Characterization of the active site of Methionine γ-Lyase from *Trichomonas vaginalis*

by

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Abstract

Resistance to, metronidazole and tinidazole, the drugs currently prescribed for the treatment of trichomoniasis, an infection of the genito-urinary tract of humans by the protozoan *Trichomonas vaginalis,* has created a need for the development of therapeutics with a different mode of action. Trifluoromethionine (TFM) has been proposed as a potential novel antiinfective prodrug that is activated by the enzyme methionine γ-lyase (MGL), which is present in *T. vaginalis* but not in the cells of the human host. The presence of closely related enzymes, such as cystathionine γ-lyase, in humans necessitates the development of TFM derivatives that are highly selective for TvMGL so that activation of the prodrug will be limited to the parasitic cells. An essential step in the development of selective TFM-based prodrugs is mapping of the TvMGL active site to identify the residues that participate in substrate binding and catalysis, particularly compared to related human enzymes.

Therefore, the primary goal of this thesis was to determine the effect of a set of 9 site-directed variants of key active-site residues on the steady state kinetic parameters of TvMGL. As a prerequisite to this study, an affinity purification protocol and continuous assays for the methionine, homocysteine and cysteine hydrolysis activities of TvMGL were developed. The Y56F, R58A, Y111F, S338A, R373A and R373K variants of TvMGL lack detectable methionine and homocysteine hydrolysis activity and the turnover rate of the I55A and D239A variants is reduced up to 14-fold, while that of L339A is within 2-fold of the wild-type TvMGL enzyme. Roles for residue R373 in binding the α-carboxylate group of the substrate and for I55 in hydrophobic packing with

the nonpolar side chain of the substrate are proposed. Residues Y56 and R58 are expected to anchor and position the PLP cofactor, through interaction with the phosphate moiety. Residue S338 may guide the catalytic lysine and Y111 is proposed to play a role in proton transfer, guided by interaction with R58, in the context of the α,γ-elimination reaction catalyzed by TvMGL.

These results further our understanding of the substrate-binding surface of the TvMGL active site, information that will assist in the development of selective TFM-based prodrugs for the treatment of trichomoniasis.

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Abbreviations

AAT	Aspartate aminotransferase
ATP	Adenosine triphosphate
CBL	Cystathionine β-lyase
CBS	Cystathionine β-synthase
CGL	Cystathionine γ-lyase
CGS	Cystathionine γ-synthase
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
E. coli	*Escherichia coli*
E.C.	Enzyme Commission
eCBL	*Escherichia coli* cystathionine β-lyase
eCGS	*Escherichia coli* cystathionine γ-synthase
E. histolytica	*Entamoeba histolytica*
hCGL	Human cystathionine γ-lyase
HicDH	D-2-Hydroxyisocaproate dehydrogenase
IPTG	Isopropyl- β-*D*-thiogalactopyranoside
L-Cth	L-Cystathionine
L-Cys	L-Cysteine
L-Hcys	L-Homocysteine
LDH	L-lactate dehydrogenase
MBTH	3-methyl-2-benzothiazolinone hydrozone hydroclorate

MGL	Methionine γ-lyase
NADH	Nicotinamide adenine dinucleotide, reduced form
Ni-NTA	Nickel-nitrilo triacetic acid
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PLP	Pyridoxal 5'-phosphate
P. putida	*Pseudomonas putida*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFM	Trifluoromethionine
T. vaginalis	*Trichomonas vaginalis*

1. Introduction

Sexually transmitted infections (STI) are a growing problem in modern society in both developed and developing countries. Despite our ever increasing knowledge of the infectious agents, how they spread and preventative measures there is an increasing number of reported cases of STIs worldwide (Harp and Chowdhury 2011). Trichomoniasis, an infection of the genito-urinary tract of humans by the protozoan *Trichomonas vaginalis*, accounts for half of the STI cases reported each year (Petrin, Delgaty *et al*. 1998, Kulda 1999, Dacks, Walker *et al*. 2008, Cudmore and Garber 2010, Harp and Chowdhury 2011) . Up to 8-10 million people in the United States and 11 million in Europe are diagnosed annually with trichomoniasis (Petrin, Delgaty et al. 1998, Harp and Chowdhury 2011).

In women, *T. vaginalis* adheres to and damages vaginal epithelial cells and causes vaginitis. Women with symptomatic thrichomoniasis have a wide range of symptoms ranging from a relatively asymptomatic state to severe inflammation. Moreover, women with thrichomoniasis have several complications associated with adverse pregnancy outcome, preterm birth or premature labor, low birth weight, atypical pelvic inflammatory diseases, amplified HIV transmission-acquisition and increased risk of cervical cancer (Moodley, Wilkinson et al. 2002, Van Der Pol, Williams et al. 2005, Van Der Pol 2007). The prevalence and spectrum of trichomoniasis in males are less characterized but the infection appears to be asymptomatic in 60% of men (Munson, Napierala et al. 2013). In sexually active men, *T. vaginalis* causes urethris, prostathis, cystitis and increased the risk of HIV transmission (Moodley, Wilkinson et al. 2002, Munson, Napierala et al. 2013).

Despite the public health risk demonstrated by high incidence level, *Trichomonas vaginalis* is one of the most poorly studied parasites with respect to its virulence properties and treatment methods. The genome of *Trichomonas vaginalis* was been sequenced and submitted to the National Center for Biotechnology Information (NCBI) in 2007 (Carlton, Hirt *et al*. 2007). It is anticipated that this information will stimulate the development of new approaches for the treatment of trichomoniasis.

1.1 Trichomonads Morphology

Trichomonads represent a distinct well defined monophyletic group of protozoan flagellates that are adapted for living in anaerobic or microaerobic environments. Some trichomonad species are pathogenic to their host, as exemplified by the sexually transmitted pathogens of the genito-urinary tract of humans (*T. vaginalis*) and cattle (*Trichomonas foetus*) (Petrin, Delgaty et al. 1998, Harp and Chowdhury 2011). Typical of the trichomonads, *T. vaginalis* possesses a hydrogenosome, an organelle which produces molecular hydrogen, glycerol, lactate, ethanol, CO_2, and acetate as an end product of fermentative energy metabolism. This double-membrane bound organelle lacks DNA, as well as cytochromes and respiratory chain enzymes, such as those of the mitochondria (Harp and Chowdhury 2011). The hydrogenosome constitutes a separate compartment of energy metabolism in *T. vaginalis* as well as in other species of trichomonads flagellates and rumen ciliates (Müller, Mentel et al. 2012). Although, hydrogenosomal functions confer an energetic advantage to the cell, at least in trichomonads, hydrogenosomal metabolism is not absolutely indispensable (Müller, Mentel et al. 2012).

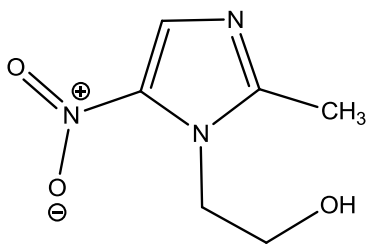
Carbohydrates are the preferred energy source of *T vaginalis*; however, under conditions of carbohydrates scarcity, amino acids can be used to sustain metabolism. When grown in the absence of maltose, a common sugar, it was shown that *T. vaginalis* consumed greater amounts of amino acids especially arginine, threonine and leucine (Rowe and Lowe 1986).

1.2 Treatments for Parasitic Infections

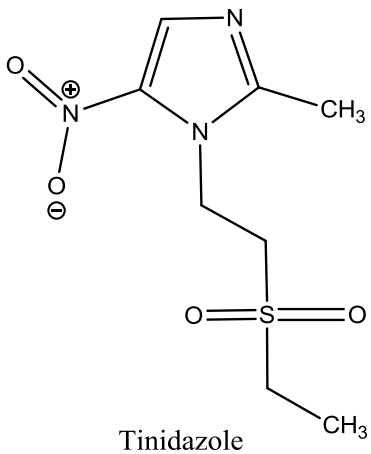
The current standard therapy for the treatment of trichomoniasis is metronidazole (Mz) and variants of this compound. The Mz prodrug is activated in the hydrogenosome to a reactive nitro-radical form which binds transiently to DNA, causing breaks in the nucleotide strands, thereby leading to cell death (Wassmann, Hellberg et al. 1999, Dunne, Dunn et al. 2003, Leitsch, Kolarich et al. 2009).

Metroinidazole possesses a heterocyclic compound imidazole ring with a nitro group at the fifth position, and is derived from the *Streptomyces* antibiotic azomycin (Tovar, Fischer et al. 1999, Leitsch, Kolarich et al. 2009). The Mz prodrug enters *T. vaginalis* through passive diffusion and forms a cytotoxic nitro radical anion following anaerobic reduction by pyruvate-ferrodoxin oxidoreductase (PFOR) (Leitsch, Kolarich et al. 2009). In this process Mz acts as an electron sink, capturing the electrons from reduced ferrodoxin which would normally be donated to hydrogen ions to form hydrogen gas in the hydrogenase reaction (Edwards 1980, Leitsch, Kolarich et al. 2009). Tinidazole is a second generation nitroimidazole with a plasma elimination half-life that is twice that of Mz and improved penetration of male reproductive tissue (Kulda, Tachezy et al. 1993, Wassmann, Hellberg et al. 1999).

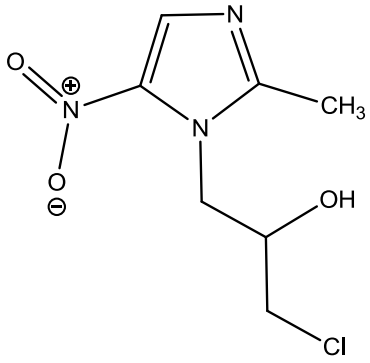
At the present Mz and tinidazole are the only approved drugs for treatment of trichomoniasis in the USA (Harp and Chowdhury 2011). The development of resistance to Mz has reduced its effectiveness in the treatment of *T. vaginalis* infections (Wassmann, Hellberg et al. 1999, Ali and Nozaki 2007). Currently, Mz is ineffective for more than 50% of trichomoniasis cases (Pal, Banerjee et al. 2009).



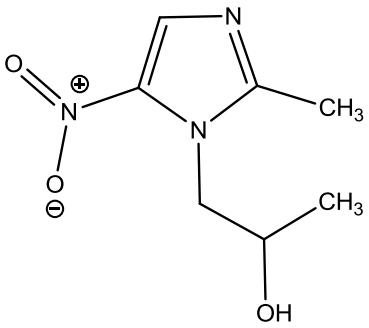
Metroinidazole



Tinidazole



Ornidazole



Secnidazole

Figure 1.1. The antiparasitic prodrug: Metroinidazole and its derivatives.

1.3 *T. vaginalis* Methionine γ-Lyase as a potential drug target for parasitic infection

A therapeutic alternative to Mz is the prodrug L-trifluoromethionine (TFM), which is activated by methionine γ-lyase (MGL) and is reported to be effective against the parasites *Trichomonas vaginalis, Entamoeba histolica, Pseudomonas putida, Clostridium pasteurianum* and *Porphyromonas gingivalis* (Alston and Bright 1983, Coombs and Mottram 2001, El-Sayed 2010). MGL is a pyridoxal-5'-phosphate (PLP)-dependent enzyme that catalyzes the hydrolysis of L-methionine, *via* an α,γ-elimination reaction, to yield α-ketobutyrate, ammonia and methanethiol (Nakayama, Esaki et al. 1984, Thong, Coombs et al. 1987, McKie, Edlind et al. 1998, Sato, Yamagata et al. 2008, Sato and Nozaki 2009).

The α,γ-elimination of trifluoromethionine (TFM) by MGL produces α-ketobuturate, ammonia and trifluoromethanethiol (CF_3SH), which is converted nonenzymatically, under physiological conditions, to thiocarbonyl difluoride (CSF_2) (Coombs and Mottram 2001). Activation of TFM to CSF_2 by MGL provides a promising therapeutic direction as this enzyme has no counterpart in mammals. Therefore, the toxic CSF_2 would be localized to the pathogen in which is produced (Coombs and Mottram 2001).

