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Characterization of Cobalamin-Independent Methionine Synthase from *Candida albicans* and *Saccharomyces cerevisiae*

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by

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Dedication

To my family, especially my son, for their support and encouragement.

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Characterization of Cobalamin-Independent Methionine Synthase from *Candida albicans* and *Saccharomyces cerevisiae*

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Methionine synthases are folate-dependent enzymes that catalyze the transfer of a methyl group from 5-methyltetrahydropteroylpolyglutamate (CH_3–$H_4PteGlu_n$), also called 5-methyltetrahydrofolate, to L-homocysteine to form L-methionine. There are two major classes of methionine synthases, the cobalamin-dependent and the cobalamin-independent methionine synthases.

The cobalamin-dependent methionine synthase is a very large, 140 kDa protein, and uses cobalamin to aid in the transfer of the methyl group from 5-CH_3–$H_4PteGlu_n$ to homocysteine. Only organisms that can synthesize or obtain cobalamin, such as mammals, use cobalamin-dependent methionine synthase.

Organisms that cannot obtain or synthesize cobalamin, such as fungi, use the cobalamin-independent methionine synthases, and some bacteria such as *E. coli* use enzymes from both classes. Proteins from the cobalamin-independent

class have a molecular weight of 86 kDa, and have no amino acid sequence homology to the cobalamin-dependent enzymes. These enzymes are zinc dependent, and kinetic analyses of the *E. coli* cobalamin-independent methionine synthase (MetEp) reveal that it will only bind polyglutamated forms of 5-CH_3–$H_4PteGlu_n$. Methionine synthases from fungi are not well characterized. They may be interesting anti-fungal drug targets because of the mechanistic differences between them and the cobalamin-dependent forms present in humans.

C. albicans resides in the normal flora of the human body. However, it is able to cause infection in immunocompromised patients. In the past two decades, *C. albicans* has become one of the most common opportunistic pathogens, particularly in hospitals. Increasing drug resistance to present drugs, and severe side effects results in the constant search for new drug targets to create better and more effective therapies.

The work presented here investigates the cobalamin-independent methionine synthase from *C. albicans* (CaMet6p) and from *S. cerevisiae* (ScMet6p). Substrate specificity for both enzymes was explored through kinetic analyses, and a strategy was implemented to study important active site residues by site-directed mutagenesis. A conditional cobalamin-independent methionine synthase (*CaMET6*) mutant in *C. albicans* was constructed, using the PCR-based gene disruption method, to assess the viability of the resulting null mutant strain.

The results from these experiments have provided new insights into enzyme function, and support the study of CaMet6p as an anti-fungal drug target.

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CHAPTER 1: INTRODUCTION

Methionine synthases catalyze the transfer of a methyl group from 5-methyltetrahydropteroylpolyglutamate (5-CH_3-H_4PteGlu$_n$) to L-homocysteine (Hcy) to form L-methionine, in the last step of methionine biosynthesis (Figure 1.1). These enzymes mark the convergence between the methionine metabolism pathway and the one-carbon metabolism pathway (Figure 1.1). Folate mediated one-carbon metabolism plays an essential role in the synthesis of nucleotides, vitamins, and some amino acids (Cossins *et al.*, 1997; Piper *et al.*, 2000). Only the tetrahydro- form of folate is active, and dihydrofolate reductase catalyzes the reduction of folic acid to dihydrofolate (DHF), and then to tetrahydrofolate (H_4PteGlu$_n$)(Figure 1.2). H_4PteGlu$_n$ has three main structural features: a pteridine ring, p-aminobenzoic acid (PABA), and a polyglutamate chain (Figure 1.3). Serine, glycine, and formate provide the one-carbon units, which are activated by attachment to tetrahydrofolate (H_4PteGlu$_n$) (Piper *et al.,* 2000). One-carbon metabolism in yeast is compartmentalized, where one-carbon units are interconverted between the mitochondria and cytoplasm (Figure 1.4). Serine hydroxymethyl transferase catalyzes the conversion of H_4PteGlu$_n$ into 5, 10 methylenetetrahydrofolate (5, 10-CH_2-H_4PteGlu$_n$) through the conversion of serine to glycine (Figure 1.4). Several pathways also involve 5, 10 methylene-H_4PteGlu$_n$. For example, 5, 10 methylenetetrahydrofolate can be used to convert dUMP to dTMP using thymidylate synthase (Figure 1.5). It can also be oxidized

to form 10-formyl-$H_4PteGlu_n$, which is used in the synthesis of purines (Figure 1.5). 5, 10-CH_2-$H_4PteGlu_n$ can also be reduced to 5-CH_3-$H_4PteGlu_n$ catalyzed by 5, 10 methylene tetrahydrofolate reductase (MTHFR). The 5-CH_3- $H_4PteGlu_n$ created is then used by methionine synthase to synthesize methionine from homocysteine (Figure 1.5).

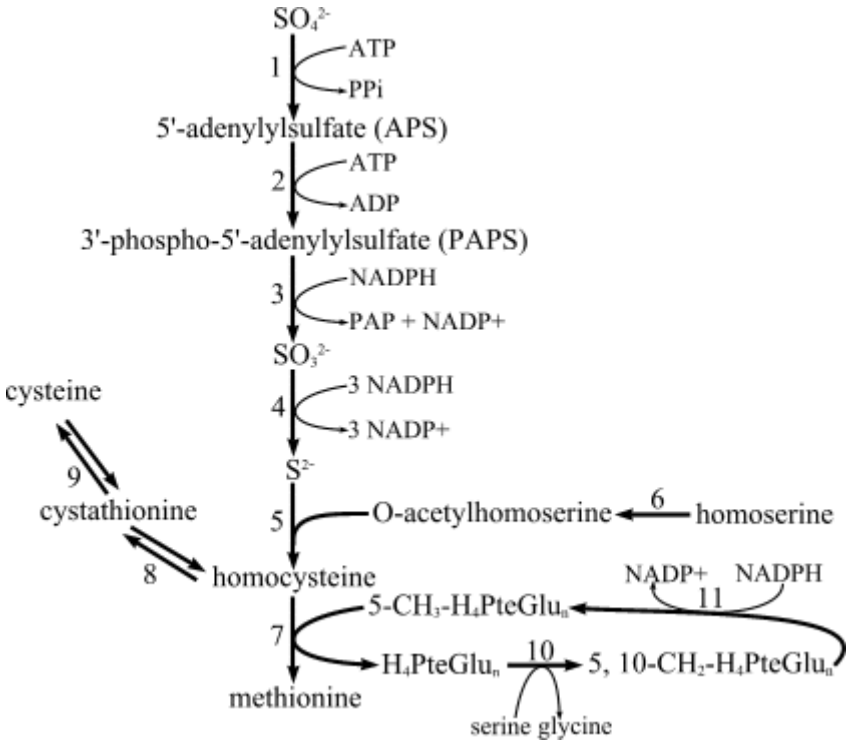

Figure 1.1 Biosynthesis of sulfur amino acids. Enzymes: 1, ATP sulfurylase; 2, APS kinase; 3, PAPS reductase; 4, sulfite reductase; 5, homocysteine synthase; 6, homoserine O-acetyltransferase; 7, methionine synthase; 8, cystathionine β-synthase; 9, cystathionine γ-lyase. Methionine synthase accepts 5-methyltetrahydrofolate (5-CH_3-$H_4PteGlu_n$) from the one carbon pathway as a substrate, and produces tetrahydrofolate ($H_4PteGlu_n$). Enzymes in folate metabolism: 10, serine transhydroxymethylase; 5, 10-methylenetetrahydrofolate reductase.

