit{NmelPMS} R310A

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure316.png}
\caption{Inhibition of \textit{NmelPMS} R310A with increasing leucine concentrations. Inhibition data found with 500 \(\mu\text{M}\) AcCoA, 550 \(\mu\text{M}\) \(\alpha\)-KIV, 20 mM MgCl\(_2\), 20 mM KCl and 500 \(\mu\text{M}\) DTP in 50 mM HEPES buffer at pH 7.5. Amount of enzyme used in each assay was 8 \(\mu\text{g}\).}
\end{figure}

Despite this \textit{NmelPMS} variant displaying a significant decrease in catalytic activity compared to the wild-type enzyme, the substrates were held at the same concentrations as the other \textit{NmelPMS} enzymes as a limited amount of AcCoA was available (therefore AcCoA was not saturating). An approximate EC50 value of 2 \(\mu\text{M}\) leucine was obtained, indicating that this mutant is slightly more sensitive to leucine than the wild-type enzyme. The enzyme reaches a maximum inhibition level of 12 \(\pm\) 4 \% remaining activity compared to the 22 \(\pm\) 4 \% remaining activity of the maximally inhibited wild-type enzyme.
3.10.2 *N*meIPMS E353A

![Graph](image)

**Figure 3.17**: Inhibition of *N*meIPMS E353A with increasing leucine concentrations. Inhibition data found with 500 µM AcCoA, 550 µM α-KIV, 20 mM MgCl₂, 20 mM KCl and 500 µM DTP in 50 mM HEPES buffer at pH 7.5. Amount of enzyme used in each assay was 1.6 µg.

An approximate EC50 value of 8 µM leucine is obtained. This is slightly higher than the value for the wild-type enzyme suggesting that this variant is slightly less sensitive to leucine compared to the wild-type enzyme. The enzyme reaches a maximum inhibition level of 28 ± 6 % remaining activity compared to the 22 ± 4 % remaining activity of the maximally inhibited wild-type.
3.10.3 *Nm*IPMS E466A

![Graph showing inhibition of NmIPMS E466A with increasing leucine concentrations.](image)

**Figure 3.18**: Inhibition of *Nm*IPMS E466A with increasing leucine concentrations. Inhibition data found with 500 µM AcCoA, 550 µM α-KIV, 20 mM MgCl₂, 20 mM KCl and 500 µM DTP in 50 mM HEPES buffer at pH 7.5. Amount of enzyme used in each assay was 1.6 µg.

An approximate EC50 value of 0.5 µM leucine is obtained. This value is much lower than that of the wild-type enzyme and shows a major change in sensitivity towards leucine associated with this substitution. The enzyme reaches a maximum inhibition level of 13 ± 6 % remaining activity compared to the 22 ± 4 % remaining activity of the maximally inhibited wild-type enzyme.
3.11 Summary of findings

The *NmelPMS* variants were successfully created, expressed and purified using IMAC and SEC. The variants created were of the expected size as shown by MS. The CD spectra showed that each of the variants were folded in a similar conformation as the wild-type enzyme, suggesting no major structural changes had occurred due to the amino acid changes.

Michaelis-Menten kinetics showed a difference in the kinetic activities of the variants where the apparent $K_m$ of AcCoA was slightly altered in the E353A and E466A variants and significantly increased in the R310A variant. Measured apparent $K_m$ values for $\alpha$-KIV were similar to that of the wild-type enzyme. Each variant also displayed a small decrease in $k_{cat}$ displaying the importance that individual amino acids play in the function of the protein as a whole. The $k_{cat}/K_m$ values were once again similar for $\alpha$-KIV but varied significantly for AcCoA. This data suggests that AcCoA binding and catalysis is highly susceptible to alteration by changes in the overall conformation of the enzyme due to changes in hydrogen bond formation. AcCoA is a large substrate and is located peripherally to the catalytic barrel (Figure 3.19). The size of AcCoA and its binding location may make it more susceptible to changes in the relative position of the catalytic barrel and subdomain I, whereas, the relatively small $\alpha$-KIV which displays similar $\alpha$-KIV binding across the variants, is largely unaffected by relative subdomain movements.

Inhibition data found with the natural inhibitor leucine showed similar sensitivity towards leucine for the R310A and E353A variants although small differences were observed. This data suggests these residues alone play a minor role in the overall transfer of regulatory signal. Interestingly these variants displayed changes to the maximal level of inhibition observed when compared to the wild-type enzyme suggesting the changes in conformation of the enzymes due to these salt-bridges being disturbed can alter the extent of which the enzyme can be inhibited. The lack of influence of a single mutation could possibly be due to multiple regulatory signal transfer pathways operating within the enzyme so that the alteration of one of these pathways
alone does not have a substantial effect. These favoured signal transfer pathways could lead to the different levels of maximal inhibition observed.

The E466A variant displayed a significant increase in sensitivity towards leucine which was unexpected as this substitution was designed to disrupt hydrogen bond formation with Ser352 thus mimicking the uninhibited state therefore it was expected to see a drop in sensitivity of the enzyme towards leucine. This unexpected change in leucine sensitivity could be due to the enzyme adopting a different conformation, leading to increased leucine inhibition via utilisation of other regulatory signal transfer pathways in the enzyme. This possibility is supported by the significant increase in the denaturation temperature observed with this variant in the presence of increasing levels of leucine (Table 3.2).

Figure 3.19: NmeIPMS homology model with AcCoA bound (red) based upon the crystal structure of LiCMS\textsuperscript{13} (PDB code 3BLI) showing the large area AcCoA interacts with when binding to the enzyme.
Denaturation temperatures found using DSF showed a general trend of increasing the melting temperature as the concentration of leucine increased for the NmelPMS wild-type and variants. The R310A variant displayed a lesser increase in protein stability upon addition of leucine than the wild-type enzyme and variants suggesting this amino acid substitution may have had a large effect on the ability of the enzyme to adopt a stable conformation upon leucine binding, as would be expected with the disruption of the hydrogen bond usually formed at this position. The E353A variant displayed similar denaturation temperatures to the wild-type suggesting the hydrogen bonds formed upon leucine binding do not play a significant role in the stability of the enzyme. The E466A variant showed a large increase in denaturation temperature of almost 20 °C was found with 5 mM leucine. Once again this variant displayed unexpected results as it was thought that the disruption of the hydrogen bond to Ser352 would be expected to result in a decrease in denaturation temperature due to the enzyme becoming more flexible. This raises the possibility that upon disruption of the Glu466-Ser352 hydrogen bond, the enzyme is free to explore different hydrogen bonding contacts which are more stabilising.
Chapter 4

Summary of thesis and future directions

The aim of this research is to increase the understanding of the allosteric inhibition of α-IPMS and probe the effect of single amino acid substitutions on inhibitor selectivity. To study this, α-IPMS enzymes from *M. tuberculosis*\(^ {23,24}\) and *N. meningitidis*\(^ {24}\) were altered using site-directed mutagenesis and characterised using a variety of methods and conditions to probe the effects on inhibitor selectivity and regulatory signal transfer.