Mammals do possess cystathionine-γ-lyase (CGL), an enzyme that catalyzes the α,γ-elimination of the amino acid L-cystathionine. However, although CGL binds TFM with high affinity, it is not a substrate for this enzyme. The ability of protozoan MGL to activate TFM, while mammalian CGL cannot, is surprising considering the 40% amino acid sequence identity between human CGL and MGL of *T. vaginalis* (McKie, Edlind et al. 1998). The structures for TvMGL (PDB 1E5F) and human CGL (PDB 2NMP) are

also remarkably similar, displaying a root mean square deviation of ~1.5 Å for the Cα of the peptide backbone.

1.4 The pathways interconverting L-homocysteine and L-cysteine

The sulfur-containing amino acids L-methionine and L-cysteine play essential roles in cellular metabolism, including protein synthesis, methylation reactions and redox homeostasis (Thomas and Surdin-Kerjan 1997, Ravanel, Gakière et al. 1998). The direction of the transsulfuration pathway, which interconverts L-cysteine and L-homocysteine, the immediate precursor of L-methionine, is species-specific. The mammalian and yeast transsulfuration pathway runs from L-homocysteine to L-cysteine and mammals must obtain L-methionine through the diet. Conversely plants and bacteria synthesize L-cysteine *de novo* and convert it to L-homocysteine and, ultimately, L-methionine.

The enzymes of the bacterial and plant transsulfuration pathway are cystathionine γ-synthase (CGS), which condenses *O*-succinylhomoserine and L-cysteine, yielding L-cystathionine, which is hydrolyzed by cystathionine β-lyase (CBL). The first enzyme of the mammalian transsulfuration pathway is cystathionine β-synthase (CBS), which catalyzes the condensation of L-homocysteine and serine to produce L-cystathionine, the substrate of cystathionine γ-lyase (CGL) to produce L-cysteine and α-ketobutyrate (Figure 1.2) (Stipanuk, 1986). In contrast, *T. vaginalis* does not possess either transsulfuration pathway, thereby providing the opportunity to target an enzyme unique to this protozoan parasite for the development of novel therapeutics, including derivatives of TFM

(Thomas and Surdin-Kerjan 1997, Ravanel, Gakière et al. 1998, Coombs and Mottram 2001, Loftus, Anderson et al. 2005, Carlton, Hirt et al. 2007).

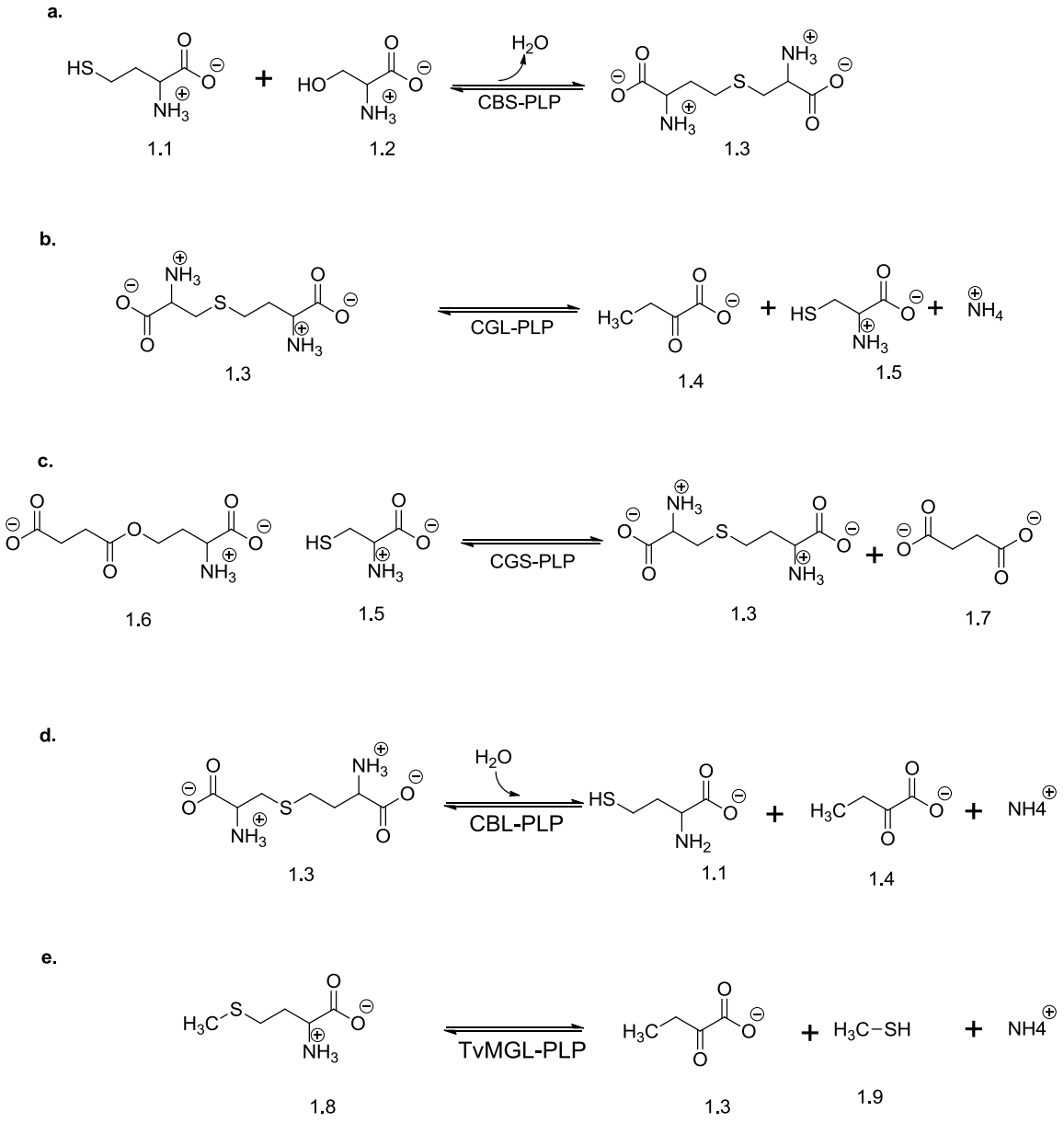


Figure 1.2. Reactions catalyzed by PLP-dependent enzymes catalyzing transformations of sulfur-containing amino acids:
a) cystathionine β-synthase (CBS), the first enzyme of the yeast and mammalian transsulfuration pathway, catalyzes the condensation of (1.1) L-homocysteine and(1.2) L-serine to yield (1.3) L-cystathionine,
b) cystathionine γ-lyase (CGL), the second enzyme of the yeast and mammalian transsulfuration pathway, hydrolyzes (1.3) L-cystathionine to produce (1.4) α-ketobutyrate, L-(1.5) cysteine and ammonia,
c) cystathionine γ-synthase (CGS), the first enzyme of the bacterial transsulfuration pathway, condenses (1.6) *O*-succinyl-L-homoserine and(1.5) L-cysteine, yielding (1.3) L-cystathionine and (1.7) succinate,
d) cystathionine β-lyase (CBL), the second enzyme of the bacterial transsulfuration pathway, hydrolyzes (1.3) L-cystathionine to produce (1.1) L-homocysteine, (1.4) α-ketobutyrate and ammonia,
e) methionine γ-lyase hydrolyzes (1.8) L-methionine to yield (1.4) α-ketobutyrate , (1.9) methathiol and ammonia

L-Cysteine is essential for growth and for tolerance to reactive oxygen species in *T. vaginalis* (Bruchhaus, Richter et al. 1998, Coombs, Westrop et al. 2004, Vicente, Ehrenkaufer et al. 2009). The absence of a transsulfuration pathway in this species could be expected to result in an accumulation of L-homocysteine and consequently interfere with cellular methylation reactions due to an imbalance in the L-methionine-to-L-homocysteine ratios. Analysis of the parasite's genome suggests that L-homocysteine cannot be converted to L-methionine because the organism lacks methionine synthase genes (Anderson and Loftus 2005, Loftus, Anderson et al. 2005, Carlton, Hirt et al. 2007).

The hydrolysis of L-homocysteine by MGL prevents its accumulation in the cell (Lockwood and Coombs 1991). This reaction also produces H_2S, which is incorporated in the *de novo* biosynthesis of L-cysteine in this species, thereby providing an alternative to the transsulfuration pathway for the transfer of the thiol group of L-homocysteine (Sato, Yamagata et al. 2008). The α-ketobutyrate product, generated by MGL, may also serve as an energy source for the organism (Samarawickrema, Brown et al. 1997).

1.5 Pyridoxal 5'-phosphate dependent enzymes

Typical of enzymes catalyzing transformations of amino acid substrates, TvMGL and the enzymes of the bacterial, plant, yeast and mammalian transsuluration pathways are dependent on the pyridoxal 5'-phosphate cofactor, a derivative of pyridoxine (vitamin B_6). The PLP cofactor is covalently bound, *via* a Schiff base linkage, with the ε-amino group of an active-site lysine residue, which is replaced by the α-amino group of the

amino acid substrate in the first step of all PLP-catalyzed reactions (Christen, Kasper et al. 1996). The carbanion resulting from the subsequent cleavage of one of the Cα bonds is delocalized and stabilized by the pyridinium ring of the cofactor (Schneider, Käck et al. 2000). The catalytic versatility of PLP, which catalyzes an array of transformations of amino acids (e.g. transamination, side-chain rearrangement, decarboxylation, racemisation) is regulated by the protein component of the enzyme, which imposes substrate and reaction specificity. Therefore, although TvMGL and yCGL both catalyze α,γ-elimination reactions, they are specific for the substrates L-methionine and L-cystathionine, respectively. PLP-dependent enzymes can be classified into five structural families based on their overall structure and the conformation of their active site (Schneider, Käck et al. 2000). The largest of these groups is classified known as fold type I. This family consists of aminotrasferases, decarboxylases as well as enzymes that catalyze α,β- or α,γ-elimination reactions, including TvMGL and, with the exception of CBS, the enzymes of transsulfuration pathways. A characteristic of fold type I enzymes is the location of the active site at the interface between the subunits comprising the catalytic dimer. Each subunit of the enzyme is composed of a large and small domain. The active site is situated at the interface of these two domains and includes residues from the adjacent monomer (Figure 1.3).

The active sites of TvMGL and the enzymes of the transsulfuration pathways share several common features and conserved amino acids (Clausen, Huber et al. 1996, Clausen, Huber et al. 1997, Aitken, Kim et al. 2003, Farsi, Lodha et al. 2009, Lodha, Jaworski et al. 2010). The active sites of *E. coli* CGS and CBL have been thoroughly