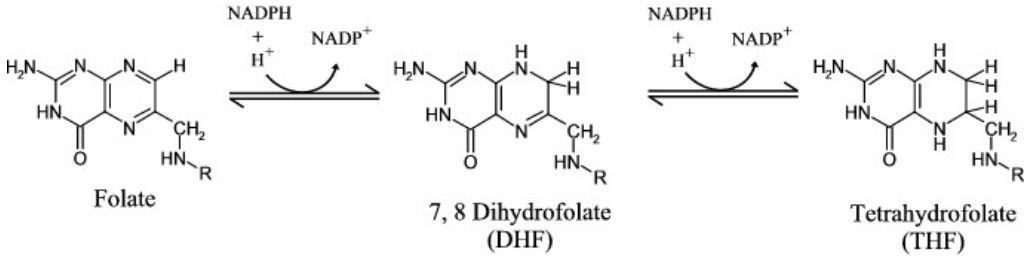

Figure 1.2 The conversion of folate to tetrahydrofolate by dihydrofolate reductase.

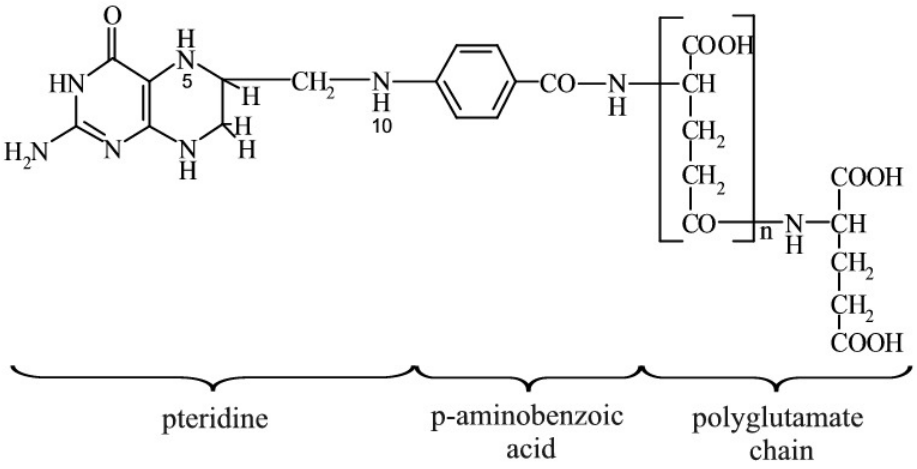

Figure 1.3 The structure of tetrahydrofolate ($H_4PteGlu_n$). The N-5 and N-10 positions can be substituted with one-carbon units.

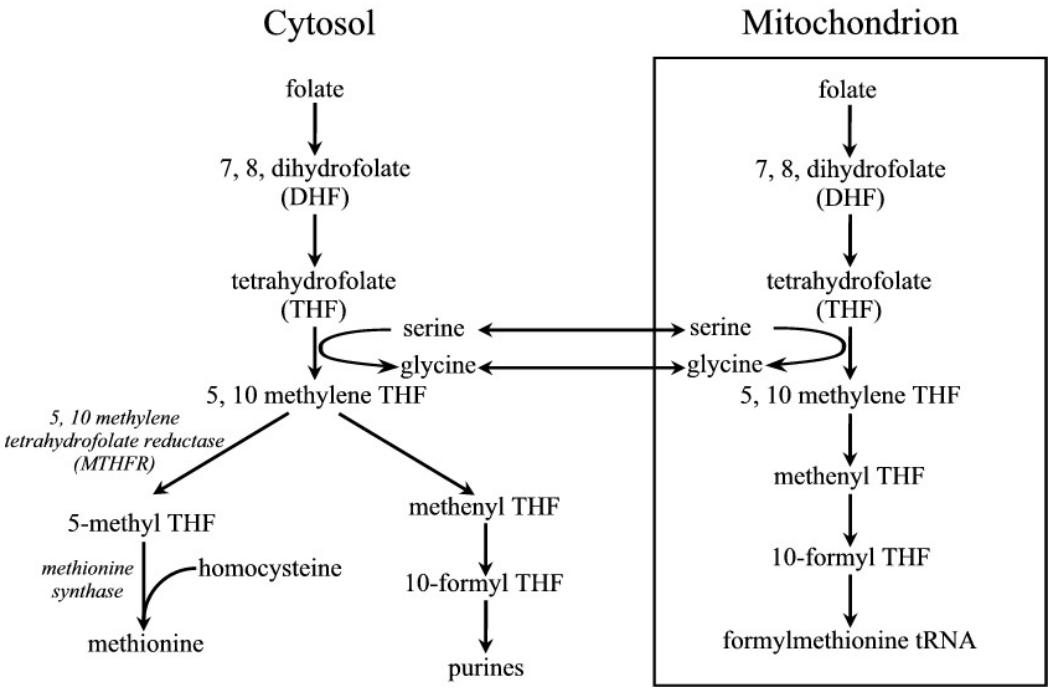

Figure 1.4 Compartmentalization of one-carbon metabolism. The metabolic system in the mitochondria and cytoplasm are similar, and one-carbon units are interconverted between the two compartments. (Adapted from Thomas *et al.*, 1997)

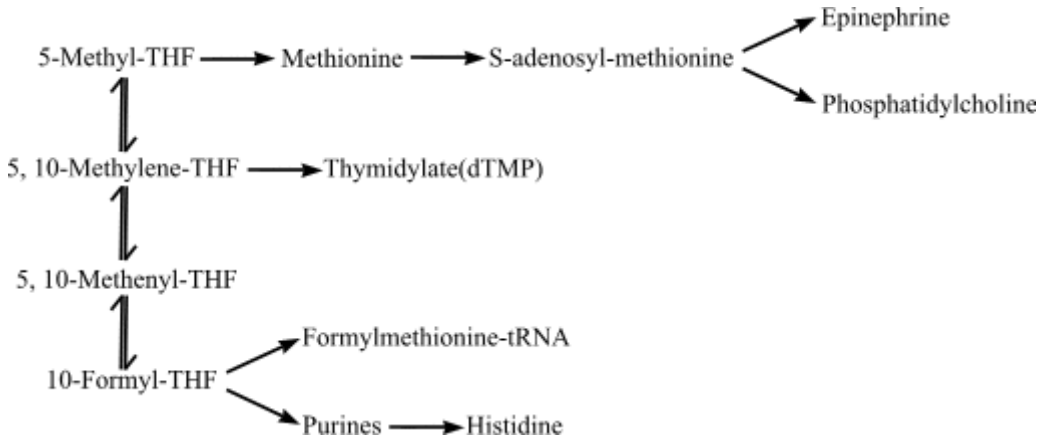

Figure 1.5 Products of one-carbon metabolism.