α-IPMS enzymes from *M. tuberculosis* and *N. meningitidis* were expressed, purified and characterised using MS, DSF and kinetic assays. α-IPMS enzymes from these organisms were chosen due to their pathogenic nature, introducing the possibility that these enzymes may be viable drug targets. The wild-type enzymes were found to have similar catalytic activity and regulation to α-IPMS enzymes previously studied,\(^ {23,30,40}\) with \(K_m\) values for the substrates α-KIV and AcCoA in the micromolar range, \(k_{cat}\) values between 3 and 15 s\(^{-1}\) and inhibition constants also in the low micromolar range.\(^ {23,30,39,40}\)
4.1 Changes to the regulatory pocket of *Mtul*PMS can have a large effect on the inhibitor selectivity and sensitivity of the enzyme

*Mtul*PMS enzymes studied are homodimeric comprising of a catalytic (β/α)_{8}-barrel domain, two subdomains and a regulatory domain comprised of a distinct (βββα)_{2} fold (Figure 1.7). The two subdomains separate the catalytic and regulatory domains and are thought to play a vital role in the transfer of the regulatory signal from the regulatory domain to the catalytic domain upon binding of the natural inhibitor, leucine. CMS is an enzyme which shares distinct similarities to α-IPMS, such as the overall structure, the reaction catalysed and the feedback regulation by a similar molecule, isoleucine (Figure 1.14-1.15).^{13,35}

Recently, studies on the regulatory site of LiCMS by Zhang *et al*^{13} where variants (based on docking studies) were created via site-directed mutagenesis to attempt to change the inhibitor selectivity of LiCMS from isoleucine to leucine. Results from this study showed that the V468A variant of LiCMS displayed significantly increased leucine sensitivity. However this variant also displayed increased inhibition to the natural inhibitor isoleucine. Other mutation in this study included Y430L, L451V, Y454A and I458A which all displayed a decrease in the catalytic efficiency of the enzyme and a decrease in sensitivity to isoleucine. The Y454A variant also displayed an increase in sensitivity towards leucine, but to a lesser extent than the V468A variant. The comparative locations of these substituted residues with *Mtul*PMS residues can be viewed in figure 2.2. In light of these studies, it was proposed that a change in inhibitor selectivity could be achieved by alteration to hydrophobic residues in the regulatory pocket of *Mtul*PMS via site-directed mutagenesis, to resemble the regulatory pocket in CMS, changing the inhibitor selectivity from leucine to isoleucine.

Variants of *Mtul*PMS in which key amino acids which line the hydrophobic pocket responsible for the interaction with the hydrophobic side chain of leucine were created to explore the effects on inhibitor sensitivity and enzyme regulation. These variants displayed similar catalytic activities to the wild-type enzymes; however, inhibition by leucine and norvaline was
significantly altered. The variants displayed similar inhibition properties with isoleucine suggesting alteration of the binding for this allosteric effector may require multiple regulatory site residue changes.

Interestingly, a single amino acid change of Ala567 to a valine was able to tune the inhibitor selectivity from leucine to norvaline showing it is indeed possible to tune inhibitor selectivity with a single amino acid substitution.

Many of the variants did display a decrease in sensitivity towards leucine while maintaining their sensitivity towards isoleucine, meaning that the relative inhibitor selectivity of the enzymes had indeed been altered from leucine to isoleucine. However, combinations of changes may well be required to effectively switch inhibitor sensitivity.

4.2 Disruption of hydrogen bonding contacts can have large consequences on enzyme activity, regulation and stability

_NmelIPMS_ was used to explore the mechanism of regulatory signal transfer between the catalytic and regulatory domain. In _α-IPMS_ the catalytic and regulatory ligand binding sites are a large distance apart relative to the overall size of the enzyme, and they are separated by subdomains I and II, which are thought to mediate regulatory signal transfer upon binding of the inhibitor, leucine. Comparisons of the ligand-free and leucine-bound _MtuIPMS_ crystal structures reveal little structural change on ligand binding.\(^{33}\) However, recent preliminary molecular dynamic simulations of _NmelIPMS_ shows considerably more flexibility and structural differences in the enzyme between the ligand-free and leucine-bound model than previously thought and raises the possibility that a conformational shift may occur between the asymmetric monomer units. Relatively short molecular dynamic studies also displayed a difference in hydrogen bond occupancies calculated over ligand-free and leucine-bound trajectories of key residues located in the subdomains (Figure 3.3-3.5). This led to the work
carried out as part of this thesis to substitute these key residues and examine the effects on catalytic ability, regulation and stability of the enzyme.

Single amino acid substituted variants of NmelIPMS were created via site-directed mutagenesis to explore the transfer of the regulatory signal from the regulatory domain to the catalytic domain upon binding of the natural inhibitor leucine. Significant differences in catalytic activities were observed, with $k_{\text{cat}}/K_m$ values for AcCoA of between $0.007 \pm 0.003$ and $0.61 \pm 0.04 \ \mu\text{M}^{-1}\text{s}^{-1}$, while maintaining relatively similar $k_{\text{cat}}/K_m$ values for the substrate $\alpha$-KIV. The E466A variant displays a significant increase in inhibition by leucine compared to the wild-type enzyme (with an EC50 value of 0.5 $\mu$M compared to 5 $\mu$M in the wild-type) while the other variants show similar inhibitory characteristics to the wild-type enzyme with the exception that the maximal level of inhibition is altered in the variants. From this data it is observed that the Glu466 residue appears to play a significant role in the regulation and thermal stability of the enzyme.

Mutation of Glu466 to an alanine resulted in an enzyme with significantly higher denaturation temperatures in the presence and absence of leucine. The R310 residue appears to play a significant role in binding AcCoA as data suggests at least a ten-fold increase in the apparent $K_m$ of AcCoA. When the E353 residue was substituted for alanine a two-fold increase in the apparent $K_m$ for AcCoA of the enzyme was observed suggesting it may also play a role in the binding of AcCoA.
4.3 Future Experiments

4.3.1 What is the effect upon inhibitor selectivity of double and triple amino acid substituted variants of MtulIPMS?

Single amino acid substitutions in MtulIPMS produced significant differences in enzyme selectivity and sensitivity with leucine and norvaline. However, no increase in the sensitivity towards isoleucine was observed. The creation of double and triple amino acid substituted variants of MtulIPMS may lead to more drastic changes in the inhibitor selectivity compared to what was observed in the work carried out in this thesis.