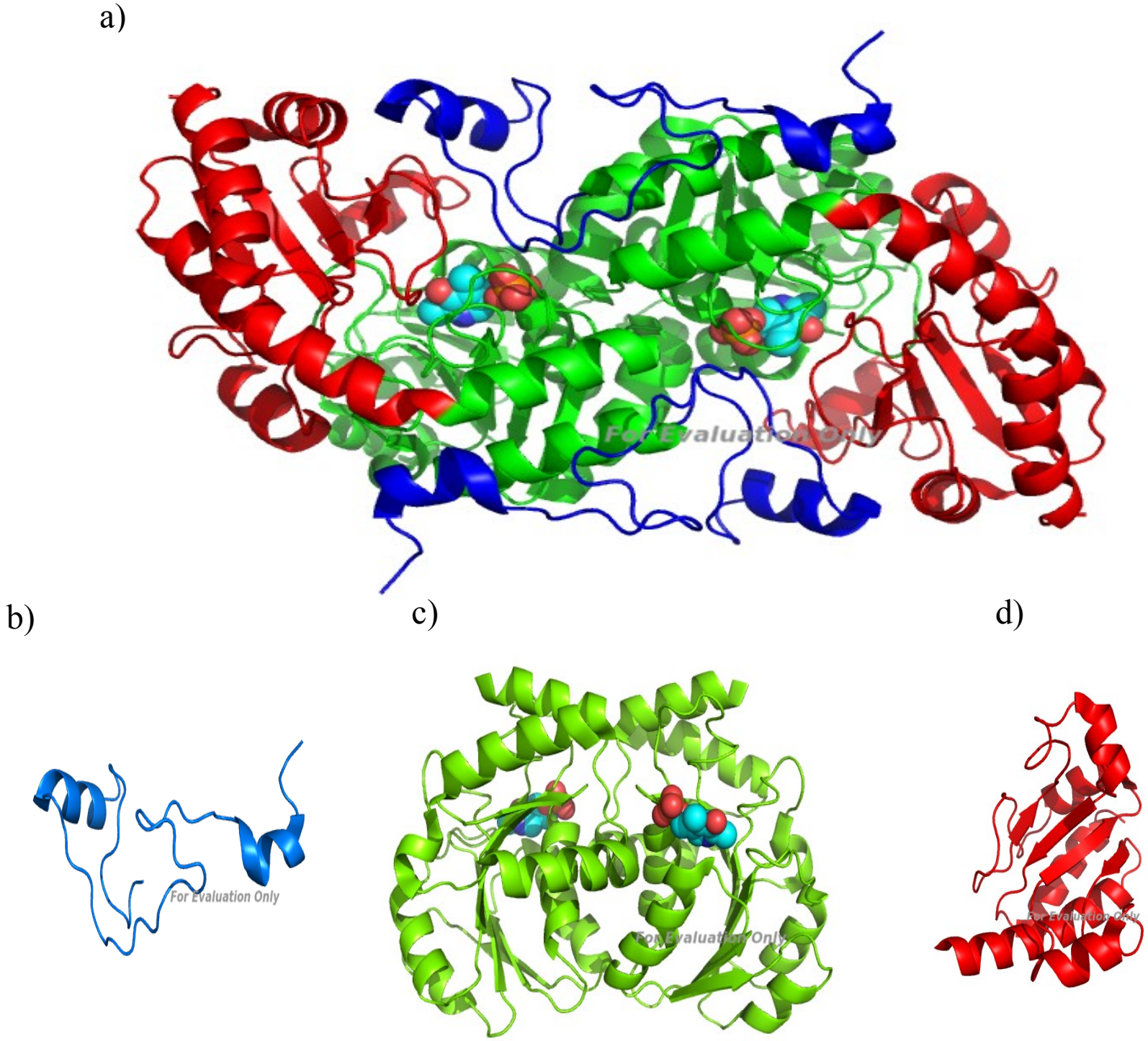


Figure 1.3. The structure of TvMGL1(PDB 1E5F) is representative of those of fold-type I of PLP-dependent enzymes. The protein backbone is shown in cartoon and the PLP cofactor in van der Waals surface representations, respectively. (a) The TvMGL1 catalytic dimer, (b) the N-terminal domain (residues 14-60), (c) the central PLP-binding domain (residues 61-258), (d) the C-terminal domain (residues 259-396).The images was rendered using the PyMOL Molecular Graphics System, Version 1.5.5.0.5 Scrodinger, LLC.

characterized and these enzymes provide a useful context for comparison of the role of active-site residues as determinants of substrate and reaction specificity in TvMGL, information that will be required for the development of MGL-specific derivatives of TFM (Farsi, Lodha et al. 2009, Lodha, Jaworski et al. 2010, Lodha and Aitken 2011). For example, arginine residues R58 and R372 of eCBL binds the distal and proximal (with respect to the cofactor) carboxylate groups of the L-cystathionine substrate (Lodha, Jaworski et al. 2010). A common role is anticipated for TvMGL-R373 and eCBL-R372, as the L-methionine and L-cystathionine substrates both possess a α-carboxylate group. However, as L-methionine does not possess a side-chain carboxylate, unlike the L-cystathionine residue R58 of TvMGL is not expected to play a direct role in substrate binding.

1.6 Methionine γ-lyase

Methionie γ-lyase (MGL) (E.C. 4.4.1.11) catalyzes the physiological hydrolysis of L-methionine and homocysteine, *via* a α,γ-elimimation mechanism, as well as the α,β-elimination of L-cysteine (Tanaka, Esaki et al. 1985, Thong, Coombs et al. 1987, McKie, Edlind et al. 1998). MGL enzymes have been characterized from bacterial (*Pseudonomas putida* and *Citrobacter freundii*), plant (*Arabidopsis thaliana*) and protist (*Enatamoeba histolica and Trichomonas vaginalis*) sources (McKie, Edlind et al. 1998, Goyer, Collakova et al. 2007, Kudou, Misaki et al. 2008, Sato, Yamagata et al. 2008, Fukumoto, Kudou et al. 2012).

Structures are available for the MGL enzymes from *P. putida*, *T. vaginalis* and *C. freundii* and all share a common homotetrameric subunit arrangement of a pair of catalytic dimers (McKie, Edlind et al. 1998, Sato, Yamagata et al. 2006, Kudou, Misaki et al. 2007). Each catalytic dimer possesses a pair of active sites, located at the subunit interface (Clausen, Huber et al. 1996, Sato, Yamagata et al. 2006, Kudou, Misaki et al. 2007).

Each subunit consists of three different domains: the N-terminal domain, the PLP-binding domain and the C-terminal domain. The N-terminal domain (residues 1-63) is composed of two α-helices which connect a long loop structure containing 25 residues, which forming part of the active site of the neighboring subunit of the catalytic dimer (Figure 1.3) (Motoshima, Inagaki et al. 2000, Kudou, Misaki et al. 2007, Morozova, Bazhulina et al. 2010). The PLP-binding domain (residues 64-259) is composed of the seven-stranded, mainly parallel, β-sheet characteristic of fold-type I PLP-dependent enzymes (Kudou, Misaki et al. 2007, Morozova, Bazhulina et al. 2010). The C-terminal domain (residues 260-398) consists of a five stranded β-sheet and five α-helixes (Motoshima, Inagaki et al. 2000, Kudou, Misaki et al. 2007). The active site is situated at the interface between the PLP-binding and C-terminal domains.

1.7 Objectives

The extensive use of Mz-based drugs and lack of alternative therapeutics for treating human protozoan infections have led to the development of drug resistance in *T. vaginalis*. Trifluoromethionine, a prodrug activated by the enzyme MGL, has been proposed as an alternative for the treatment of human protozoan infections. The goals of this study are to:

1. develop an affinity purification system for TvMGL,
2. further characterize the substrate specificity of the wild-type TvMGL enzyme and
3. Investigate the role of the following active-site residues I55A,Y56F, R58A, Y111F, D239A, S338A, L339A, R373A, R373K, which correspond to residues shown to be involved in substrate binding in the closely related enzymes yCGL, eCBL and eCGS.

This knowledge will contribute to the development of variants of the TFM prodrug, for the treatment of trichomoniasis, with high specificity for activation by TvMGL nut not similar enzymes in humans.

2. Methods

2.1 Reagents

L-Methionine and L-cysteine were purchased from Fisher Scientific. DL-Homocysteine and β-nicotinamide adenine dinucleotide (β-NADH, reduced form) were Sigma-Aldrich products. The Ni-nitrilotriacetic acid (Ni-NTA) chromatography resin was obtained from Qiagen. The hydroxyisocaproate dehydrogenase (HicDH) coupling enzyme was purified as previously described by Aitken, Kim et al. (2003). A plasmid containing the TvMGL1 coding sequence (pQE60-TvMGL1) was obtained from Dr. G. Coombs (University of Strathclyde). The oligonucleotide primers were synthesized by Integrated DNA Technologies and all constructs were sequenced by BioBasic.

2.2 Preparation of the TvMGL expression construct and site-directed mutants

Bacterial cells containing the pQE60-TvMGL1 plasmid were streaked on Luria-Bertani (LB) broth containing 1.5% agar and 100 μg/mL ampicillin and a colony was selected to grow in a 10-mL liquid culture for isolation of the plasmid. The plasmid was purified using the Wizard Plus SV miniprep DNA purification kit (Promega) and sequenced using the primers pSEC60seqF, pSEC60seqR, TvMGLseq1, TvMGLseq2, TvMGLseq3, TvMGLseq4 and TvMGLseq4r (Table 2.1). The sequences were assembled using CAP3 (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::cap3) and found to be identical to that reported for TvMGL (Genbank accession number XM_001309487).

The TvMGL coding sequence was amplified from the vector pQE60 using the forward primer TvMGL1-Fc and the reverse primer TvMGL1-Rc, which incorporate *NdeI* and *PstI* restriction sites, respectively, to facilitate insertion into the pTrc99-AF

expression vector (Farsi, Lodha et al. 2009). This vector which encodes a 6-His affinity tag thereby resulting in an N-terminally His-tagged TvMGL. The resulting plasmid was used to transform cells of *Escherichia coli* strain ER1821, *via* the heat shock method (Farsi, Lodha et al. 2009). The presence of the pTrc99AF-TvMGL plasmid in the cells was verified by colony PCR.

Table 2.1. Primers used in the construction of the site-directed mutants of *Trichomonas vaginalis* methionine γ-lyase (TvMGL1)

Primer	Sequence
I55A[a]	5'-gaatccggctacgcctacacacgtctcg
Y56F[a]	5'-tccggctacatcttcacacgtctcggc
R58A[a]	5'-ctacatctacacagctctcggcaacccaac
R58K[a]	5'-ctacatctacacaaaactcggcaacccaac
Y111F[a]	5'-gatgagtgccttttcggctgcacacatgc
D239A[a]	5'-gttggtatcaaggcgatcacaggatctg
S338A[a]	5'-gttggtatcaaggcgatcacaggatctg
L339A[a]	5'-cttgcagtttccgctggtggctgcgag
R373A[a]	5'-gatggcatgatcgcgctttctgtcgg
R373K[a]	5'-cagatggcatgatcaaactttctgtcgg
pSEQ60seqF	5'-cgaaaagtgccacctgacgtc
pSEQ60seqR	5'-cgccaagctagcttggattctcacc
TvMGL1seq1	5'-acgtctcggcaacccaacagtttcaa
TvMGL1seq2	5'-agtgcctttatggctgcacacatgct
TvMGL1seq3	5'-ttggcgtcgatgttgttgtccactct
TvMGL1seq4	5'-acacttgcagtttcccttggtggct
TvMGL1seq4r	5'-agccaccaagggaaactgcaagtgt
TvMGL1-Rc	5'-tgacctgcagttataaaagagcgtcaaggcc
TvMGL1-Fc	5'-gtcacatatgtctcacgagagaatgacc
pSECseq0	5'-ggcgtcaggcagccatcggaagctg
pSECseq7r	5'-gcccgccaccctccgggccgttgcttcgc

[a]Second mutagenic primers are the reverse complement of the sequenced listed

2.3 Purification of the recombinant, 6-His tagged TvMGL1

The expression and purification of the recombinant wild-type TvMGL1 protein and the site-directed variants started with inoculation of 100 mL of LB media, containing 100 µg/mL of ampicillin, with the *E. coli* ER1821 cells containing the pTrc99AF-TvMGL construct and grown overnight at 200 rpm and 37 °C. A 25-mL aliquot of the overnight culture was used to inoculate each of six 2.8-L baffled Fernbach flasks, containing 1 L of LB media and 100 µg/mL ampicillin in each. The cultures were grown at 200 rpm and 37 ºC until the optical density at 600 nm (OD_{600}) reached 0.60, induced with a final concentration of 0.18 mM IPTG and incubated for a further 16 hours at 200 rpm and 30ºC. The cells were then harvested by centrifugation at 5,000 rpm and 4 ºC for 10 min (Sorvall RC 6+ centrifuge, Thermo Scientific). The pellet was resuspended in 0.85% NaCl, transferred to 50-mL Falcon tubes and centrifuged for 10 min at 5,000 rpm. The supernantant was discarded and the pellet was resuspended in buffer A (50 mM sodium phosphate, pH 7.8, 300 mM NaCl, 20 mM Imidazole, 20 µM PLP) containing 1 mg/mL lysosyme solution (1mg/ml) and incubated at room temperature for 30 minutes before sonication, on ice, for eight 30-s intervals. The sonicated cells were centrifuged at the at 15,000 rpm and 4 ºC for 45 minutes and again at 18,000 rpm and 4ºC for 35 minutes (Sorvall RC 6+ centrifuge, Thermo Scientific). The resulting supernatant was loaded on a chromatography column (1 x 10cm) containing 3 mL of Ni-NTA resin, equilibrated with buffer A. The column was washed with 20 column volumes buffer B (50 mM sodium phosphate, pH 7.8, 300 mM NaCl, 10% v/v glycerol, 40 mM imidazole, 20 µM PLP) and the TvMGL protein eluted with a linear gradient of 40-250 mM

imidazole in buffer B. The fractions were assessed by SDS-PAGE and those containing >90% pure protein, by visual inspection, were pooled, concentrated and stored at -80 °C.