Methionine is one of two sulfur-containing amino acids, and it is the first amino acid in most protein, since its DNA codon is usually the point of translation initiation. More importantly, methionine is activated with ATP to form S-adenosylmethionine (AdoMet), by the enzyme methionine adenosyltransferase (Figure 1.6). S-adenosylmethionine is an important methyl donor, and is the second most commonly used metabolic cofactor, the first being ATP (Kozbial *et al*., 2005). Each cellular organism has several AdoMet-utilizing enzymes (Kozbial *et al*., 2005), with which AdoMet donates methyl groups for covalent modification of a variety of substrates. Methylation of DNA is important for the regulation of gene expression, and methylation regulates hormones, neurotransmitters, and signal-transduction systems (Fontecave *et al.*, 2004). S-adenosylhomocysteine (AdoHcy) is a product of these methyl transfers (Figure 1.6). AdoHcy is then hydrolyzed by S-adenosylhomocysteine hydrolase to form homocysteine, which can re-enter the methionine metabolism pathway through the action of methionine synthase (Figure 1.6).

There are two major classes of methionine synthases, cobalamin-dependent, and cobalamin-independent methionine synthases (Gonzalez *et al.*, 1992; Matthews *et al.*, 2003). Organisms that can acquire, or synthesize cobalamin use cobalamin-dependent methionine synthases, and organisms that cannot acquire or synthesize cobalamin use cobalamin-independent methionine synthases. Mammals use only cobalamin-dependent methionine synthase, and

yeasts and plants use only the cobalamin-independent methionine synthase. Some bacteria, such as *E. coli*, use both forms of the enzyme.

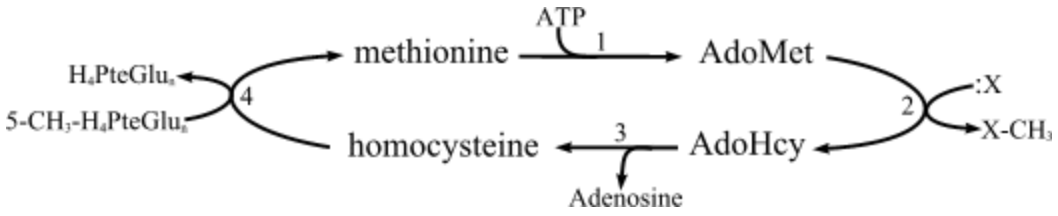

Figure 1.6 The S-adenosylmethionine (AdoMet) cycle. X represents a methyl acceptor. Methionine reacts with ATP to form AdoMet catalyzed by (1) S-adenosylmethionine synthetase; (2) methyltransferases transfer the methyl group from AdoMet to a methyl acceptor forming S-adenosylhomocysteine (AdoHcy); (3) AdoHcy is hydrolyzed by S-adenosylhomocysteine hydrolase to form homocysteine (Hcy); (4) methionine is regenerated when methionine synthase methylates homocysteine.

COBALAMIN-DEPENDENT METHIONINE SYNTHASE

The most extensively studied cobalamin-dependent methionine synthase is the MetH enzyme from *Escherichia coli* (Matthews, 2001). Cobalamin-dependent methionine synthase catalyzes the conversion of homocysteine and 5-CH_3-$H_4PteGlu_n$ to methionine and $H_4PteGlu_n$ in two steps (Figure 1.7). In the first step, the methyl group is transferred from 5-CH_3-$H_4PteGlu_n$ to cob(I)alamin, producing methylcobalamin and $H_4PteGlu_n$. The methyl group is then transferred from methylcobalamin to homocysteine forming methionine and cob(I)alamin. Occasionally cob(I)alamin is oxidized to the inactive cob(II)alamin form, which is

then reactivated by reductive methylation where flavodoxin is the electron donor and AdoMet is the methyl donor (Figure 1.7) (Goulding *et al.*, 1997). The cobalamin-dependent methionine synthases are very large proteins, roughly 140 kDa. They appear to have four major domains, which function in binding L-homocysteine, CH_3–$H_4PteGlu_n$, cobalamin, and AdoMet respectively (Goulding *et al.*, 1997).

The X-ray structure of the two amino terminal domains of the enzyme from *Thermotoga maratima* has been reported (Evans *et al.*, 2004), as have structures for the cobalamin-binding domain (Drennan *et al.*, 1994) and the AdoMet binding domain of the *E. coli* enzyme (Dixon *et al*., 1996). The structures show that each domain is activated by its own substrate-binding site. Three of the domains are involved in methyl transfer and the fourth domain is involved in AdoMet binding (Evans *et al.*, 2004). Kinetic analysis shows that the turnover number for MetHp is 1100/min (Banarjee *et al*., 1990).

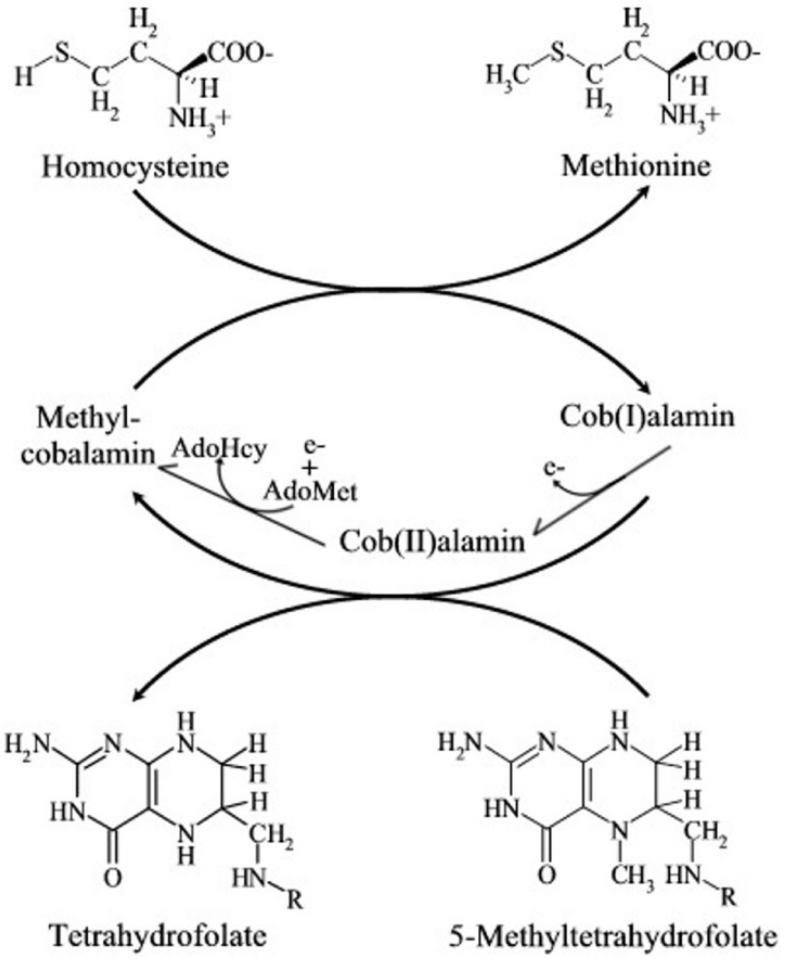

Figure 1.7 The enzymatic reactions catalyzed by MetHp. A methyl group is transferred from CH_3-H_4folate to the cob(I)alamin cofactor bound to MetHp, forming H_4-folate and methylcobalamin. The methyl group is then transferred from methylcobalamin to homocysteine, forming methionine and regenerating cob(I)alamin. (Adapted from Evans *et al.*, 2004)