For example, the A567V variant displayed significantly increased sensitivity to norvaline while maintaining leucine sensitivity. The A558V variant displayed a decrease in regulation with all tested inhibitors. It is possible that creating a double amino acid substituted variant with these changes would lead to an enzyme that displays high sensitivity towards norvaline and loses sensitivity toward leucine. Another viable change would be the further increase in side chain length of Val551 and adding a bulky substituent such as glutamic acid to select for isoleucine, which lacks the C4 methyl group of leucine, while shortening the side chain of Ile627 to an alanine to make room for the C3 methyl group of isoleucine (Figure 4.1). Results from these experiments would increase the understanding of how the regulatory binding site residues interact with the inhibitors and lead to potential new studies into the regulation of α-IPMS.
4.3.2 Does removing multiple possible regulatory signal transfer pathways reduce enzyme regulation in *Nme*IPMS?

Single amino acid substitutions to disrupt hydrogen bond interactions deemed as significant by molecular dynamic modelling had a large effect of the binding affinity for AcCoA and enzyme regulation with leucine. It would be interesting to see the effects on the regulation, catalytic activity and stabilisation of *Nme*IPMS variants with multiple hydrogen bond disruptions run over longer simulation trajectories (up to µs in length). This may help elucidate the mechanism of the regulatory signal transfer throughout the enzyme and establish if a monomer conformation interconversion through a symmetrical for of the enzyme occurs.
4.4 Concluding Remarks

The understanding of the inhibitor binding selectivity, sensitivity and signal transfer is vital to the overall understanding of enzymatic function. *Mtu*IPMS and *Nmel*IPMS have provided an insight into these mechanisms. Further studies are required to gain a full understanding of these functions.
Chapter 5

Materials and Methods

5.1 General methods

5.1.1 Water

All buffers and solutions used in this research were made using water treated with a Millipore Milli-Q system prior to use.

5.1.2 Determination of pH

The pH of relevant solutions was determined using either the Denver Instruments UB-10 Ultra-Basic pH meter or Mettler Toledo SevenCompact pH meter. Solutions were made acidic via the addition of HCl and basic by addition of NaOH.

5.1.3 Protein structure viewing and images

Structural representations of proteins were generated using the program “PyMOL™” Molecular Graphics System, version 1.5.0.3. (Schrödinger, LLC).

5.1.4 Sequence alignments

All sequence alignments were performed using the Clustal Omega tool (www.ebi.ac.uk/Tools/msa/clustalo/).
5.2 Site-directed mutagenesis

5.2.1 Primers

Primers were designed using PrimerX (www.bioinformatics.org/primerx) and synthesised by GeneWorks. Primers were stored at 100 µM in TE buffer (10mM Tris-HCl, 0.1mM EDTA). A full list of primers used can be found in table 5.1.

Table 5.1: Primers used to generate variants in *Mtu*IPMS and *Nme*IPMS. Mutated bases are shown in bold.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>PCR primer 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mtu</em>IPMS A558I</td>
<td>Forward: GACTACTACGAGCACATTATGAGCGCCGGCGCA&lt;br&gt;Reverse: GTCGCCGGCGCTCATATAATGCTCTGCTAGTAGTC</td>
</tr>
<tr>
<td><em>Mtu</em>IPMS A558V</td>
<td>Forward: CTACTACGAGCAGTTGAGCGCCGGCGAG&lt;br&gt;Reverse: CGCCGGCGCTCATACGCTGCTGCTAGTAG</td>
</tr>
<tr>
<td><em>Mtu</em>IPMS V551L</td>
<td>Forward: GTTTGACGTGGCCTGCTGGACTACTAC&lt;br&gt;Reverse: GTCAGTAGCTCGAAGGACGCACGTCACAAAC</td>
</tr>
<tr>
<td><em>Mtu</em>IPMS A567V</td>
<td>Forward: GACGACGCTCACGTTGGCCTGATGAGCTG&lt;br&gt;Reverse: CACATACGCGCCACCTGAGCGTCGTC</td>
</tr>
<tr>
<td><em>Mtu</em>IPMS I627A</td>
<td>Forward: CGCACCAGTCAGCGACCACCGCGTCGCTGCTGCGAC&lt;br&gt;Reverse: GGCACCAGCGACCCGCGCTGCTGCTGCGAC</td>
</tr>
<tr>
<td><em>Nme</em>IPMS R310A</td>
<td>Forward: GACGGGGTGCTGAAACACGCAGAACATTACGAGATTATG&lt;br&gt;Reverse: CATATTCTCGTAAGTTTCCGGCGTGCTTCAGCACCAC</td>
</tr>
<tr>
<td><em>Nme</em>IPMS E353A</td>
<td>Forward: GTTGGAAAGCCGAGGAAGCTGAC&lt;br&gt;Reverse: GTTCAGTGCTCCTCCGGCGCCTTTCAAC</td>
</tr>
<tr>
<td><em>Nme</em>IPMS E466A</td>
<td>Forward: GAAAGCCAAGGCGGCACAGCTGCTCGTCGTC&lt;br&gt;Reverse: GACCGACGCTGGTCGGCCTTGCTGCTTTC</td>
</tr>
</tbody>
</table>

5.2.2 PCR equipment

PCR reactions were performed using a Veriti® 96-well Thermal Cycler (Applied Biosystems).
5.2.3 Mutagenesis of *Mtul*PMS variants

Amino acid-substituted *Mtul*PMS variants A558I, A558V, A567V, V551L and I627A were generated by creating mutations in the plasmid pProExHTa-LeuA. Mutagenesis was performed using either a QuikChange XL II Site-directed Mutagenesis Kit, QuikChange Lightning Site-directed Mutagenesis Kit or a *Pfu*Ultra High-Fidelity DNA polymerase (Agilent Technologies©), using cycling protocols and a reaction volume of 50µL. Reaction components used as recommended by each manufacturer.

After PCR the template DNA was digested using a High-Fidelity *Dpn*1 restriction enzyme (Stratagene) for 5 min at 37°C.

5.2.4 Mutagenesis of *Nmel*PMS variants

*Nmel*PMS variants R310A, E353A and E466A were generated by creating mutations in the plasmid pET-151. Mutagenesis was performed using a QuikChange Lightning Site-directed Mutagenesis Kit containing *Pfu*Ultra High-Fidelity DNA polymerase, using cycling protocols and a reaction volume of 50 µL. Reaction components used as recommended by the manufacturer.

After PCR the template DNA was digested using a High-Fidelity *Dpn*1 restriction enzyme (Stratagene) for 5 min at 37°C.

5.2.5 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using either self-poured 1% agarose gels or pre-cast E-Gel® 1.2% (w/v) agarose gels (Invitrogen).

E-Gel® agarose gels were used following the manufacturers guidelines. Samples were loaded directly to the gel with no dye. Gels were run using an E-Gel® iBase™ Safe Imager™.