2.4 SDS-Polyacrylamide gel electrophoresis

Samples were collected throughout the protein purification process, including supernatant, column wash and eluted fractions, and 15 µl of protein sample were mixed with 15 µl of 1X protein loading buffer (50 mM Tric-HCl pH 6.8, 1% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol]), and heated at 95ºC for 3 minutes. The samples were loaded onto an SDS polyacrylamide gel and run at 140 V for 90 minutes. The resolving gel was composed of 14% (w/v) acrylamide, 380 mM Tris-base pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) ammoniumpersuflate, and 0.04% (v/v) tetramethylethylenediamine (TEMED). The stacking gel was composed of 5% (w/v) acrylamide, 130 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persuflate and 0.1% (v/v) TEMED. The protein bands were visualized by staining the gel with Gel Code Blue. The gels were recorded with Alpha imaginer model 2200.

2.5 Determination of kinetic parameters

Enzyme activity was measured in a volume of 1.5 mL at 25ºC and detected using an Agilent 8453 UV-visible spectrophotometer using a quartz cuvette with continuous stirring at 300 rpm. The assay buffers were comprised of 50 mM potassium phosphate, pH 6.5 or 6.8, and 50 mM HEPES, pH 8.0, containing 20 µm PLP. The γ-elimination of L-methionine and L-homocysteine by TvMGL was measured using the HicDH assay in

which reduction of the α-ketobutyrate product is monitored as the concomitant oxidation of NADH monitored at 340 nm (ε_{340}=6200 M^{-1} cm^{-1}) (Aitken, Kim et al. 2003). The coupling enzyme LDH was employed in place of HicDH for the β-elimination of L-cysteine by TvMGL as the catalytic efficiency of LDH and HicDH are highest for pyruvate and α-ketobutyrate, respectively (Aitken, Kim et al. 2003). A background reading was recorded prior to initiation of the reaction by the addition of TvMGL. Data were fit using the Michaelis-Menten equation (1) to obtain the values for K_m and k_{cat}. The catalytic efficiency (k_{cat}/K_m) was obtained independently using equation (2). Data were fit by nonlinear regression with Kaleidagraph 4.0 (Synergy Software).

$$\frac{v}{[E]} = \frac{Kcat\ X\ [S]}{Km+[S]} \text{ (eq.1)}$$

$$\frac{v}{[E]} = \frac{\frac{Kcat}{Km}\ x\ [S]}{1+[S]/Km} \text{ (eq.2)}$$

2.6 Measuring pH dependence of TvMGL

The pH dependence of the specific activity of L-methionine hydrolysis by TvMGL was determined using the continuous HicDH assay. Activity was measured between pH 5.5-9.0 in a three component buffer comprising 50 mM citrate (pK_a= 4.7), 50 mM phosphate (pK_a= 7.2) and 50 mM bicine (pK_a= 8.3) with 20 μM PLP, 5 mM L-methionine, 250 μM NADH and 34.5 μM HicDH. Each reaction was initiated by the addition of 100 μL of 1.1 μM of TvMGL enzyme were fit to the bell shape curve defined

by equation 3, in which k_{cat}/K_m^{max} is the upper limit for k_{cat}/K_m at the pH optimum (Aitken, Kim et al. 2003).

$$\frac{Kcat}{Km} = \frac{\frac{Kcat}{Km^{max}}}{1+10^{pKa1-pH}+10^{pH-pKa2}} \quad \text{(eq.3)}$$

3. Results

3.1 Constructing TvMGL1 site-directed mutants

The TvMGL1 coding sequence in the pQE60 plasmid was verified by sequencing and is identical to that reported for TvMGL (Genbank accession number XM_001309487) (Carlton, Hirt et al. 2007). The TvMGL1 coding sequence was amplified with the TvMGL1f-*NdeI* and TvMGL1r-*PstI* primers, introducing *Nde*I and *Pst*I restriction sites at the start codon and immediately 3' of the stop codon, and inserted between these sites of the pTrc99AF expression plasmid, which encodes an N-terminal 6-His_6 affinity tag (Farsi, Lodha et al. 2009). Overlap extension PCR was employed to generate site-directed mutants of the TvMGL1 coding sequence. The amplicons of the 5' to 3' segments, which overlap by the mutagenic primer sequence, produced in the first round of PCR are shown in Figure 3.1. The reassembled amplicons (Figure 3.2) were inserted between the NdeI/PstI sites of the pTrc99AF vector and used to transform the *E. coli* competent cell line ER1821. The pTrc99AF-TvMGL construct, and site-directed variants derived from it, were sequenced to verify that no mutations had been introduced during amplification.

3.2 Purification of TvMGL1 and active-site variants via affinity chromatography

The wild-type TvMGL1 and a series of nine active-site variants (S338A, L339A, R373A, Y111F, D239A, I55A, Y56F and R58A) were expressed and purified using Ni-NTA affinity chromatography. The expression system from pTrc3 vector is to generate a six histidine residues tag to the *N*-terminal of the expressed protein. An example of the

SDS-PAGE gels of the Ni-NTA purification process of TvMGL1-L339A is shown in (Figure 3.3).

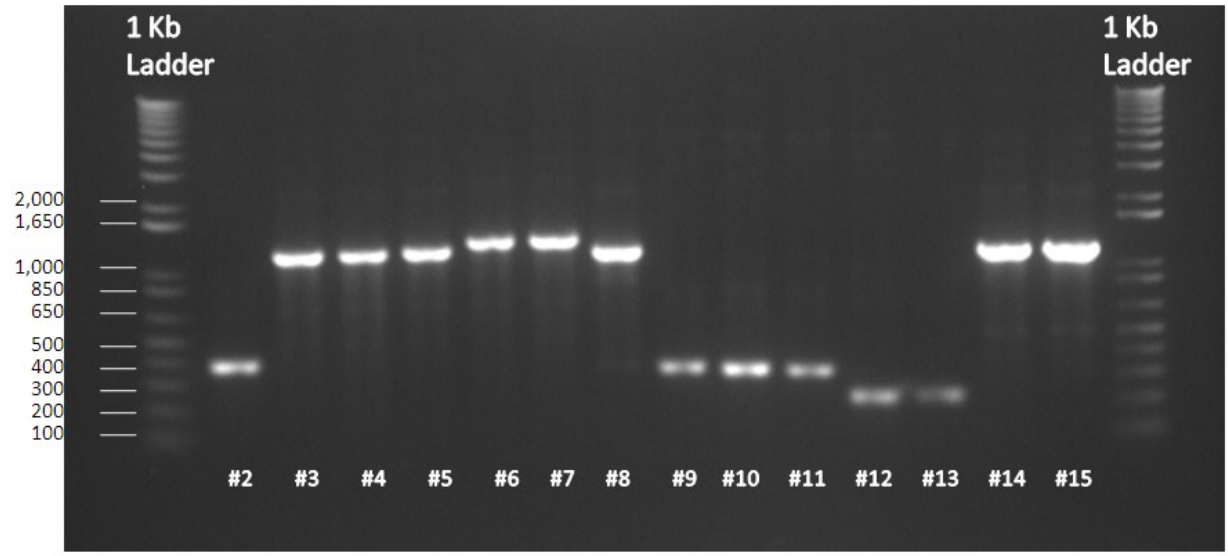


Figure 3.1 Agarose (1%) gel electrophoresis the 5' and 3' amplicons produced by overlap-extension PCR: L59A (lane 2 -5' segment and lane 8 -3'), V337A (lane 3 -5'segment and lane 9 -3'), S338A (lane 4 -5' segment and lane 10 -3'), L339A (lane 5 5-' segment and lane 11 -3'), R373A (lane 6 -5' segment and lane 12 -3'), R373K (lane 7 -5' segment and lane 13 -3'). Lane #14 and #15 are the products of amplification of the TvMGL1 coding sequence with the TvMGL1-*NdeI* and TvMGL1-*PstI* primers.

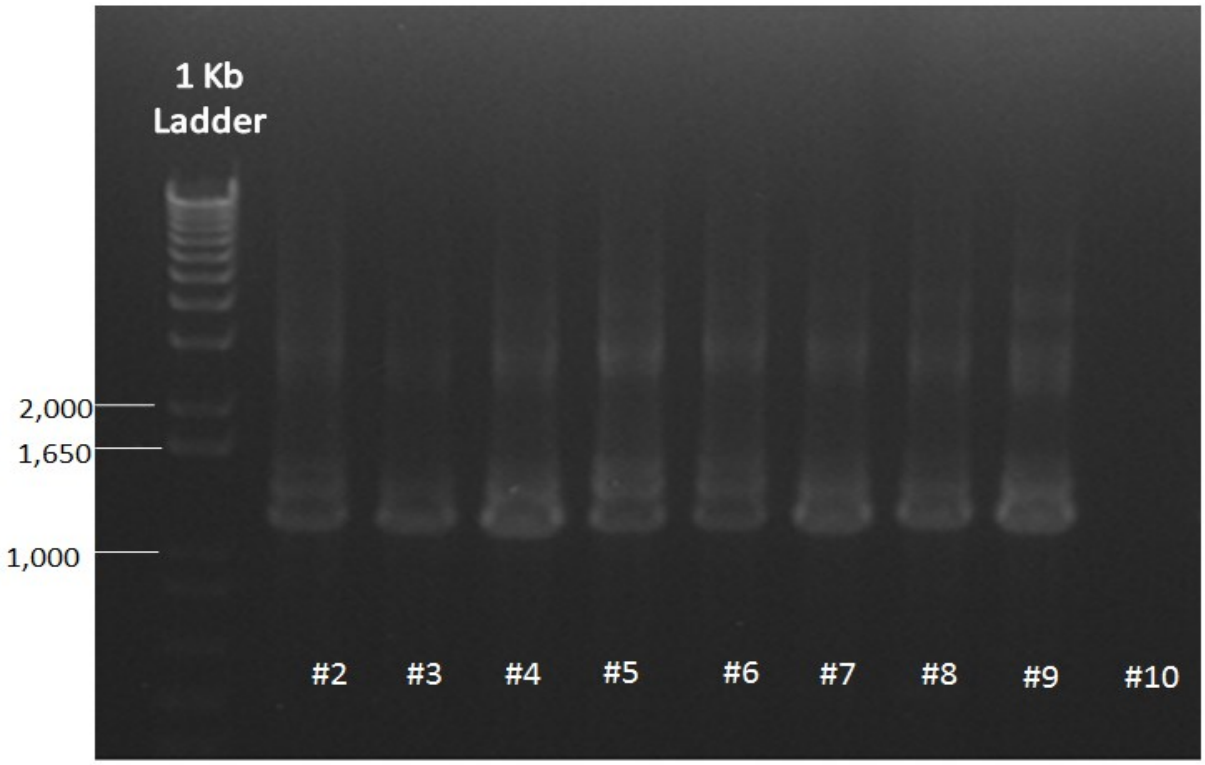


Figure 3.2 Digital image of reassembly PCR on 1% w/v agarose stained with ethidium bromide. Lanes #2 to #9 represent the mutants L59A, V337A, S338A, L339A, R373A, R373K, Y111F and D239A. Lane 10 contained the negative control reaction.