HUMAN METHIONINE SYNTHASE

In human metabolism methionine is an essential amino acid, and sufficient amounts are generally obtained through diet. Methionine synthase is one of only two known cobalamin-dependent enzymes, the other being methylmalonyl-CoA mutase (Kolhouse *et al*., 1977). Human methionine synthase is widely distributed in tissues, and serves as part of the methionine salvage pathway (Figure 1.8) (Stipanuk *et al.*, 2004). Homocysteine is a byproduct of methionine metabolism, but increased homocysteine levels in the blood, due to defects in this pathway, cause human disease. Accumulated homocysteine is associated with cardiovascular disease, and promotes arteriosclerosis (Jakubowski *et al.*, 2004). Homocysteine build up is implicated as a cause for neural tube defects, Alzheimer's disease, dementia, and blood clotting disorders (Selhub, 1999).

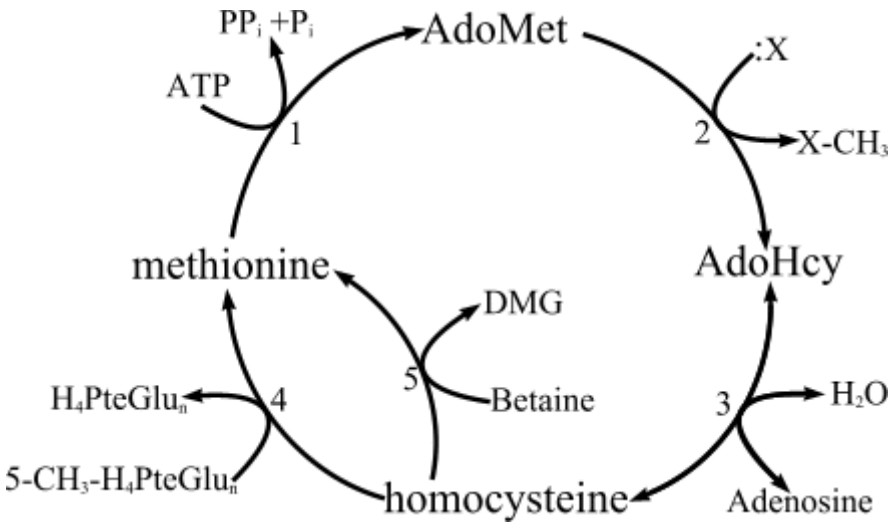

Figure 1.8 Methionine Salvage Pathway Enzymes: 1, methionine adenosyltransferase; 2, methyltransferase requiring AdoMet as the methyl donor; 3, S-adenosylhomocysteine methyltransferase; 4, cobalamin-dependent methionine synthase; 5, betaine homocysteine methyltransferase (BHMT).

BETAINE HOMOCYSTEINE METHYLTRANSFERASE

A second methyltransferase involved in human methionine metabolism, called betaine homocysteine methyltransferase (BHMT), does not require cobalamin. BHMT is a hexamer of 45 kDa subunits (Millian *et al.*, 1998), and is present only in the liver, kidneys, and lens of humans (Stipanuk, 2004). BHMT is a zinc metalloenzyme catalyzing the transfer of the methyl group from betaine to homocysteine, forming dimethylglycine and methionine (Figure 1.9). A proposed transition-state structure is shown in Figure 1.10, along with a transition-state analog (Evans *et al.*, 2002; Castro *et al*., 2004). This transition-state analog was used in crystal structures to define contacts for both substrates (Evans *et al.*, 2002).

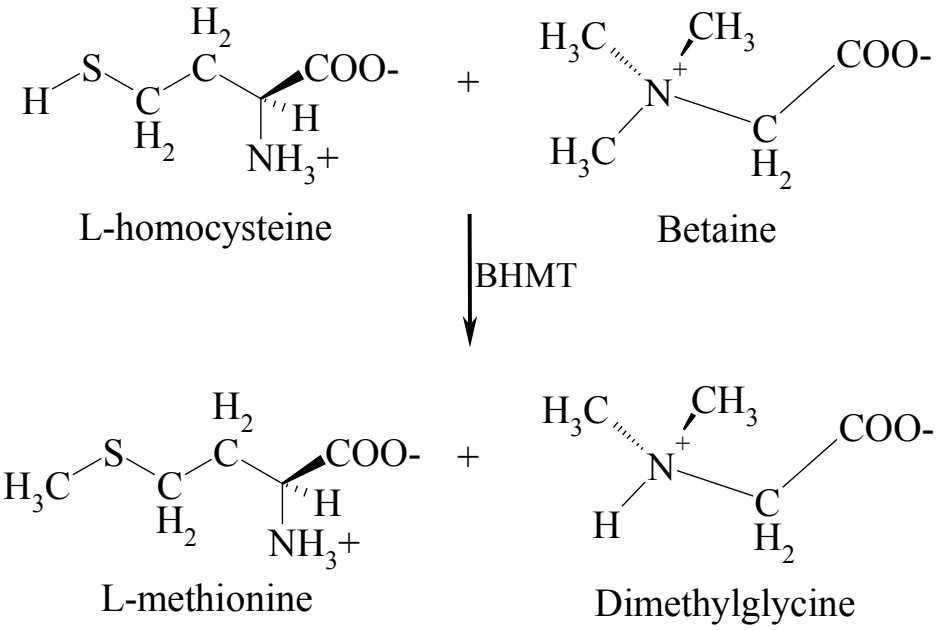

Figure 1.9 The reaction catalyzed by betaine homocysteine methyltransferase (BHMT). A methyl is transferred from betaine to L-homocysteine, forming L-methionine and dimethylglycine.