Self-poured agarose gels were made by heating 0.4 g agarose is 40 mL tris-acetate-EDTA (TAE) buffer until dissolved. The solution was then cooled to ~50°C and 4 µL of SYBR Safe® DNA stain was added. The solution was then poured into a mould. Samples to be run on self-poured gels
were mixed with a 6x loading dye. Electrophoresis was run at 85 V for 60 min with TAE for running buffer using a Mini-Sub™ Cell GT (Bio-Rad).

Gels were viewed and photographed under UV light using a Molecular Imager™ Gel Doc™ XR (Bio-Rad).

TAE buffer: 60mM Tris-HCl, 1mM EDTA, 20mM acetic acid.

Sample loading dye (6x): 60mM Tris-HCl, 60mM EDTA, 0.2% (w/v) orange G, 0.05% (w/v) xylene cyanol ff, 60% (v/v) glycerol.

### 5.2.6 Chemical transformation

Transformations were performed by thawing 50 µL or 100 µL samples of chemically competent cells on ice for 15 min then adding 2-5 µL plasmid (30-200 ng/µL). Samples were then incubated for a further 20 min on ice followed by a heat-shock step for 45 s at 42°C. Samples were then place back on ice for 2-4 min after which 250 µL super optimal broth (SOC) medium was added to the cells. The cells were then grown at 37°C for 1 h with shaking. After growth, 20-200µL of the cells was spread on LB-agar plates containing the appropriate antibiotics. These plates were then incubated at 37°C for 12-18 h.

SOC medium: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgSO₄ and 20mM glucose.

### 5.2.7 Plasmid preparation and purification

Plasmids were extracted and purified from 5 mL cell cultures grown at 37°C overnight (containing the appropriate antibiotics) of OneShot TOP10 or XL1Blue cell lines. Cells were harvested and then plasmids were extracted using a Roche High Pure Plasmid Isolation Kit. The plasmid extraction and purification was carried out following the kit manual.
5.2.8 DNA sequencing

DNA sequencing was carried out by Canterbury Sequencing on an ABI3100 Genetic Analyzer (Applied Systems Inc.) using a procedure based on Sanger chain-determination protocol. Double-stranded plasmid samples were prepared as 6µL aliquots of 20-80 ng/µL plasmid per sequencing run. Sequencing primers were provided at 3.2µM. A list of sequencing primers used can be found in table 5.2.

Table 5.2: Sequencing primers used throughout this study. The pProEx primers were used to sequence MtU PMS and T7 primers were used for Nme1PMS sequencing.

<table>
<thead>
<tr>
<th>Sequencing primer</th>
<th>Sequence of primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>pProEx Forward</td>
<td>AGCGGATAACAATTTTACACACAAAAAGC</td>
</tr>
<tr>
<td>pProEx Reverse</td>
<td>ATCTGTATCAGGCTGAAAATC</td>
</tr>
<tr>
<td>T7 Forward</td>
<td>TAATACGACTCACTATAGGG</td>
</tr>
<tr>
<td>T7 Reverse</td>
<td>GCTAGTTATTGCTCAGCGG</td>
</tr>
</tbody>
</table>

5.3 Cell cultures

5.3.1 *Escherichia coli* cell lines

*E. coli* OneShot TOP10 cell lines were used for plasmid propagation, and *E. coli* BL21(DE3)Star for protein expression.

5.3.2 Glycerol stocks

All *E. coli* strains created during this study were stored at -80°C as 1.2 mL samples containing 20% v/v glycerol. Cells were grown for 12-16 h before addition of glycerol and flash-freezing.
5.3.3 Antibiotics

All plasmids used in this study carry resistance to ampicillin. Ampicillin was included in all cell cultures at a concentration of 0.1 mg/mL. Antibiotics were stored at -80°C at 1000x the desired final concentration dissolved in water.

5.3.4 LB media

Cell cultures were grown in liquid LB broth which was autoclaved before use. The appropriate antibiotic was added before use.

LB-agar was stored at room temperature and melted via microwave before use. After melting the solution was allowed to cool to around 50 °C then the appropriate antibiotic was added for selection. The solution was then poured into round petri dishes.

Liquid LB: 20 g/L Lennox-L broth base

LB-agar: 20 g/L Lennox-L broth base, 15 g/L agar

5.3.5 Protein expression

All α-IPMS enzymes were expressed and purified using similar protocols. A typical expression consisted of two batches of 1 L LB medium containing the appropriate antibiotic inoculated by a small scale pre-growth which was in turn inoculated by a scraping of frozen glycerol stock. The 1 L cultures were grown at 37 °C until an OD₆₀₀ of 0.5-0.7 AU was reached at which time IPTG was added to a concentration of 0.25 mM. Cultures were then grown overnight at 23 °C.

5.3.6 Cell harvesting

Cell cultures were harvested in 1 L flasks by centrifugation at 14,000 g for 10 min at 4 °C. Small cultures of less than 5 mL were harvested in 1.6 mL micro-centrifuge tubes at 17,000 g for 5 min.
5.4 Purification

5.4.1 Cell lysis

Before lysis, cells were resuspended in 15 mL equilibration buffer. Lysis was performed using an Omni-Ruptor 4000 Ultrasonic Homogenizer (Omni International) or a detergent based method using Bugbuster®.

Sonication was performed on ice. Typical lysis consisted of 4 repeats of 70 % power at 35 Hz for 5 min. The sample temperature was checked after each repeat to ensure over-heating did not occur.

Small scale growths were lysed using Bugbuster® following their provided protocol.

After lysis the solution was centrifuged at 44,000 g for 45 min to separate the soluble fraction from the insoluble. Chromatography was performed without delay to minimise proteolysis.

5.4.2 Chromatography equipment

All chromatography was performed using either a GE Healthcare AKTApurifier™ 10 or a Bio-Rad BioLogic DuoFlow. All buffers used were filtered using a 0.2 µm prior to being injected into a 50 mL Superloop™ (GE Healthcare). Protein elution was monitored at 260 nm and 280 nm and peaks at these wavelengths were analysed for protein of interest using SDS-PAGE gels. Chromatography was performed at 4°C for overnight protocols and room temperature for protocols under 3 hours.

5.4.3 Immobilised metal affinity chromatography

All protein purified in this thesis underwent of two IMAC steps. The first of these chromatography steps separated the His-tagged protein from soluble *E. coli* background proteins. The second step separated the untagged protein from the His-tag and TEV protease after tag cleavage.
IMAC was performed using either a 5 mL Histrap® column (GE healthcare) or a 5 mL Talon® Metal Affinity column (GE Healthcare). In both protocols the columns were equilibrated using 5 column volumes (CV) of equilibration buffer. Elution was performed via a 50:50 equilibration: elution buffer gradient over 10 CV for *Mtu*IPMS. For *Nme*IPMS a single step elution buffer change was used to elute bound protein for a length of 6 CV. After use all columns were washed through for 5 to 10 CV of low-salt buffer and stored in ethanol.