3.3 A continuous assay for determining kinetic parameters of TvMGL

TvMGL1 catalyzes the hydrolysis of L-methionine to yield methanethiol (CH_3-SH), α-ketobutyrate and ammonia (NH_3^+). A continuous assay for TvMGL was developed which employs the coupling enzyme hydroxyisocaproate dehydrogenase (HicDH) to reduce the α-ketobutyrate product of MGL to 2-hdroxybutyrate, with the concomitant oxidation of NADH to NAD^+ (ε_{340}= 6200 $M^{-1}cm^{-1}$). This assay is based on the coupled assay described by Aitken and colleagues for the hydrolysis of *O*-succinylhomeserine by *E. coli* cystathionine γ-synthase, which also follows an α, γ – elimination mechanism to produce α-ketobutyrate (Aitken, Kim et al. 2003).

The concentrations of HicDH and TvMGL1 enzymes in the continuous assay were optimized to ensure rates measured were not limited by insufficient coupling enzyme in the assay (Fig. 3.4 and 3.5). TvMGL1 also catalyzes the hydrolysis of L-homocysteine to yield free thiol (SH), α-ketobutyrate and ammonia (NH_3^+), allowing the same continuous assay to be employed for both substrates. The enzyme concentrations for the assay were optimized for each substrate at pH 6.5, 6.8 and 8.0 (Figures 3.4 and 3.5).

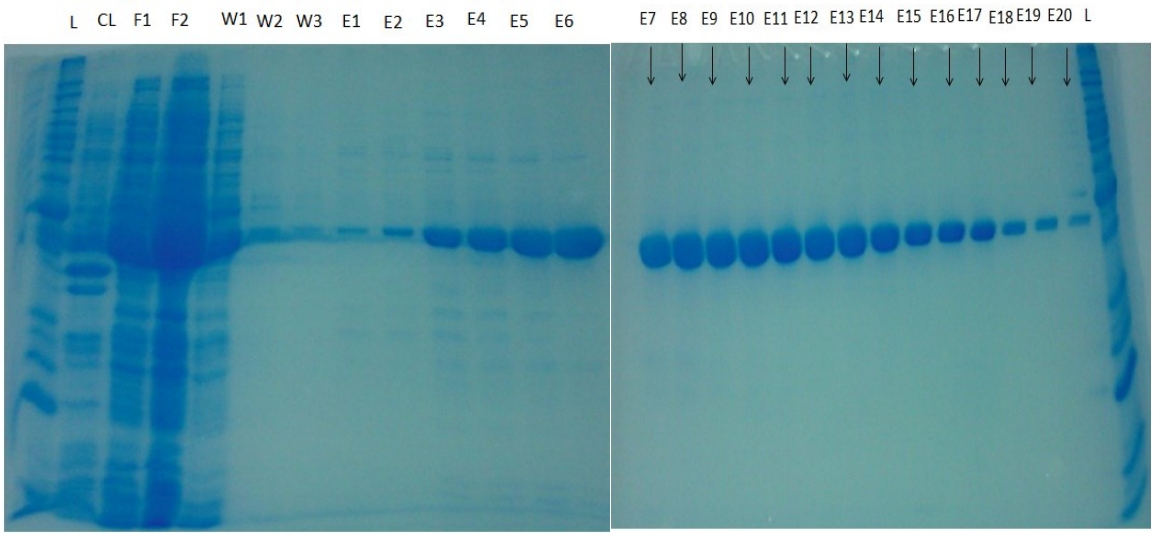


Figure 3.3 SDS-PAGE gel of the fractions from the purification of the TvMGL1-L339A variant. Lanes: L – the protein ladder; CL – cell lysate; F1 – flow-through following loading of the cell lysate on the column (10X dilution); F2 – flow-through loading the cell lysate on the column (without dilution); W1 – collected after 50 mL of the buffer B was run through the column; W2 – collected after a second 50 mL of buffer B; W3 – collected after a third 50 mL volumes of buffer B; E1-E20 are fractions – collected during elution with 40-250 mM linear gradient imidazole in buffer B.

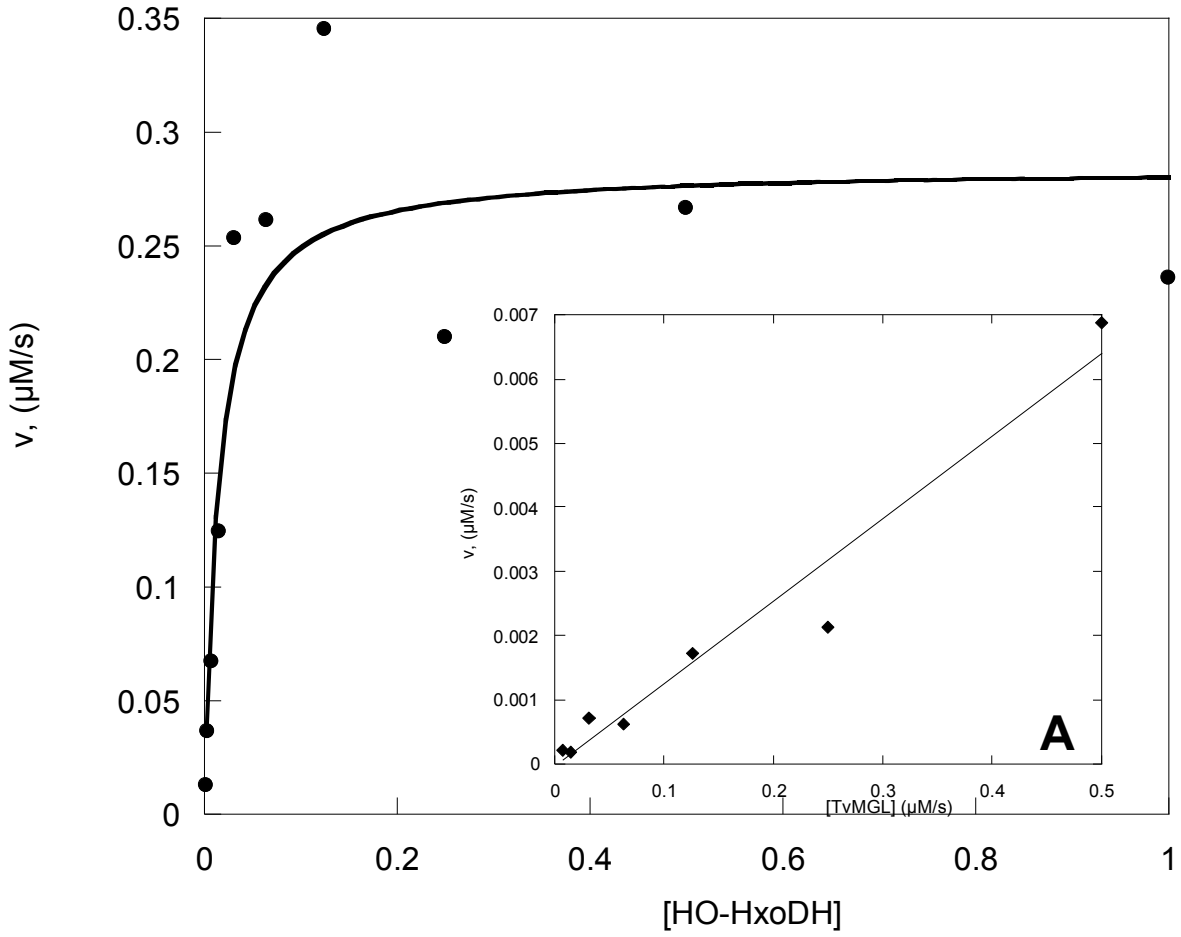


Figure 3.4 Optimization of enzyme concentrations for the continuous TvMGL1 assay for L-Met hydrolysis. Dependence of the rate of NADH oxidation on (inset A) TvMGL and (main panel) HicDH concentration in the coupled, HicDH-based assay for the hydrolysis of L-Met by TvMGL1. Reactions were carried out in a volume of 1.5 mL at 25 ºC and monitored at 340 nm for 500 s at 5 s intervals. Conditions: 50 mM phosphate buffer, pH 6.5, 20 µM PLP, 250 µM NADH and 7 mM of L-Met with (inset A) 34.5 µM HicDH and 0.27-4.37 µM TvMGL or (main panel) 1.1 µM TvMGL and 0.058 – 7.44 µM HicDH.

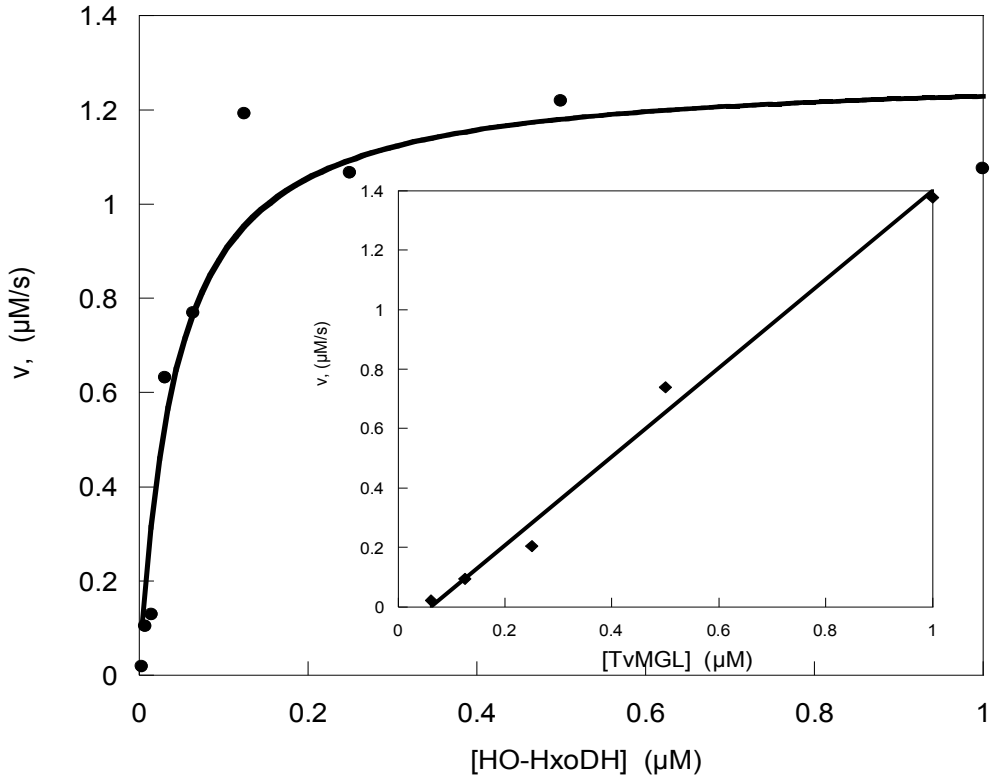


Figure 3.5 Optimization of enzyme concentrations for the continuous TvMGL1 assay for L-Hcys hydrolysis. Dependence of the rate of NADH oxidation on (inset A) TvMGL and (main panel) HicDH concentration in the coupled, HicDH-based assay for the hydrolysis of L-Hcys by TvMGL. Reactions were carried out in a volume of 1.5 mL at 25 ºC and monitored at 340 nm for 500 s at 5 s intervals. Conditions: 50 mM phosphate buffer, pH 6.5, 20 µM PLP, 250 µM NADH and 7 mM of L-Hcys with (inset A) 34.5 µM of HicDH and 0.27-4.37 µM of TvMGL or (main panel) 1.1 µM TvMGL and 0.058 – 7.44 µM HicDH.