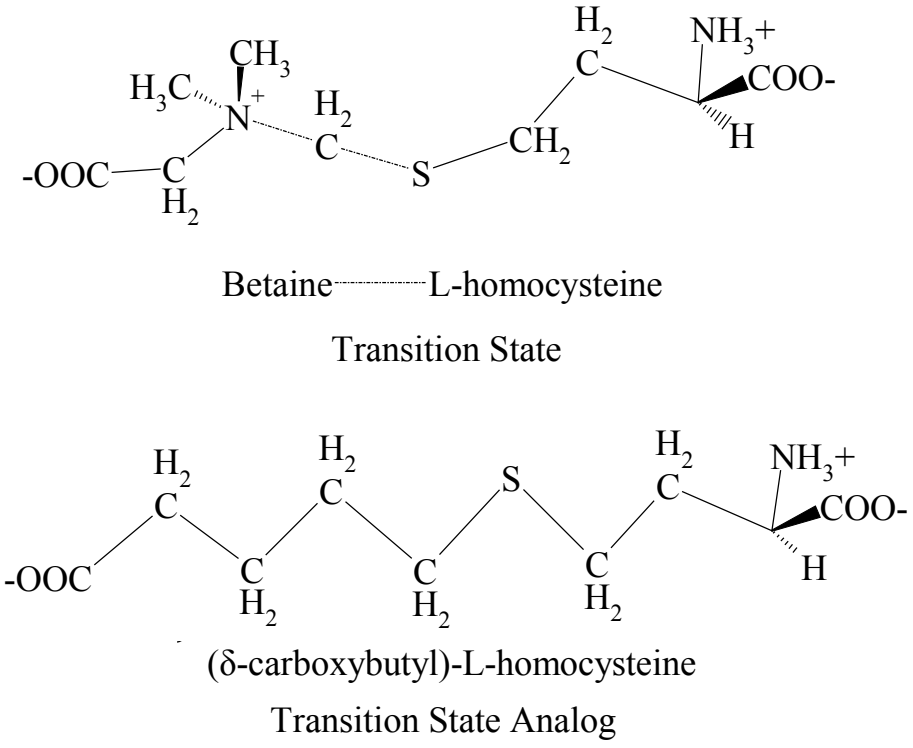

Figure 1.10 The proposed transition state structure, and a transition state analog (*S*-(δ-carboxybutyl)-L-homocysteine) for the BHMT reaction.

COBALAMIN-INDEPENDENT METHIONINE SYNTHASE

Cobalamin-independent methionine synthases are large enzymes, generally around 86 kDa. They have no obvious amino acid sequence homology with the cobalamin-dependent enzymes (Gonzales *et al*., 1992). Cobalamin-independent methionine synthases catalyze the conversion of 5-CH_3-H_4PteGlu$_n$ and L-homocysteine to L-methionine and H_4PteGlu$_n$, as shown in equation 1. 5-CH_3-H_4PteGlu$_n$ acts as the methyl donor and L-homocysteine is the methyl acceptor.

$$5\text{-}CH_3\text{-}H_4PteGlu_n + \text{L-homocysteine} \leftrightarrows H_4PteGlu_n + \text{L-methionine} \quad (1)$$

Cobalamin-independent methionine synthases are zinc dependent enzymes (Gonzales *et al.*, 1996; Zhou *et al.*, 1999); they use the active site zinc to bind and activate the L-homocysteine substrate (Matthews *et al.*, 2003). The most studied cobalamin-independent methionine synthase is MetEp from *E. coli*. Inductively coupled plasma-atomic emission spectrometry (ICP) showed that MetEp has one equivalent of zinc for each protein subunit (Gonzalez *et al*., 1996). A conserved cysteine in the C-terminal half of MetEp, Cys726, is required for zinc binding, which was determined through site-directed mutagenesis. The mutant enzyme, Cys726Ser, showed no bound zinc using ICP and was inactive (Gonzalez *et al.*, 1996). Kinetic analyses of the cobalamin-independent enzyme from *E. coli* show

that it will accept only polyglutamated forms of 5-CH_3-H_4PteGlu_n. The turnover number for MetEp is 23/min, which is about 50-fold lower than the turnover number determined for MetHp, the cobalamin-dependent enzyme (Gonzalez *et al*., 1996).

CURRENT COBALAMIN-INDEPENDENT METHIONINE SYNTHASE STRUCTURES

Currently there are two three-dimensional crystal structures of cobalamin-independent methionine synthases. The first structure reported is the 765 amino acid enzyme from the higher plant *Arabidopsis thaliana,* AtMetEp (Ferrer *et al.*, 2004)(Sequence alignment Figure 1.11). This structure can be found in the RCSB Protein Data Bank (http://www.rcsb.org/pdb/) with ID numbers of 1U1H, 1U1J, 1U1U, and 1U22. AtMetEp crystals reveal a monomeric protein in which each monomer is composed of two domains that create a deep groove at the interface (Figure 1.12) (Ferrer *et al.*, 2004). The N-terminal domain extends from residues 2 to 391, called the folate barrel, and the C-terminal domain extends from residues 392 to 765, called the homocysteine barrel. The two domains are connected by loop 390-395. Both domains are structurally similar, and form an eightfold (βα) repeat. This structure is also known as a TIM (triose phosphate isomerase) barrel; each domain consists of eight parallel β-sheets surrounded by eight α-helices (Wierenga *et al*., 2001). The TIM barrel fold is the most common fold among all the known protein structures. In AtMetEp, the two TIM barrels

face each other forming one active site between the domains, and forming a cleft that allows the substrates to enter (Ferrer *et al*., 2004).

The active site of AtMetEp is located in the C-terminal domain. The active site zinc of AtMetEp binds to the sulfur atom of Cys 649, Cys 733, and to the side chain nitrogen of His 647 (Figure 1.13). These residues are homologous to the zinc binding residues determined for *E. coli* by site-directed mutagenesis (Zhou *et al*., 1999; Peariso *et al*., 2001). There is a water molecule, W1, in close proximity to the active site zinc, 2-3 Å. Homocysteine was observed at a distance of 3.6-4.4Å from the active site zinc atom, and the sulfur of homocysteine does not interact strongly with the zinc atom (Figure 1.14) (Ferrer *et al*., 2004). The pteridine ring of CH_3-$H_4PteGlu_5$ protrudes toward the zinc atom, and makes stacking interactions with Trp567 (Figure 1.15). The methyl group from CH_3-$H_4PteGlu_5$ is about 7Å from the sulfur of homocysteine. The p-aminobenzoate moiety had weak electron density and could not be modeled (Ferrer *et al*., 2004). Only the first glutamyl residue of CH_3-$H_4PteGlu_5$ appeared in the electron density map, and is bound in a positively charged binding pocket created with loop 507-529 (Figure 1.15)(Ferrer *et al*., 2004). The glutamyl residue has ionic interactions with the side chain of Arg521 and hydrogen bonds to the backbone of Cys522.