Histrap equilibration buffer: 50 mM KPO$_4$, 25 mM Imidazole, 250 mM NaCl at pH 7.5

Histrap elution buffer: 50 mM KPO$_4$, 400 mM Imidazole, 250 mM NaCl at pH 7.5

Talon equilibration buffer: 50 mM KPO$_4$, 300 mM KCl, 10 mM Imidazole at pH 8.0

Talon elution buffer: 50 mM KPO$_4$, 300 mM KCl, 150 mM Imidazole at pH 8.0

5.4.4 TEV protease treatment

To cleave the His-tag from the protein of interest protein from the first IMAC step was diluted ten-fold in SEC buffer containing no imidazole. TEV protease was then added to the protein containing solution and incubated at 4 °C overnight. This reaction also contained 1 mM DTT to reduced disulfide bonds. 1 mg of TEV protease was added to each sample.

5.4.5 Size-exclusion chromatography

The final purification stage of all proteins was performed using a Sephacryl® S-200 26/60 HiPrep column (GE Healthcare). The column was equilibrated with 1 column volume (CV) of SEC buffer before protein injection. The protein was then eluted with 1 CV SEC buffer and stored back in ethanol.

*Mtu*IPMS SEC buffer: 50 mM KPO$_4$, 250 mM NaCl at pH 7.5

*Nme*IPMS SEC buffer: 50 mM BTP at pH 7.0
5.4.6 Concentration of protein samples

All protein samples were concentrated at 4 °C using a 10000 Da molecular weight cut-off centrifugal concentrator (GE Healthcare or Millipore).

5.4.7 Determination of protein concentration

The concentration of protein containing solutions were determined using a Nanodrop ND-1000 spectrophotometer which measured the absorbtion at 280 nm. Protein concentration was then calculated using the Beer-Lambert Law and calculated extinction coefficients from Protparam (http://web.expasy.org/protparam/).

5.4.8 Buffer exchange

Exchanging buffers for protein solutions was done via successive concentration and dilution with desired buffer using a 10000 Da molecular weight cut-off centrifugal concentrator (GE Healthcare or Millipore) at 4 °C.

5.4.9 Protein storage

Purified protein was stored at -80 °C in 100 µL aliquots at 1-10 mg/mL. All aliquots were flash-frozen using liquid nitrogen prior to storage.
5.5 Protein characterisation

5.5.1 Protein parameters

Protein parameters were calculated from sequence data using the ProtParam tool (http://web.expasy.org/protparam/). All sequences included the additional residues GA (for *MtulPMS* enzymes) and GIDPFT (for *NmelPMS* enzymes) which are added to the N-terminal.

5.5.2 Polyacrylamide gel electrophoresis

SDS-PAGE was performed using either NuPAGE® 10 % Bis-Tris 12-well pre-cast gels (Invitrogen) or Bolt® 12% Bis-Tris 12-well gels. NuPAGE® gels were run in NuPAGE® MES SDS running buffer (Invitrogen). Bolt® gels were run in Bolt® MES SDS running buffer. Samples were mixed with 4x sample buffer and DTT then boiled for 5 min.

Protein molecular weight standards were separated on the gel along with the protein samples. The protein marker used was Novex® Sharp Pre-Stained Protein Standards (Invitrogen).

Gels were viewed by staining with a solution of 1 % Coomassie brilliant blue R-250, 40 % methanol and 10 % glacial acetic acid. Gels were then destained using a solution of 40 % methanol and 10 % glacial acetic acid. Both staining and destaining steps were gels were heated in the microwave with the appropriate solution. Gels were left on shaker for 15 min to stain and 1 hour to destain.

Gels were viewed and photographed using a Molecular Imager Gel Doc XR (Bio-Rad).

5.5.3 Circular dichroism spectrometry

CD spectra measurements were performed using a JASCO J-815 Spectropolarimeter. Wavelength scans were carried out in double-distilled water (pH 7.0) between wavelengths 180-240 nm, with 0.5 data pitch, 1 seconds response and at 1 mm bandwidth at room temperature. Samples were prepared at an enzyme concentration of 0.2 mg/mL.
5.5.4 Mass spectrometry

Protein masses were measured using a Bruker maXis 3G.

5.5.6 Differential scanning fluorimetry

DSF was performed using a Bio-Rad iCycler iQ5™ Multicolour Real-Time PCR Detection System. 25 µL samples were prepared in a 96-well plate as follows: 20 µL BTP buffer (pH 7.5 for NmelPMS or 8.0 for MtuIPMS) including ligands, 1 µL 250x SYPRO Orange dye and 4 µL of protein at 1 mg/mL. Ligand concentrations displayed in relevant chapters.

The 96-well plated was sealed and subjected to a thermal melt program from 20 to 95 °C in 0.2 °C increments holding each step for 20 s. Each sample was measured in triplicate and compared to a control containing everything but protein.

5.6 Activity assays

5.6.1 Kinetic assay equipment

All kinetic assays were performed using a Varian Cary 100 UV-visible spectrophotometer using stoppered quartz cuvettes with a pathlength of 1 cm or 0.2 cm.

Reaction rates were measured by calculating a least-squares fit of rate data in Cary WinUV Kinetic Application (Version 3, Varian).

5.6.2 4-4’-Dithiodipyridine-coupled assays at 324 nm

All activity assays were performed via a coupled assay using DTP to detect the formation of the CoA product at 324 nm at 25 °C (ε = 1.98 x 10⁴ M⁻¹ cm⁻¹) based on the method of de Carvalho et al. Reaction mixtures are described in the relevant chapter.

All kinetic measurements were performed in duplicate or triplicate with errors typically under 10%.
5.6.3 Substrate concentration determination

The concentrations of the solutions of substrates AcCoA and α-KIV were determined using DTP-coupled assay at 324 nm. Limiting amounts of substrate to be measured were added to the assay cuvette while the other substrate was held at excess concentration. The change in absorbance (ΔAbs) was measured in triplicate. A sample containing no substrates was used to measure the change in absorbance due to the increase in enzyme (ΔAbs_{enz}). The corrected change in absorbance was then calculated as ΔAbs - ΔAbs_{enz}. The corrected absorbance was then converted to the concentration of the limiting substrate using the Beer-Lambert Law with an extinction coefficient of 1.98 x 10^{4} \text{M}^{-1}\text{cm}^{-1}.

5.6.4 Michaelis-Menten kinetics

Assays to determine apparent $K_{m}$ and $k_{cat}$ values for all enzymes used the DTP-coupled assay at 324 nm. Apparent $K_{m}$ values were determined using SigmaPlot 11.0 by fitting to the Michaelis-Menten equation.