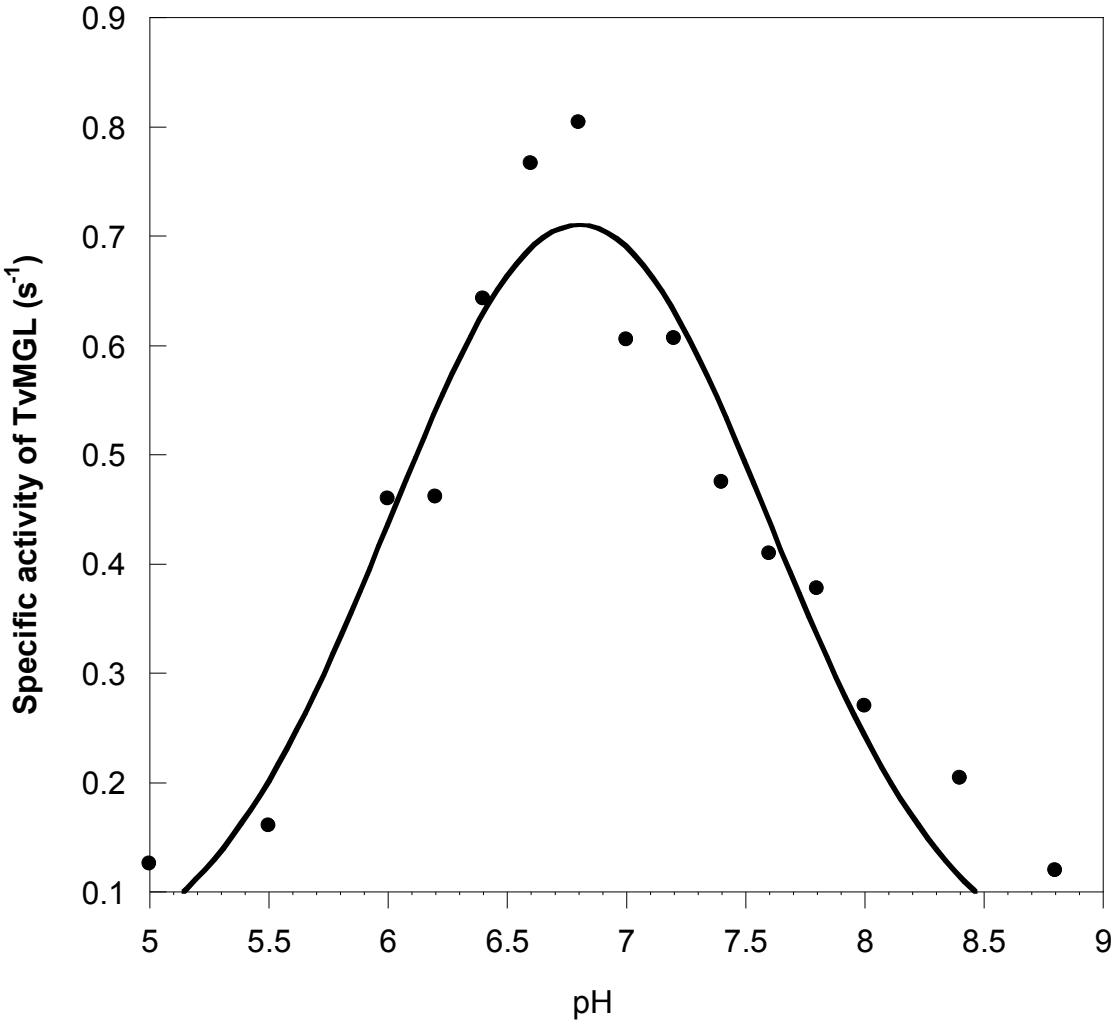


Figure 3.6 pH dependence of the specific activity of TvMGL for the hydrolysis of L-Met

Reactions were carried out in 1.5 mLvolume at 25 ºC, and the rates were monitored at 340 nm for 500 s at 5-s intervals. Conditions: 50 mM three component buffer (citrate pK_a =4.7, phosphate pK_a=7.2, bisine pK_a=8.3), 250 µM NADH, 20 µM PLP, 5 mM L-Met and 4.5 µM HicDH.

The $K_m^{L\text{-}Met}$ (0.27 ± 0.07 mM) for the hydrolysis of L-methionine by TvMGL1 is within ~2-fold of the values of 0.65 mM and 0.20 mM reported by the McKie et al. (1998) and Moya et al. (2011), respectively. However, measured k_{cat} for L-methionine (0.47 ± 0.04 s^{-1}) at pH 6.5 is 16-fold lower than that the reported value of 7.6 s^{-1} (McKie, Edlind et al. 1998). The measured k_{cat} for L-methionine is reduced 2-fold at pH 8.0 (0.23 ± 0.01 s^{-1}) compared to pH 6.5 (0.47 ± 0.04 s^{-1}), indicating that the affinity tagged TvMGL1 enzyme also has a low pH optimum (Table 3.1). The effect of pH on the specific activity of L-methionine hydrolysis by TvMGL1 was determined to be between 6.5-6.8 (Fig. 3.6). This finding is in agreement with the reported pH optimum of 6.6 (Faleev, Alferov et al. 2009).

The catalytic efficiency of TvMGL at pH 6.8 for L-homocysteine ((9 ± 2) x 10^3 $M^{-1}s^{-1}$)) is 14-fold greater than that of L-methionine ((6.6 ± 1.0) x 10^2 $M^{-1}s^{-1}$)). This preference for L-homocysteine is not unique toTvMGL1 as MGL from other species, such as *Pseudonomas putida*, *Citrobacter freundii*, *Entamoeba histolitica* also demonstrate a greater catalytic efficiency for this substrate (McKie, Edlind et al. 1998, Inoue, Inagaki et al. 2000, Sato, Yamagata et al. 2008). In contrast, the catalytic efficiency of TvMGL for the α,γ-elimination substrate, L-methionine is 6-fold greater than that of the α,β-elimination substrate L-cysteine.

Table 3.1 Kinetic parameters of wild-type TvMGL for L-methionine, L-homocysteine and L-cysteine

Substrate	pH	k_{cat} (s^{-1})[a]	K_m (mM)[a]	k_{cat}/K_m ($M^{-1}s^{-1}$)[b]
L-methionine → α-ketobutyrate + CH_3SH + NH_3^+				
Methionine	6.5	0.47 ± 0.04	0.27 ± 0.07	(2.2 ± 0.4) x 10^3
	6.8	0.51 ± 0.04	0.90 ± 0.07	(6.6 ± 1.0) x 10^2
	8.0	0.23 ± 0.01	0.30 ± 0.06	(8.0 ± 1.0) x10^2
L-homocysteine → α-ketobutyrate + SH + NH_3^+				
Homocysteine	6.5	2.74 ± 0.2	0.34 ± 0.09	(8 ± 2.) x 10^3
	6.8	3.7 ± 0.3	0.4 ± 0.14	(9. ± 2.) x 10^3
	8.0	1.10 ± 0.08	0.28 ± 0.08	(4 ± 1) x 10^3
L-cysteine → pyruvate + SH + NH_3^+				
Cysteine	6.5	0.3 ± 0.24	0.29 ± 0.09	(1.1 ± 0.3) x 10^3
	6.8	0.36 ± 0.05	3 ± 1	(1.1 ± 0.3) x 10^2
	8.0	0.140 ± 0.006	0.19 ± 0.03	(8 ± 1) x 10^2

*Kinetic measurements were carried out in 50 mM phosphate buffer (pH 6.5 or 6.8) or 50 mM HEPES buffer (pH 8.0) containing 20 μM PLP, 0.25 mM NADH, 34.5 μM HicDH, 4.3 μM TvMGL1 and a range of 0.05-7.0 mM of L-Met, L-Hcys and L-Cys at 25 ºC.
[a]Data were fitted to Eq. (1)
[b]Data were fitted to Eq. (2)

3.4 The Y56F, R58A, Y111F, S338A, R373A and R373K variants

No enzyme activity was observed for the Y56F, R58A, Y111F, S338A, R373A and R373K variants of TvMGL for the hydrolysis of L-Met or L-Hcys. Similarly, a 900-fold decrease in activity was reported by Inoue et al. (2000) and Fukomoto et al. (2012) for the Y111F variant of MGL of *P. putida* and the R58A and R373A variants of eCBL are inactive (Lodha, Jaworski et al. 2010).

3.5 The I55A, D239A and L339A variants

The k_{cat} values for L-Met and L-Hcys hydrolysis are decreased 14- and 11-fold, respectively, by the I55A substitution (Table 3.2 and Table 3.3). Similarly, replacement of D239 with alanine decreases the k_{cat} value for both L-Met and L-Hcys hydrolysis by ~10 fold. The catalytic efficiency of the L339A variant is within 3-fold of the wild-type for both substrate (Tables 3.2 and 3.3).

Table 3.2 Kinetic parameters for the hydrolysis of L-Met by TvMGL and site-directed variants

Enzyme	k_{cat} (s^{-1})[a]	K_m (mM)[a]	k_{cat}/K_m ($M^{-1}s^{-1}$)[b]
L-methionine → α-ketobutyrate + CH_3SH + NH_3^+			
TvMGL (wt)	0.51 ± 0.06	0.9 ± 0.3	(6 ± 1) x 10^2
I55A	0.037 ± 0.002	0.7 ± 0.16	50 ± 9
Y56F	n.a.	n.a.	n.a.
R58A	n.a.	n.a.	n.a.
Y111F	n.a.	n.a.	n.a.
D239A	0.052 ± 0.003	0.9 ± 0.14	39 ± 7
S338A	n.a.	n.a.	n.a.
L339A	0.26 ± 0.03	0.6 ± 0.2	(4 ± 1) x 10^2
R373A	n.a.	n.a	n.a.
R373K	n.a.	n.a.	n.a.

n.a. no activity detected

*Kinetic measurements were carried out in 50 mM Phosphate buffer pH 6.8, containing 20 μM PLP, 02.5 mM NADH, 4.3 μM HicDH, 4.3 μM TvMGL1 and a 0.05-7.0 mM of L-Met at 25 ºC.

[a]Data were fitted to Eq. (1)

[b]Data were fitted to Eq. (2)

Table 3.3 Kinetic parameters for the hydrolysis of L-Hcys by TvMGL and site-directed variants

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
L-homocysteine → α-ketobutyrate + ^{-}SH + NH_3^{+}			
TvMGL (wt)	3.7 ± 0.3	0.4 ± 0.14	(9 ± 2) x 10^3
I55A	0.34 ± 0.04	0.7 ± 0.2	(5 ± 1) x 10^2
Y56F	n.a.	n.a.	n.a.
R58A	n.a.	n.a.	n.a.
Y111F	n.a.	n.a.	n.a.
D239A	0.68 ± 0.09	0.7 ± 0.34	(1.0 ± 0.4) x 10^3
S338A	n.a.	n.a.	n.a.
L339A	2.1 ± 0.2	0.84 ± 0.3	(2.45 ± 0.7) x 10^3
R373A	n.a.	n.a.	n.a.
R373K	n.a.	n.a.	n.a.

n.a. no activity detected
*Kinetic measurements were carried out in 50 mM Phosphate buffer pH 6.8 containing 20 μM PLP, 0.25 mM NADH, 4.3 μM HicDH, 4.3 μM TvMGL1 and a range of 0.05-7.0 mM of L-Hcys at 25 ºC.
[a]Data were fitted to Eq. (1)
[b]Data were fitted to Eq. (2)

4. Discussion

The extensive use of metronidazole-based drugs for the treatment of human protozoan infections has led to the development of drug resistance. TFM, a prodrug activated by MGL, has been proposed as an alternative for the development of a novel class of therapeutics for the treatment of trichomoniasis, a STI caused by *Trichomonas vaginalis*. Specificity of TFM-derivatives for TvMGL is essential in order to ensure they are activated by this enzyme within the parasite and not activates by the closely related PLP-dependent enzymes of the human host cells.

Refinement of TFM, by development of derivatives, is required to enhance the TvMGL specificity of this potential therapeutic. Therefore, characterization of the TvMGL active site is a necessary step in the development of safe and effective TFM-based it will enable the design of TFM derivatives optimized for binding to this enzyme. The closely related PLP-dependent enzymes of the bacterial, yeast and human transsulfuration pathways (CGS, CBL and CGL) have been thoroughly characterized and provide the context for comparison of the results of this study probing the active site of TvMGL (Aitken, Kim et al. 2003, Farsi, Lodha et al. 2009, Lodha and Aitken 2011, Jaworski, Lodha et al. 2012).

The focus of this study is the investigation of conservative substitutions of nine TvMGL1 active sites residues on the steady state kinetic parameters of this enzyme. The results will guide future experiments probing the binding contacts in the TvMGL active site, with the goal of developing the understanding required for refinement of TFM

derivatives (e.g. would the addition of a methyl or hydroxyl group at a given position enhance selectivity for TvMGL compared to related host enzymes).