Figure 1.11


ScMet6 -MVQSAVLGFPRIGPNRELKKATEGYWNGKITVDELFKVGKDLRTQNWKLQKEAGVDIIP 59
CaMet6 -MVQSSVLGFPRIGGQRELKKITEAYWSGKATVEELLAKGKELREHNWKLQQKAGVDIIP 59
EcMetE MTILNHTLGFPRVGLRRELKKAQESYWAGNSTREELLAVGRELRARHWDQQKQAGIDLLP 60
AtMetE --MASHIVGYPRMGPKRELKFALESFWDGKSTAEDLQKVSADLRSSIWKQMSAAGTKFIP 58
TmMetE --MKAYAFGFPKIGEKREFKKALEDFWKGKITEEQFEEEMNKLRMYMVENYRKN-VDVIP 57
ScMet6 SNDFSFYDQVLDLSLLFNVIPDRYTKYDLSP-IDTLFAMGRGLQRKATETEKAVDVTALE 118
CaMet6 SNDFSYYDQVLDLSLLFNAIPERYTKFDLAP-IDVLFAMGRGLQKKATETQAAVDVTALE 118
EcMetE VGDFAWYDHVLTTSLLLGNVPARHQNKDGSVDIDTLFRIGRGRAPTGEP------AAAAE 114
AtMetE SNTFAHYDQVLDTTAMLGAVPPRYGYTGGEIGLDVYFSMARGNAS----------VPAME 108
TmMetE SNELSYYDFVLDTAVMVGAVPERFGEYRGLS---TYFDMARG------------GKALE 101
ScMet6 MVKWFDSNYHYVRPTFSKTTQFKLN---GQKPVDEFLEAKELGIHTRPVLLGPVSYLFLG 175
CaMet6 MVKWFDSNYHYVRPTFSHSTEFKLNTAAGIKPVDEFNEAKALGVQTRPVILGPVSYLYLG 178
EcMetE MTKWFNTNYHYMVPEFVKGQQFKLT---WTQLLDEVDEALALGHKVKPVLLGPVTWLWLG 171
AtMetE MTKWFDTNYHYIVPELGPEVNFSYA---SHKAVNEYKEAKALGVDTVPVLVGPVSYLLLS 165
TmMetE MTKFFNTNYHYLVPEIETEEFYLLE----NKPLEDYLFFKSKGIETAPWVIGPFTFLYLS 157
ScMet6 KADKDS-LDLEPLS-LLEQLLPLYTEILSKLASAGATEVQIDEPVLVLDLPANAQAAIKK 233
CaMet6 KADKDS-LDLEPIS-LLPKILPVYKELLQKLKEAGAEQVQIDEPVLVLDLPEAVQSKFKE 236
EcMetE KVKGE---QFDRLS-LLNDILPVYQQVLAELAKRGIEWVQIDEPALVLELPQAWLDAYKP 227
AtMetE KAAKGVDKSFELLS-LLPKILPIYKEVITELKAAGATWIQLDEPVLVMDLEGQKLQAFTG 224
TmMetE KRNGEWIRRPNQMEKLLESLVSVYKEVFEKLVENGCKEILVNEPAFVCDLEKAHWDLILN 217
ScMet6 AYTYFGEQSNLPKITLATYFGTVVPN-LDAIKGLP-VAALHVDFVRAPEQFDEVVAA-IG 290
CaMet6 AYDALVG-ADVPELILTTYFGDVRPN-LKAIENLP-VAGFHFDFVRVPEQLDEVASI-LK 292
EcMetE AYDALQG---QVKLLLTTYFEGVTPN-LDTITALP-VQGLHVDLVHGKDDVAELHKR-LP 281
AtMetE AYAELESTLSGLNVLVETYFADIPAEAYKTLTSLKGVTAFGFDLVRGTKTLDLVKAG-FP 283
TmMetE VYRELSE----FPLTVFTYYDSVSDY--EACVSLP-VKRLHFDFVSNEENLKNLEKHGFP 270
ScMet6 NKQTLSVGIVDGRNIWKNDFKKSSAIVNKAIEKLGADRVVVATSSSLLHTPVDLNNETKL 350
CaMet6 DGQTLSAGVVDGRNIWKTDFAKASAVVQKAIEKVGKDKVVVATSSSLLHTPVDLESETKL 352
EcMetE SDWLLSAGLINGRNVWRADLTEKYAQIK---DIVGKRDLWVASSCSLLHSPIDLSVETRL 338
AtMetE EGKYLFAGVVDGRNIWANDFAASLSTLQALEGIVGKDKLVVSTSCSLLHTAVDLINETKL 343
TmMetE EDKKLVAGVINGRQPWKVDLRKVASLVEK----LGAS--AISNSCPLFHLPVTLELENNL 324
ScMet6 DAEIKGFFSFATQKLDEVVVITKNVSGQDVAAALEANAKSVESRGKSKFIHDAAVKARVA 410
CaMet6 DAVIKDWFSFATQKLDEVVVIAKNVSGEDVSKQLEANAASIKARSESSITNDPKVQERLT 412
EcMetE DAEVKSWFAFALQKCHELALLRDAVNSGDTAALAEWSAP-IQARRHSTRVHNPAVEKRLA 397
AtMetE DDEIKSWLAFAAQKVVEVNALAKALAGQKDEALFSANAAALASRRSSPRVTNEGVQKAAA 403
TmMetE PGGLKEKLAFAKEKLEELKMLKDFLEG---------KTFDLPNVSFEDFAVDLQAVERVR 375
ScMet6 SIDEKMSTRAAPFEQRLPEQQKVFNLPLFPTTTIGSFPQTKDIRINRNKFNKGTISAEEY 470
CaMet6 TINEALATRKAAFPERLTEQKAKYNLPLFPTTTIGSFPQTKDIRINRNKFAKGQITAEEY 472
EcMetE AITAQDSQRANVYEVRAEAQRARFKLPAWPTTTIGSFPQTTEIRTLRLDFKKGNLDANNY 457
AtMetE ALKGSDHRRATNVSARLDAQQKKLNLPILPTTTIGSFPQTVELRRVRREYKAKKVSEEDY 463
TmMetE NLPEDSFRREKEYTERDRIQRERLNLPLFPTTTIGSFPQTPEVRKMRSKYRKGEISKEEY 435

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ScMet6          EKFINSEIEKVIRFQEEIGLDVLVHGEPERNDMVQYFGEQINGYAFTVNGWVQSYGSRYV 530
CaMet6          EAFINKEIETVVRFQEEIGLDVLVHGEPERNDMVQYFGEQLNGFAFTTNGWVQSYGSRYV 532
EcMetE          RTGIAEHIKQAIVEQERLGLDVLVHGEAERNDMVEYFGEHLDGFVFTQNGWVQSYGSRCV 517
AtMetE          VKAIKEEIKKVVDLQEELDIDVLVHGEPERNDMVEYFGEQLSGFAFTANGWVQSYGSRCV 523
TmMetE          EAFIKEQIKKAIELQEEIGLDVLVHGEFERTDMVEFFAEKLNGIATTQNGWVLSYGSRCY 495
                   * ..*: .:  **.:.:******* **.***::*.*::.* . * **** *****

ScMet6          RPPIIVGDLSRPKAMSVKESVYAQSITSKPVKGMLTGPITCLRWSFPRDDVDQKTQAMQL 590
CaMet6          RPPIIVGDVSRPKAMTVKESVYAQSITSKPMKGMLTGPVTILRWSFPRDDVSGKIQALQL 592
EcMetE          KPPIVIGDISRPAPITVEWAKYAQSLTDKPVKGMLTGPVTILCWSFPREDVSRETIAKQI 577
AtMetE          KPPVIYGDVSRPKAMTVFWSAMAQSMTSRPMKGMLTGPVTILNWSFVRNDQPRHETCYQI 583
TmMetE          RPPIIYGTVTRPEPMTLKEITYAQSLTEKPVKGMLTGPVTIMSWSYYREDIPEREIAYQI 555
                :**:: * ::** .:::     ***:*.:*:*******:* : **: *:*   .  . *:

ScMet6          ALALRDEVNDLEAAGIKVIQVDEPALREGLPLREGTERSAYYTWAAEAFRVATSGVANKT 650
CaMet6          GLALRDEVNDLEGAGITVIQVDEPAIREGLPLRAGKERSDYLNWAAQSFRVATSGVENST 652
EcMetE          ALALRDEVADLEAAGIGIIQIDEPALREGLPLRR-SDWDAYLQWGVEAFRINAAVAKDDT 636
AtMetE          ALAIKDEVEDLEKGGIGVIQIDEAALREGLPLRK-SEHAFYLDWAVHSFRITNCGVQDST 642
TmMetE          ALAINEEVKDLEEAGIKIVQIDEPAFREKAPIKK-SKWPEYFEWAINAFNLAAN-ARPET 613
                .**:.:** *** .** ::*:**.*:**  *::  ..   *  *. .:*.:    .  .*

ScMet6          QIHSHFCYSDLDP--NHIKALDADVVSIEFSKKDDANYIAEFKN---YPNHIGLGLFDIH 705
CaMet6          QIHSHFCYSDLDP--NHIKALDADVVSIEFSKKDDPNYIQEFSE---YPNHIGLGLFDIH 707
EcMetE          QIHTHMCYCEFNDIMDSIAALDADVITIETSR-SDMELLESFEEFD-YPNEIGPGVYDIH 694
AtMetE          QIHTHMCYSHFNDIIHSIIDMDADVITIENSR-SDEKLLSVFREGVKYGAGIGPGVYDIH 701
TmMetE          QIHAHMCYSDFNEIIEYIHQLEFDVISIEASR-SKGEIISAFENFKGWIKQIGVGVWDIH 672
                ***:*:**..::   . *  :: **::** *: .. : :  * :   :   ** *::***

ScMet6          SPRIPSKDEFIAKISTILKSYPAEKFWVNPDCGLKTRGWEETRLSLTHMVEAAKYFREQY 765
CaMet6          SPRIPSKQEFVSRIEEILKVYPASKFWVNPDCGLKTRGWPEVKESLTNMVEAAKEFRAKY 767
EcMetE          SPNVPSVEWIEALLKKAAKRIPAERLWVNPDCGLKTRGWPETRAALANMVQAAQNLRRG- 753
AtMetE          SPRIPSSEEIADRVNKMLAVLEQNILWVNPDCGLKTRKYTEVKPALKNMVDAAKLIRSQL 761
TmMetE          SPAVPSINEMREIVERVLRVLPKELIWINPDCGLKTRNWDEVIPSLRNMVALAKEMREKF 732
                ** :** : :   :.        . :*:********* : *.  :* :**  *: :*