All Michaelis-Menten data was obtained using volumes of 1 mL in 1 cm pathlength cuvettes.

5.6.5 MtuIPMS wild-type and variant Michaelis-Menten conditions

All MtuIPMS $K_{m}$ values were found under the same conditions of 500 µM DTP, 3 mM MgCl$_2$ and 20 mM KCl. While obtaining apparent $K_{m}$ values for AcCoA, α-KIV was held at 400 µM. While obtaining apparent $K_{m}$ values for α-KIV, AcCoA was held at 500 µM. Assays were carried out in 50 mM Tris-HCl buffer at pH 8.0.
5.6.6 *Nmel*IPMS wild-type and variant Michaelis-Menten conditions

**Nmel**IPMS wild-type

The wild-type *Nmel*IPMS apparent AcCoA $K_m$ was found under the following conditions: 500 µM α-KIV, 500 µM DTP, 20 mM MgCl$_2$ and 20 mM KCl in 50 mM HEPES buffer at pH 7.5. The apparent α-KIV $K_m$ was found under the following conditions: 190 µM AcCoA, 500 µM DTP, 20 mM MgCl$_2$ and 20 mM KCl in 50 mM HEPES buffer at pH 7.5.

**Nmel**IPMS R310A

*Nmel*IPMS R310A parameters were unable to be determined due to running out of the substrate AcCoA before an accurate value was obtained. Results did however show the catalytic activity of this variant was significantly impaired with an estimated apparent AcCoA $K_m$ of 750 µM. This was found under the following conditions: 500 µM α-KIV, 500 µM DTP, 20 mM MgCl$_2$ and 20 mM KCl in 50 mM HEPES buffer at pH 7.5.

**Nmel**IPMS E353A

The apparent AcCoA $K_m$ for *Nmel*IPMS E353A was found under the following conditions: 450 µM α-KIV, 500 µM DTP, 20 mM MgCl$_2$ and 20 mM KCl in 50 mM HEPES buffer at pH 7.5. The apparent α-KIV $K_m$ was found under the following conditions: 150 µM AcCoA, 500 µM DTP, 20 mM MgCl$_2$ and 20 mM KCl in 50 mM HEPES buffer at pH 7.5.

**Nmel**IPMS E466A

The apparent AcCoA $K_m$ for *Nmel*IPMS E353A was found under the following conditions: 450 µM α-KIV, 500 µM DTP, 20 mM MgCl$_2$ and 20 mM KCl in 50 mM HEPES buffer at pH 7.5. The apparent α-KIV $K_m$ for *Nmel*IPMS E353A was found under the following conditions: 450 µM α-KIV, 500 µM DTP, 20 mM MgCl$_2$ and 20 mM KCl in 50 mM HEPES buffer at pH 7.5.
5.6.7 Inhibition assays

All inhibition data obtained was carried out using the DTP-coupled assay in 250 µL cuvettes with a pathlength of 0.2 cm. Inhibition data was plotted and analysed using SigmaPlot 11.0 or Microsoft Excel 2010.

*Mtu*IPMS inhibition with L-leucine, L-isoleucine and L-norvaline was examined in this thesis in the relevant chapter. Concentrations up to 5 mM of each of the inhibitors was tested.

Rates of time-dependant inhibition effects of *Mtu*IPMS were measured for times up to two minutes.
Appendix A

Multiple sequence alignments

The following sequence alignments were performed using Clustal Omega. The sequence alignments were used in determination of MtuPMS variants.

**Mtu**PMS and **Li**CMS sequence alignment

<table>
<thead>
<tr>
<th>MtuPMS</th>
<th>LICMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTTSES</td>
<td>PIRLNR</td>
</tr>
<tr>
<td>TEDAY</td>
<td>TWP-DR</td>
</tr>
<tr>
<td>T-</td>
<td>VDRL</td>
</tr>
<tr>
<td>Q</td>
<td>49</td>
</tr>
<tr>
<td>P</td>
<td>Q</td>
</tr>
<tr>
<td>V</td>
<td>P</td>
</tr>
<tr>
<td>E</td>
<td>A</td>
</tr>
<tr>
<td>I</td>
<td>S</td>
</tr>
<tr>
<td>P</td>
<td>I</td>
</tr>
<tr>
<td>I</td>
<td>N</td>
</tr>
<tr>
<td>P</td>
<td>E</td>
</tr>
<tr>
<td>I</td>
<td>A</td>
</tr>
<tr>
<td>P</td>
<td>R</td>
</tr>
<tr>
<td>I</td>
<td>D</td>
</tr>
<tr>
<td>P</td>
<td>E</td>
</tr>
<tr>
<td>I</td>
<td>A</td>
</tr>
<tr>
<td>P</td>
<td>K</td>
</tr>
<tr>
<td>I</td>
<td>A</td>
</tr>
<tr>
<td>P</td>
<td>A</td>
</tr>
<tr>
<td>I</td>
<td>A</td>
</tr>
</tbody>
</table>

116
MTUIPMS  EASVTIAASPAQPGEAGRHASDFTIASTPAQPGEAGRHASDFTIASTPAQPGEAGRHASDFTIASTPAQPGEAGRHASDFTIASPTAS  631
LICMS  TWNKS---------------------------------------------LDEEDQTPKTGRRHPTVAA  499
MTUIPMS  LRAVSVAVNRAAR--------------------------------------  644
LICMS  VHATEKMLNQILQPWQI-----------------------------------  516

= Location of amino acid substitutions  = Catalytic domain  = Subdomain I  = Subdomain II  = Regulatory domain