4.1 The wild-type TvMGL1

Trichomonas vaginalis and *Entamoeba histolitica* possess two genes that encode methionine γ-lyase (MGL). McKie and colleagues reported that both isosymes from *T. vaginalis* (TvMGL1 and TvMGL2) catalyze the γ- and β-elimination reactions for L-methionine, L-homocysteine and L-cysteine. They do not, however, appear to catalyze these reactions for L-cystathionine (McKie, Edlind et al. 1998). The same results were reported for EhMGL1 and EhMGL2 of *E. histolitica* (Sato, Yamagata et al. 2008).

McKie and colleagues employed a discontinuous, end point assay in which the α-ketoacid product is derivatized with 3-methyl-2-benzothiazolinone hydrazone hydroclorate (MBTH) (McKie, Edlind et al. 1998). The MBTH assay is laborious, because it involves long incubation periods and multiple time points must be sampled per reaction. In contrast, a continuous assay enables enzyme activity to be monitored in real time, thereby precluding the need for time points and incubations. Therefore, continuous assays for the hydrolysis of L-cysteine (α,β-elimination producing pyruvate) and L-homocysteine or L-methionine (α,γ-elimination producing α-ketobutyrate) were developed. These assays are based on those described by Aitken and colleagues for cystathionine γ-synthase and rely on lactate dehydrogenase (LDH) and hydroxyisocaproate dehydrogenase (HicDH) for reduction of pyruvate or α-ketobutyrate, respectively (Aitken, Kim et al. 2003).

An alternative assay based on detection of the hydrogen sulfide or methanethiol products of these reactions with 5,5'-(dithiobis-(2-nitrobenzoic acid) (DTNB) was

deemed impractical as it would require sealing of cuvettes to prevent escape of these gaseous products.

TvMGL1 catalyzes the hydrolysis of L-homocysteine and L-methionine (Table 3.1). The rate constant for L-homocysteine hydrolysis $k_{cat}^{L\text{-}Hcys} = (3.7 \pm 0.3\ s^{-1})$ is ~7 fold larger than for L-methionine hydrolysis $k_{cat}^{L\text{-}Met}$ $(0.51 \pm 0.06\ s^{-1})$. Similarly, McKie et al. (1998) reported that the turnover rate of TvMGL for hydrolysis of L-homocysteine is 37 times greater than that of L-methionine. The 5-fold difference in the preference for L-homocysteine may be due to the presence of the N-terminal affinity tag added to the enzyme to facilitate purification in this study. L-Homocysteine and L-methionine differ only by the presence of a methyl group at the ε-position of the side chain. Therefore, the two substrates are expected to bind similarly in the TvMGL active site and the greater k_{cat} of L-homocysteine may be due to the properties of its hydrogen sulfide leaving group, compared to the methanethiol leaving group of methionine.

4.2 Architecture of the active site of TvMGL1 and characterization of key residues

The TVMGL1 active site is located at the subunit interface in the catalytic dimer with the cofactor supported in a place by a network of hydrogen bonds, which is characteristic of other fold-type I PLP-dependent enzymes. (Clausen, Huber et al. 1996, Lodha, Jaworski et al. 2010, Jaworski, Lodha et al. 2012).

Substrates bind to the active site and replace the active-site lysine in Schiff base linkage with the PLP cofactor. The α-carboxylate group of the substrate is expected to form a salt bridge with Arg373, a residue conserved in all fold-type I PLP-dependent enzymes, including the closely related enzymes CBL, CGS, CGL, which also catalyze

α,β-elimination and α,γ-elimination reactions of similar sulfur-containing amino acid substrates (Clausen, Huber et al. 1996, Lodha and Aitken 2011, Jaworski, Lodha et al. 2012).

Substrates access the active site via a channel, comprised of residues of adjacent subunits, which runs along the dimer interface (Motoshima, Inagaki et al. 2000, Kudou, Misaki et al. 2007). Residues F47 and L59 are proposed to maintain this channel and, together with residues I55 and V373, form a hydrophobic patch that may be involved in binding the side chain of the amino acid side chain. This is an important distinction of TvMGL as CBL, CGL and CGL all bind substrates with a distal carboxylate moiety (Kudou, Misaki et al. 2007). Residues R58 and Y56 both form hydrogen bonds with the phosphate group of the PLP co-factor (Motoshima, Inagaki et al. 2000, Kudou, Misaki et al. 2007, Kudou, Misaki et al. 2008).

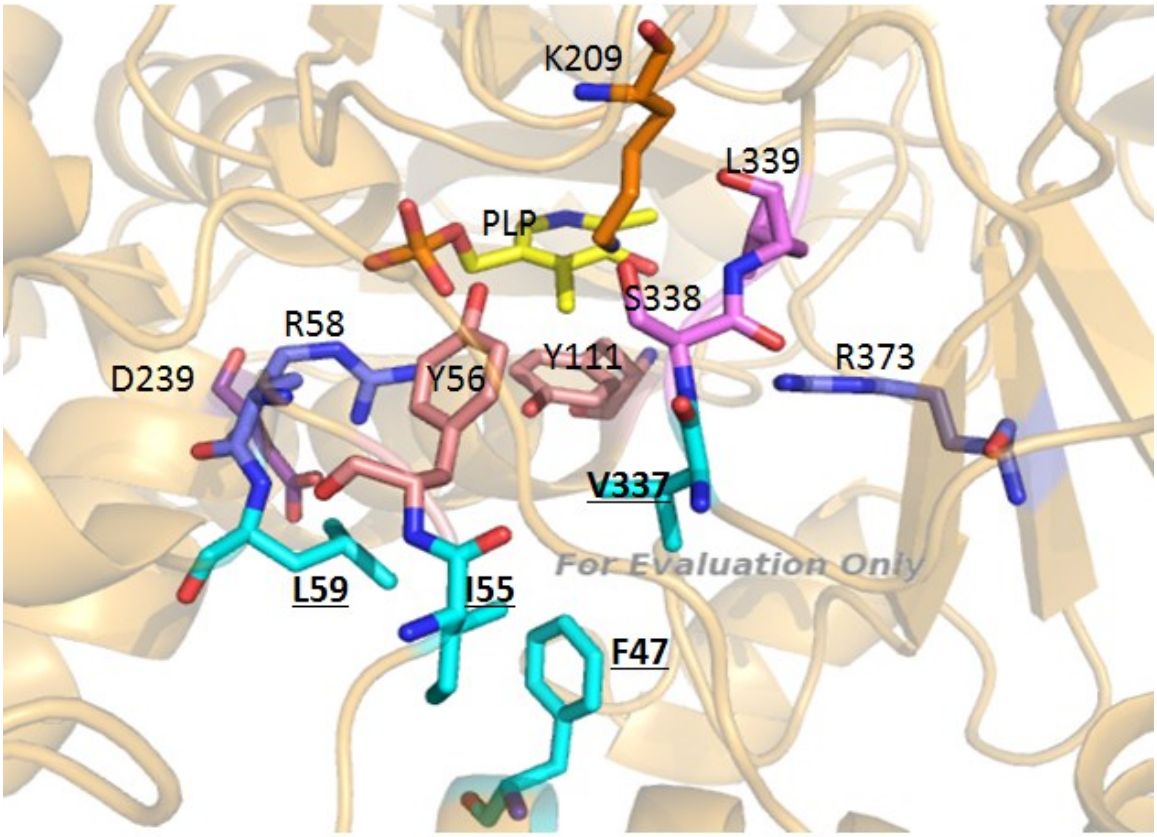


Figure 4.1 The active-site channel of TvMGL is lined by residues F47, I55, V337 and L59. The image was render in PyMOL Molecular Graphics System, Version 1.5.5.0.5 Schrödinger, LLC.

4.2.1 The Y111F variant

Replacement of Y111 with phenylalanine removes only the hydroxyl group of the side chain, conserving the packing interactions of the side chain with the aromatic ring of the cofactor (Figure 4.2). The lack of detectable methionine or homocysteine hydrolysis activity for the Y111F variant suggests a catalytic role for the hydroxyl moiety of this residue, which is conserved in the related enzymes of the mammalian and bacterial transulfuration pathways. Inoue et al. (2000) employed hydrogen exchange in D_2O, monitored using ^1H nuclear magnetic resonance spectrometry, and reported a role for the corresponding residue *of P. putida* MGL in nucleophilic attack on L- methionine (Inoue, Inagaki et al. 2000). In contrast, Lodha and Aitken et al. (2011) and Jaworski et al. (2012) studied the corresponding substitutions in the context of eCBL and eCGS, respectively, and concluded that Y111 residue plays a role in the proper orientation of the cofactor, it does not play an essential role in catalysis. Therefore, the suggestion of a catalytic role for Y111 in the context of MGL provides insight into the mechanisms that underlie reaction specificity in these closely related enzymes.

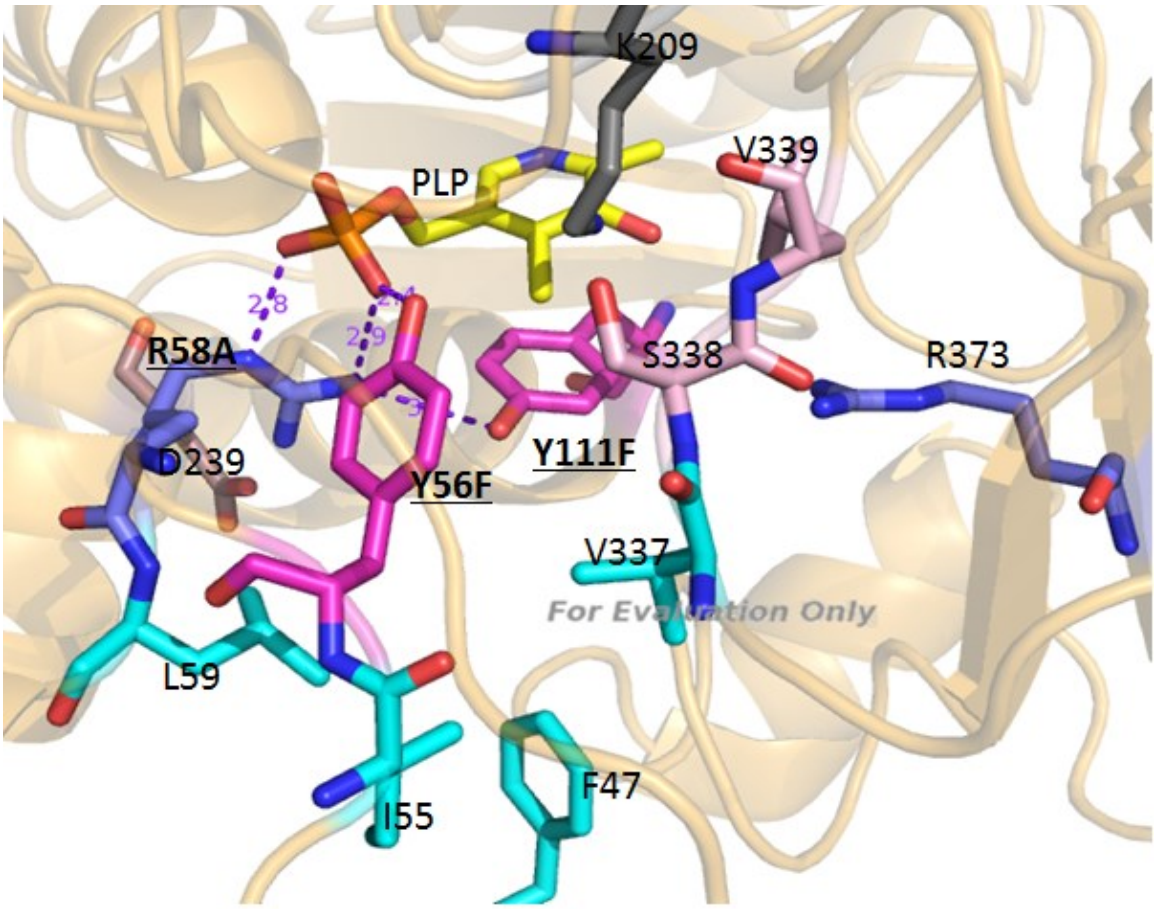


Figure 4.2 Proposed hydrogen bonding interactions of Y56 and R58 with the phosphate group of the cofactor and of R58 with the side chain of Y111. The image was rendered in PyMOL Molecular Graphics System, Version 1.5.5.0.5 Schrödinger, LLC.