α-IPMS multiple sequence alignment

---

**Catalytic domain**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CUR</td>
<td>------MSNTDSFISAPAQPQTPNQFADQPAQKNQKRUNQKPSRNLPMTRRLPQ 55</td>
</tr>
<tr>
<td>CJK</td>
<td>-----------------------------------------------MPHRQPEGGEVEITLPD 20</td>
</tr>
<tr>
<td>CDI</td>
<td>-----------------------------------------------MAVRNYPKHEVENIDLPD 20</td>
</tr>
<tr>
<td>CEF</td>
<td>-----------------------------------------------MPKRYLPEVEVIDLPD 20</td>
</tr>
<tr>
<td>SEN</td>
<td>MSIPPEP--QTPISNRKVSRPAPDQQFNPQGQGSPMSFHRYPHEKEVSVLPD 56</td>
</tr>
<tr>
<td>MLE</td>
<td>-----------------------------------------------MPFVRYPFADEVEPLVD 20</td>
</tr>
<tr>
<td>MTU</td>
<td>MTSPEEIDAITEEGHRSRAFSQAQPQISNRPSNPRKASSPRKPSRSAEVEFLPA 69</td>
</tr>
<tr>
<td>RHA</td>
<td>------MPA-ADAFTSIGTTRITPPSKPAQPPHQPENNTQKNSMHTEYRSFSEVEVSLPD 55</td>
</tr>
<tr>
<td>MSM</td>
<td>MNTDST-S-DATFST-GRTITPPSQAHPFQQPANNTQGSSMPVYRSFPADEVEKSLPD 58</td>
</tr>
<tr>
<td>SAQ</td>
<td>-----------------------------------------------MAHVNYTPAETDPPAPQPSRMPYHRQPYQQFMHRTLPLDP 41</td>
</tr>
<tr>
<td>LXX</td>
<td>-----------------------------------------------MPHKEYRPFHEQI-AVDLPD 19</td>
</tr>
<tr>
<td>KRA</td>
<td>-----------------------------------------------MQNTQTPSPMPFMRPQIQI-BVELPD 28</td>
</tr>
<tr>
<td>RSA</td>
<td>-----------------------------------------------MRNAKQSGMPVHRYPEFQI-EVELPD 28</td>
</tr>
<tr>
<td>AAN</td>
<td>-----------------------------------------------MRNAKQPSGMPHRYQPYQQF 28</td>
</tr>
<tr>
<td>ART</td>
<td>-----------------------------------------------MRNAKQPSGMPHRYMPQIQI-TVELPD 28</td>
</tr>
</tbody>
</table>

---

**Catalytic domain**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CUR</td>
<td>RTWPDKVDRAPQWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 115</td>
</tr>
<tr>
<td>CJK</td>
<td>RTWPDKVDRAPQWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 80</td>
</tr>
<tr>
<td>CDI</td>
<td>RTWPDKRINTAPWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 80</td>
</tr>
<tr>
<td>CEF</td>
<td>RTWPDKITQPWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 80</td>
</tr>
<tr>
<td>SEN</td>
<td>RTWPDNRTAPWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 116</td>
</tr>
<tr>
<td>MLE</td>
<td>RTWPDKVDRAPWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 80</td>
</tr>
<tr>
<td>MTU</td>
<td>RTWPDKVDRAPWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 120</td>
</tr>
<tr>
<td>RHA</td>
<td>RTWPDKVDRAPWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 115</td>
</tr>
<tr>
<td>MSM</td>
<td>RTWPDKVDRAPWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 118</td>
</tr>
<tr>
<td>SAQ</td>
<td>RTWPDKVDRAPWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 101</td>
</tr>
<tr>
<td>LXX</td>
<td>RTWPDKVDRAPWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 79</td>
</tr>
<tr>
<td>KRA</td>
<td>RTWPDKVDRAPWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 88</td>
</tr>
<tr>
<td>RSA</td>
<td>RTWPDKVDRAPWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 88</td>
</tr>
<tr>
<td>AAN</td>
<td>RTWPDKVDRAPWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 88</td>
</tr>
<tr>
<td>ART</td>
<td>RTWPDKVDRAPWCAVLDRLGQAMLIDDSPKRRMFHMDLMVELMGEKEIEVGFPSAQ 88</td>
</tr>
</tbody>
</table>

---
Catalytic domain

CUR  DYDFVREIIENKIPDDVTIQVLVQAREHLIRRTFEACAGAKNVIVHFYNSTSLQRVRV  175
CJK  DFNFVREIENKIPDDVTIQVLVQAREHLIRRTFEACAGAKNVIVHFYNSTSLQRVRV  140
CDI  DFDFVREIIENKIPDDVTIQVLVQAREHLIRRTFEACAGAKNVIVHFYNSTSLQRVRV  140
CEF  DFDFVREIIENKIPDDVTIQVLVQAREHLIRRTFEACAGAKNVIVHFYNSTSLQRVRV  140
SEN  DFDFVREIDAVPDVRVLQTVRCTLRELPSFASLEGAAEKVIVHFYNSTSLQRVRV  176
MLE  DFDFVREIIENKIPDDVTIQVLVQAREHLIRRTFEACAGAKNVIVHFYNSTSLQRVRV  140
MTU  DFDFVREIIENKIPDDVTIQVLVQAREHLIRRTFEACAGAKNVIVHFYNSTSLQRVRV  180
RHA  DFDFVREIIENKIPDDVTIQVLVQAREHLIRRTFEACAGAKNVIVHFYNSTSLQRVRV  175
MSM  DFDFVREIIENKIPDDVTIQVLVQAREHLIRRTFEACAGAKNVIVHFYNSTSLQRVRV  178
SAQ  DFDFVREIIENKIPDDVTIQVLVQAREHLIRRTFEACAGAKNVIVHFYNSTSLQRVRV  161
LXX  DFDFVREIIENKIPDDVTIQVLVQAREHLIRRTFEACAGAKNVIVHFYNSTSLQRVRV  139
KRA  DFDFVREIIENKIPDDVTIQVLVQAREHLIRRTFEACAGAKNVIVHFYNSTSLQRVRV  148
RSA  DFDFVREIIENKIPDDVTIQVLVQAREHLIRRTFEACAGAKNVIVHFYNSTSLQRVRV  148
AAU  DFDFVREIIENKIPDDVTIQVLVQAREHLIRRTFEACAGAKNVIVHFYNSTSLQRVRV  148
ART  DFDFVREIIENKIPDDVTIQVLVQAREHLIRRTFEACAGAKNVIVHFYNSTSLQRVRV  148

*:***:*  :*:** ****.*.* .** **: :   * ..:**:***** *** **

Catalytic domain

CUR  FKDKEGIKKLATDAALIKEIAQD-YPDTNWRWEYSPESFTGTEIEYAKEVCDAVGEVI  234
CJK  FKDKRAIKKLATDAALIKEIAQD-YPDTNWRWEYSPESFTGTEIEYAKEVCDAVGEVI  199
CDI  FKDKAIAIKKLATDAALIKEIAQD-YPDTNWRWEYSPESFTGTEIEYAKEVCDAVGEVI  199
CEF  FKDKQVKTDAALIKEIAQD-YPDTNWRWEYSPESFTGTEIEYAKEVCDAVGEVI  199
SEN  FREEREGIKKIATMGAEMALEFAGK-YPDTDFRFQYSPESFYTGTELEYSAYAEVCAVGEVI  235
MLE  FRAQATVKAATDDGCKVEEAFK-YPYHTFRFYSPESFTGTELYAAEVCDAVGEVI  199
MTU  FRAQATVKAATDDGCKVEEAFK-YPYHTFRFYSPESFTGTELYAAEVCDAVGEVI  239
RHA  FKAERDVIAIKKIATDAALIKEIAQD-YPDTNWRWEYSPESFTGTEIEYAKEVCDAVGEVI  234
MSM  FRAKIAAIAIKKLATDAALIKEIAQD-YPDTNWRWEYSPESFTGTEIEYAKEVCDAVGEVI  237
SAQ  FGLDRGIDTTAATGARLQCKYAEIHTPDTDHYEYSPESFTGTELYALEVCAVIEV  221
LXX  FSRDQVQDIALAGARLCRQFEAL-APGTIEYYESFTGTELEFAICNQALEVF  198
KRA  FGMDREGIDTAMLQGARLCRKEET-IPGTIVYYESFTGTELEFAICNQALEVF  207
RSA  FMAEQGDMALQGARLCRKEET-IPGTIVYYESFTGTELEFAICNQALEVF  207
AAU  FNQEDGGMALQGARLCRKEYET-IPGTIVYYESFTGTELEYAARVCAVIEV  207
ART  FNQEDGGMALQGARLCRKEYET-IPGTIVYYESFTGTELEYAARVCAVIEV  207

*:***:*  :*:** ****.*.* .** **: :   * ..:**:***** *** **

Catalytic domain

CUR  DPTPENPIIIINLPSTVEMITPNVYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  294
CJK  DPTPENPIIIINLPSTVEMITPNVYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  259
CDI  DPTPENPIIIINLPSTVEMITPNVYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  259
CEF  DPTPENPIIIINLPSTVEMITPNVYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  259
SEN  QPTPENPIIIINLPSTVEMITPNVYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  295
MLE  QTDPENPIIIINLPSTVEMITPNVYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  259
MTU  QTDPENPIIIINLPSTVEMITPNVYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  299
RHA  QPTPENPIIIINLPSTVEMITPNVYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  294
MSM  QPTPENPIIIINLPSTVEMITPNVYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  297
SAQ  DPTPENPIIIINLPSTVEMITPNVYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  281
LXX  EPTDPHRLVNLQATPMVIPYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  258
KRA  EPTPQVQVNLQATPMVIPYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  267
RSA  EASNDQVIINLQATPMVIPYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  267
AAU  EASADQVNLQATPMVIPYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  267
ART  EASADQVNLQATPMVIPYADIEWHRNLNRDSIILSLPHDRGEVAAEEL  267

*:***:*  :*:** ****.*.* .** **: :   * ..:**:***** *** **
### Regulatory domain

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUR</td>
<td>NRALNS...............</td>
<td>613</td>
</tr>
<tr>
<td>CJK</td>
<td>NRSQDAAGVSQGV------</td>
<td>579</td>
</tr>
<tr>
<td>CDI</td>
<td>NRALS...............</td>
<td>570</td>
</tr>
<tr>
<td>CEF</td>
<td>NRALDFKHQQLONG--GV</td>
<td>581</td>
</tr>
<tr>
<td>SEN</td>
<td>NRAGR...............</td>
<td>596</td>
</tr>
<tr>
<td>MLE</td>
<td>NRAMPR...............</td>
<td>564</td>
</tr>
<tr>
<td>MTU</td>
<td>NRAAR...............</td>
<td>614</td>
</tr>
<tr>
<td>RHA</td>
<td>NRAH...............</td>
<td>602</td>
</tr>
<tr>
<td>MSM</td>
<td>NRAARA...............</td>
<td>602</td>
</tr>
<tr>
<td>SAQ</td>
<td>NRTR...............</td>
<td>584</td>
</tr>
<tr>
<td>LXX</td>
<td>NRSVRASASSVPAELAGV</td>
<td>581</td>
</tr>
<tr>
<td>KRA</td>
<td>NRALR...............</td>
<td>575</td>
</tr>
<tr>
<td>RSA</td>
<td>NRAIRDNQVD-------</td>
<td>582</td>
</tr>
<tr>
<td>AAU</td>
<td>NRAVRDAQA--------</td>
<td>579</td>
</tr>
<tr>
<td>ART</td>
<td>NRAIRDQA--------</td>
<td>579</td>
</tr>
</tbody>
</table>

**:**

- **=** MtuPMS
- **=** Catalytic domain
- **=** Subdomain I
- **=** Residue conservation
- **=** Subdomain II
- **=** Regulatory domain

---

121
Appendix B
Michaelis-Menten plots for α-IPMS variants

### MtuIPMS V551L

<table>
<thead>
<tr>
<th></th>
<th>α-KIV $K_m$ (µM)</th>
<th>AcCoA $K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtuIPMS V551L</td>
<td>9.1 ± 0.8</td>
<td>46 ± 3</td>
<td>3.6 ± 0.1</td>
</tr>
</tbody>
</table>

### MtuIPMS A558I

<table>
<thead>
<tr>
<th></th>
<th>α-KIV $K_m$ (µM)</th>
<th>AcCoA $K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtuIPMS A558I</td>
<td>9.7 ± 1.0</td>
<td>72 ± 5</td>
<td>3.9 ± 0.1</td>
</tr>
</tbody>
</table>
**MtulPMS A558V**

![Graphs showing enzyme activity vs substrate concentration for MtulPMS A558V.](image)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-KIV $K_m$ (µM)</td>
<td>$6.7 \pm 0.6$</td>
</tr>
<tr>
<td>AcCoA $K_m$ (µM)</td>
<td>$53 \pm 3$</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$4.0 \pm 0.1$</td>
</tr>
</tbody>
</table>

**MtulPMS A567V**

![Graphs showing enzyme activity vs substrate concentration for MtulPMS A567V.](image)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-KIV $K_m$ (µM)</td>
<td>$5.0 \pm 0.4$</td>
</tr>
<tr>
<td>AcCoA $K_m$ (µM)</td>
<td>$56 \pm 3$</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$4.1 \pm 0.1$</td>
</tr>
</tbody>
</table>
### MtuPMS I627A

<table>
<thead>
<tr>
<th></th>
<th>$\alpha$-KIV $K_m$ (µM)</th>
<th>AcCoA $K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.2 ± 0.4</td>
<td>40 ± 3</td>
<td>3.2 ± 0.1</td>
</tr>
</tbody>
</table>

### NmeIPMS E353A

<table>
<thead>
<tr>
<th></th>
<th>$\alpha$-KIV $K_m$ (µM)</th>
<th>AcCoA $K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>29 ± 2</td>
<td>19 ± 1</td>
<td>11.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>$\alpha$-KIV $K_m$ ((\mu M))</td>
<td>AcCoA $K_m$ ((\mu M))</td>
<td>$k_{cat}$ (s(^{-1}))</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------</td>
<td>---------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td></td>
<td>29 ± 3</td>
<td>79 ± 3</td>
<td>9.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>750 ± 260</td>
<td>5.1 ± 0.7</td>
</tr>
</tbody>
</table>
Bibliography

(1) Furst, P.; Stehle, P. What are the essential elements needed for the determination of amino acid requirements in humans, *Journal of Nutrition*. 2004, 134, 1558S.