4.2.2 The R58A variant

Substitution of R58 with alanine eliminates the detectable hydrolysis of L-methionine and L-homocysteine. An arginine residue at this position is conserved in fold type I PLP-dependent enzymes, as exemplified by eCGS-R49 and eCBL-R58. Lodha et. al. (2010) suggested that the corresponding eCBL-R58 is involved in modulating the nucleophilic character of residue Y111 and in binding the distal carboxylate group of the cystathionine substrate. The interaction between R58 and Y111 may modulate the pK_a of the latter, thereby facilitating a role for Y111 in proton transfer (Clausen, Huber et al. 1996, Clausen, Huber et al. 1997). The same interaction between R58 and Y111 is observed in TvMGL1 (Figure 4.2). In the context of MGL R58 would not be required to bind a substrate carboxylate group and may function primarily to modulate the pK_a of Tyr111 and position its side chain with respect to the PLP cofactor in the active site (Figure 4.2).

4.2.3 The R373A/K variant

An arginine residue at the position corresponding to TvMGL-R373 is conserved in all fold type I PLP-dependent enzymes. The primary function of this residue is to coordinate the α-carboxylate group of the substrate. Substitution of A373 with alanine in TvMGL1 eliminates catalytic activity for hydrolysis of L-methionine and L-homocysteine. Similar results were observed for removal of the hydrogen-bonding capacity of the arginine side chain of the corresponding R372 of eCBL (eCBL-R372A/L) (Lodha, Jaworski et al. 2010). The conservative TvMGL-R373K substitution, which possesses a

positively charged ε-amino group but is 2 Å shorter than arginine, also lacks detectable activity (Tables 3.2 and 3.3). A 220-fold decrease of k_{cat} was reported for the corresponding eCGS-R361K variant, compared to a 4-fold decrease of k_{cat} for eCBL-R372K suggesting a difference in the role of this residue between enzymes catalyzing α,γ (eCGS and TvMGL) versus α,β-eliminations (eCBL) (Lodha, Jaworski et al. 2010, Jaworski, Lodha et al. 2012). Positioning of the substrate with respect to catalytic residue(s), via interaction between the α-carboxylate moiety and an arginine residue, may be more important in the context of an α,γ-elimination due to the greater number of electron and proton transfers required, compared to the facile α,β-elimination catalyzed by eCBL.

4.2.4 The Y56F variant

The Y56F variant of TvMGL lacks detectable activity for the hydrolysis of L-methionine and L-homocysteine. Residue Y56 residue is conserved through fold-type I PLP-dependent enzymes and is proposed to have a structural role in orienting the PLP cofactor via a hydrogen bond with the phosphate group, an interaction conserved in TvMGL1. The corresponding substitutions in eCBL (Y56F) and eCGS (Y47F) reduce the k_{cat} by 7 and 20-fold, respectively, demonstrating that this cofactor-positioning interaction is more important in the context of enzymes catalyzing α,γ (eCGS and TvMGL) than α,β (eCBL) eliminations (Lodha and Aitken 2011, Jaworski, Lodha et al. 2012).

4.2.5 The S338A variant

Removal of the side-chain hydroxyl moiety by the TvMGL-S338A substitution eliminates detectable hydrolysis of L-methionine or L-homocysteine (Tables 3.2 and 3.3). A 5600-fold reduction in k_{cat} was reported for the corresponding S339A variant of eCBL (Lodha and Aitken 2011). In the context of the α,β-elimination catalyzed by eCBL, S339 is required to tether the catalytic lysine residue to restrict proton abstraction to the Cα position and prevent proton transfers between C4' of the cofactor and C_β of the substrate (Lodha and Aitken 2011). The role of the residue corresponding to eCBL-Ser339 (TvMGL-S338) in the context of enzymes catalyzing α,γ-elimination reactions has not been demonstrated. Although the hydroxyl groups of the side chains of residues S338 and Y56 are not within hydrogen bonding distance of the catalytic lysine (K209) in the internal aldimine form of the TvMGL structure, these interactions may occur when the lysine side chain is released from Schiff base linkage with the cofactor, upon substrate binding (Figure 4.3).

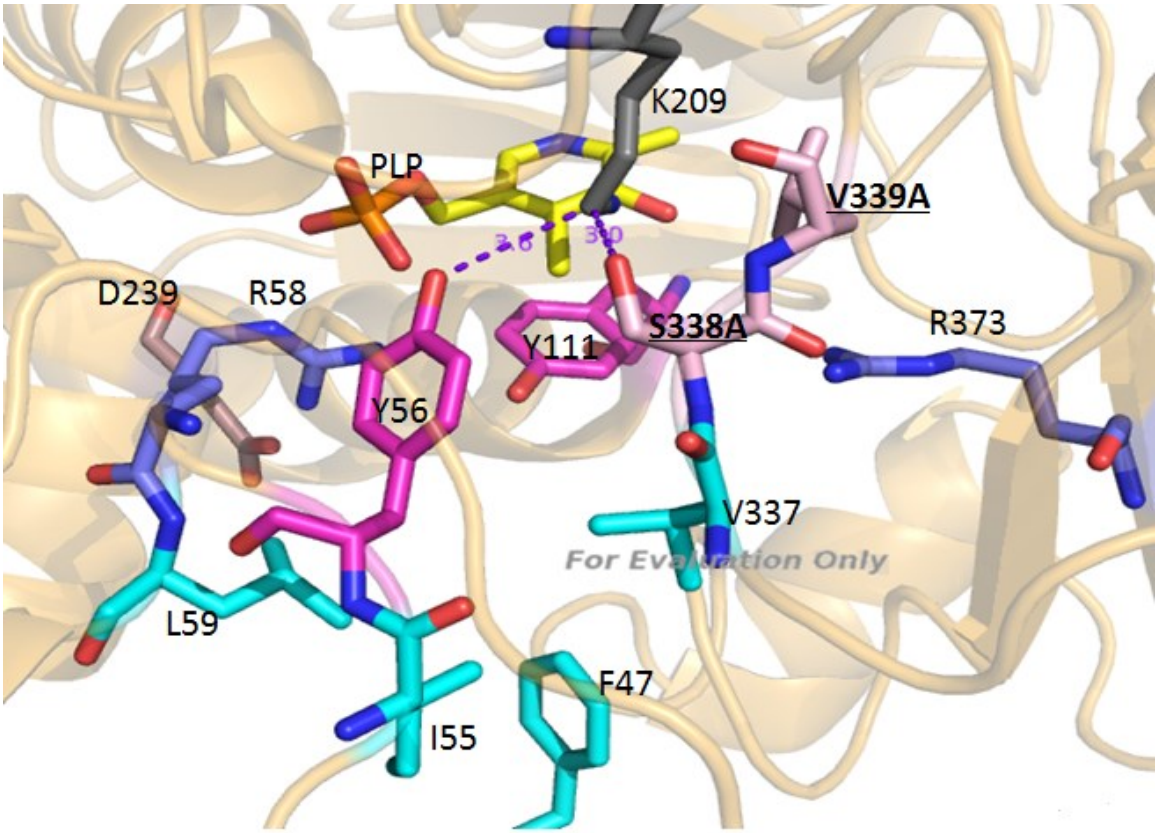


Figure 4.3. Proposed hydrogen bonding interactions of S338 and Y56 with K209 in the active site of TvMGL. The image was rendered in PyMOL Molecular Graphics System, Version 1.5.5.0.5 Schrödinger, LLC.

4.2.6 The D239A variant

D239 of TvMGL is not conserved among the closely related enzymes of the transsulfuration pathways. Its counterparts in bacterial CBL and CGS enzymes are tyrosine(Y238) and asparagines (N227) (Clausen, Huber et al. 1996, Lodha and Aitken 2011, Jaworski, Lodha et al. 2012). In eCBL Y238 was postulated to bind to distal carboxylate group of the L-cystathionine substrate, a role not required in TvMGL as the side chain of the methionine substrate is nonpolar (Lodha and Aitken 2011). The 5 and 10-fold reductions in the k_{cat} of L-homocysteine and L-methionine hydrolysis, respectively by TvMGL-D239A are likely the result of subtle changes in active-site architecture resulting from removal of the Asp239 side-chain carboxylate group (Table 2.2 and 2.3).

4.2.7 The I55A variant

Residue I55, together with V337, F47 and L59, form a hydrophobic channel that is proposed to guide the substrate into the TvMGL1 active site and assist in substrate positioning, with respect to the cofactor and catalytic residues (Figure 4.1). Substitution of TvMGL-I55 with alanine decreases the k_{cat} for L-methionine and L-homocysteine hydrolysis by 11 and 14-fold, respectively (Table 3.2 and 3.3). I55 is not conserved among the closely related enzymes of the mammalian, yeast or bacterial transsulfuration pathways, suggesting a specific role as a determinant of active-site architecture in the context of MGL. Altering the conformation of the channel by reducing the size of the side chain at position 55 suggests that this residue plays a role in maintaining the substrate in a catalytically competent position within the active site.

4.2.8 The L339A variant

The leucine at position 339 of TvMGL is conserved among the transsulfuration enzymes catalyzing α,γ-elimination (CGS and CGL), but not α,β-elimination (CBL) reactions. However, this residue has not been investigated previously. Replacement of TvMGL-L339 with alanine results in a negligible 2-fold decrease in the k_{cat} for hydrolysis of L-methionine and L-homocysteine, demonstrating that this residue does not play a role in substrate binding or catalysis. In contrast, the corresponding eCBL-W340 interacts with the α-carboxylate group of the substrate. Therefore, similar to several of the other residues investigated in this study, TvMGL-L339 may play a subtle role as a determinant of α,γ versus α,β-elimination activity (Lodha, Jaworski et al. 2010).

5. Conclusion

The objectives of this thesis are three fold: 1) to develop a method for the purification method of the TvMGL1 enzyme; 2) to characterize the substrate specificity of this enzyme; and 3) to investigate the role of the following mutations in the active site, I55A, Y56F,R58A, Y111F, D239A, S338A, L339A, R373A, and R373K.

The affinity chromatography Ni-NTA was found to be a reliable method to purify TvMGL1 and the variants. The six histidine residues attached to the TvMGL1 do not interfere in the activity of this enzyme or of the express mutants. Storing of TvMGL1 and the site-directed variants at -80 ºC did not elicit the precipitation of the enzymes over time.

TvMGL1 successfully hydrolyzed L-methionine, L-homocysteine and L-cysteine in phosphate buffer at an optimal pH of 6.8. The coupling enzymatic assay with HicDH and LDH is a reliable method to quantify the activity of the TvMGL1 wild-type and the corresponding site-directed variants. TvMGL1 favors L-homocysteine over L-methionine and prefers to hydrolyze α,γ-elimination reactions rather than α,β-elimination reactions.

It was found that the PLP cofactor is oriented in the active site through hydrogen bonding between PLP phosphate group and residues R58 and Y56. R58 also has a role in modulating the pK_a of Y111 after proton transfer from the substrate. It is concluded that D239 is involved in establishing the architecture of the active site. Moreover, I55 plays an essential role in maintaining the substrate in a catalytically competent position within the TvMGL1 active site.

L339 does not play a role in the substrate binding or catalysis. S338 and Y56 residues interact with the side chain of K209 after the release of the Schiff base linkage with the cofactor upon substrate binding.

The TvMGL1 enzyme presents a lot of similarity in the architecture of the active site with other PLP-dependent enzymes that catalyze α,γ-elimination reactions. However, there is a notable difference in the catalytic mechanism, as evidenced by the observed differences of the residues in the catalytic active site.

Thus, this thesis achieved its stated objectives of establishing a method to purify the TvMGL1 enzyme, characterizing the substrate specificity of this enzyme, and determining the effects of introducing mutations at several residues.

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