ScMet6          KN-- 767
CaMet6          ----
EcMetE          ----
AtMetE          ASAK 765
TmMetE          ES-- 734
```

Figure 1.11 (continued). Amino acid sequences of cobalamin-independent methionine synthases. The sequences from *S. cerevisiae* (ScMet6p), *C. albicans* (CaMet6p), *E. coli* (EcMetEp), *A. thaliana* (AtMetEp), and *T. maritima* (TmMetEp) are shown. The symbols are defined as follows: identical residues (*), conserved residues (:), and semi-conserved residues (.).

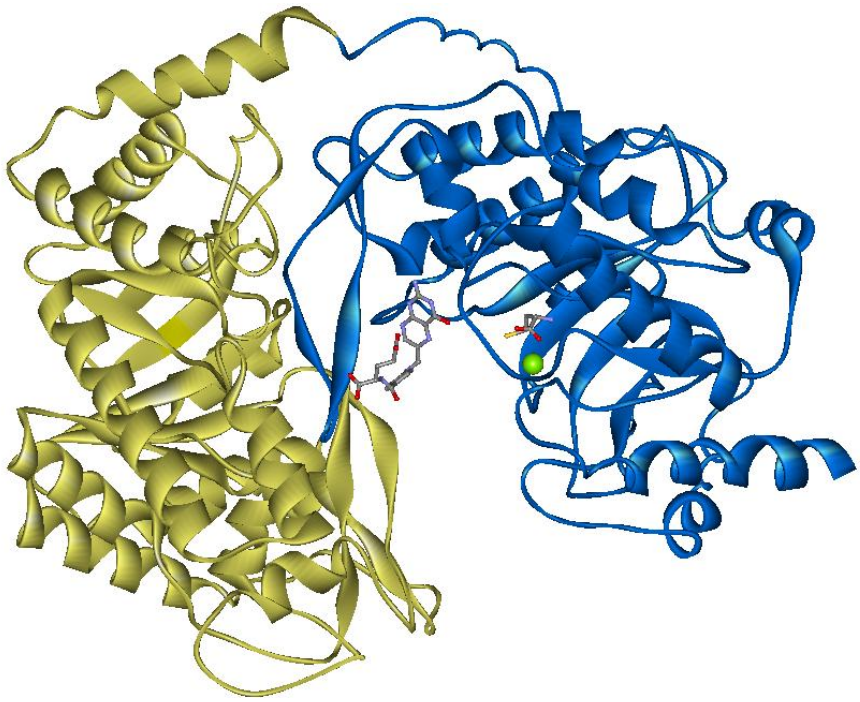


Figure 1.12 A ribbon diagram of the structure of cobalamin-independent methionine synthase from *Arabidopsis thaliana* (AtMetEp). Homocysteine and $PteGlu_5$ molecules are depicted as balls and sticks. The N-terminal domain (folate barrel) is in yellow and the C-terminal domain (homocysteine barrel) is in blue. The active site zinc is represented as a green sphere. (Ferrer *et al*, 2004).

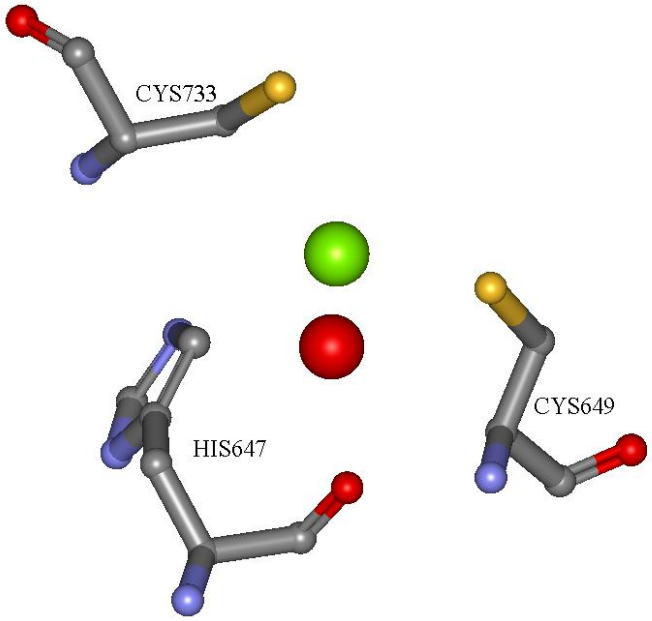


Figure 1.13 A close up view of the AtMetEp active site zinc binding residues. The His647, Cys649, and Cys733 residues are represented in the ball and stick format, and the side chains are colored according to atom type (nitrogen: blue, oxygen: red, carbon: gray and sulfur: yellow). The active site zinc is represented as a green sphere, and water 1 (W1) is represented as a red sphere. (Ferrer *et al*, 2004).

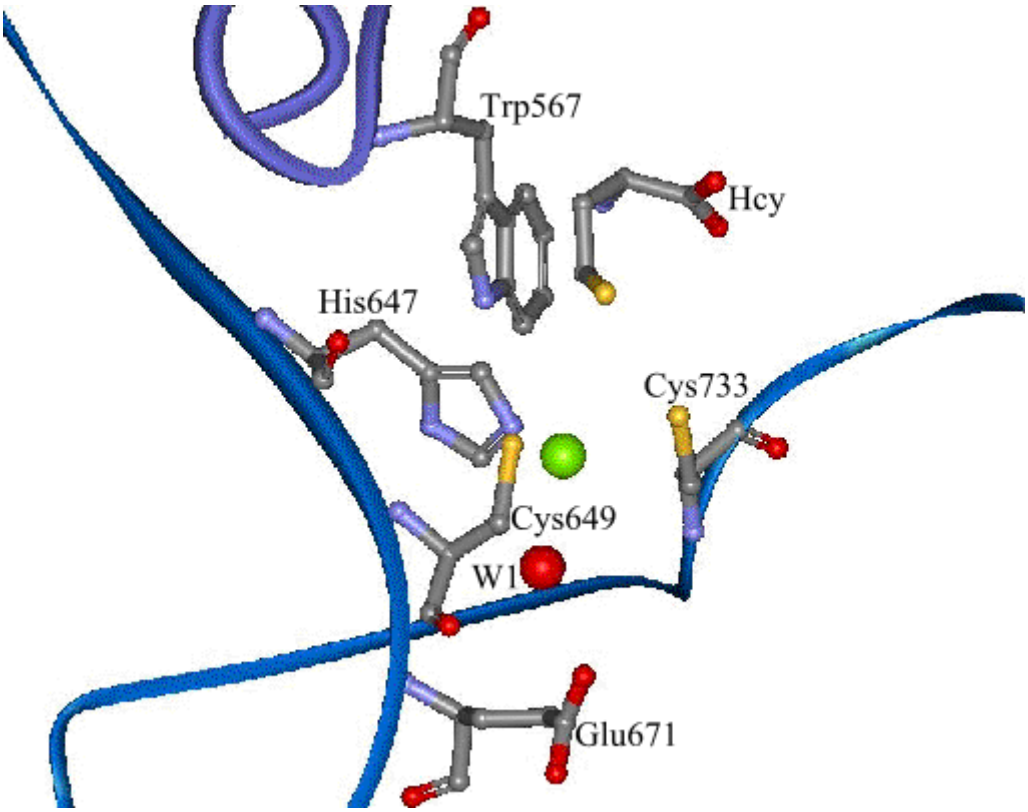


Figure 1.14 A close up view of homocysteine binding in the AtMetEp active site. (Ferrer *et al*, 2004).

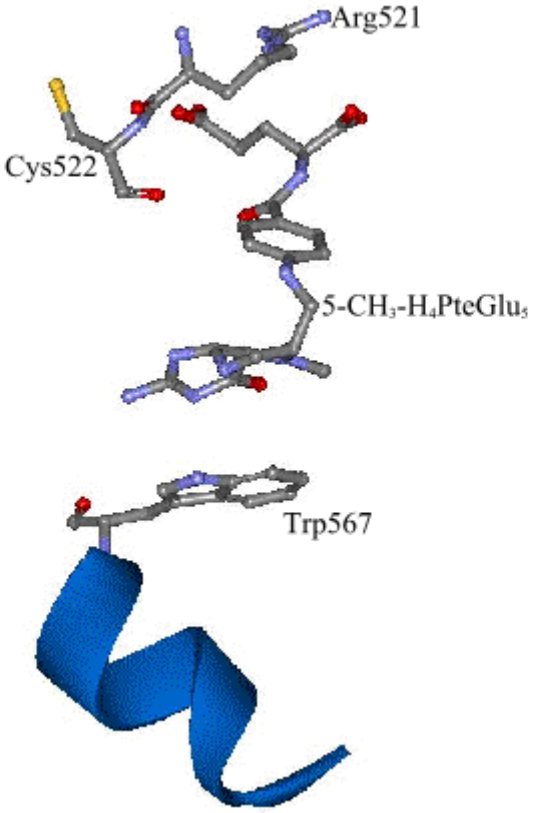


Figure 1.15 A close up view of 5-CH_3-H_4PteGlu$_5$ binding in the AtMetEp active site. The pteridine ring forms stacking interactions with Trp567. The glutamyl residue has ionic interactions with the side chain of Arg521 and hydrogen bonds to the backbone of Cys522. (Ferrer *et al*, 2004).

The three-dimensional crystal structure of the cobalamin-independent methionine synthase from *Thermotoga maratima* (TmMetEp) was determined by Robert Pejchal and Martha Ludwig (Pejchal *et al.*, 2004). This structure can be found in the RCSB Protein Data Bank (http://www.rcsb.org/pdb/) with ID numbers 1T7L, 1XDJ, 1XPG, and 1XR2. TmMetEp is a 734 amino acid enzyme, and has a similar structure to AtMetEp (Pejchal *et al*., 2004). Residues 1-351 form the N- terminal folate $(\beta\alpha)_8$ barrel, and residues 387-734 form the C-terminal homocysteine $(\beta\alpha)_8$ barrel. A linker joins the two domains with the active site formed between the domains (Figure 1.16). The N-terminal and C-terminal domains are similar in structure, and the N-terminal domain is thought to have evolved from the C-terminal domain by gene duplication, where the N-terminal domain lost the ability to bind zinc and homocysteine (Pejchal *et al*., 2004). The active site zinc is coordinated to four conserved residues, His618, Cys620, Cys704, and Glu642 (Figure 1.17). The amino group of homocysteine makes hydrogen bonds with Asp577, Glu462, and Ile409 (Figure 1.18). The carboxyl group of homocysteine binds to Ser411, and the sulfur coordinates to zinc, at a distance of 3.15Å (Figure 1.18). The pteridine ring of 5-CH_3-H_4PteGlu$_3$ stacks against a conserved Trp539, and makes hydrogen bond interactions with Lys104 and a water molecule (Figure 1.19). Glu583 interacts with 2-NH2 and N3 groups of the pteridine ring (Figure 1.19). The first glutamyl residue, of the polyglutamate chain of 5-CH_3-H_4PteGlu$_3$, binds to Arg15, Lys18, Arg493, and

Arg496. The orientation of 5-CH_3-H_4PteGlu$_3$ is not in an optimal position for the transfer of the methyl group to homocysteine, and may indicate that a conformational change is needed for catalysis to occur (Pejchal *et al*., 2004). The methyl faces away from homocysteine, and there is a distance of 11Å between the methyl and the sulfur of homocysteine (Pejchal *et al*., 2004).

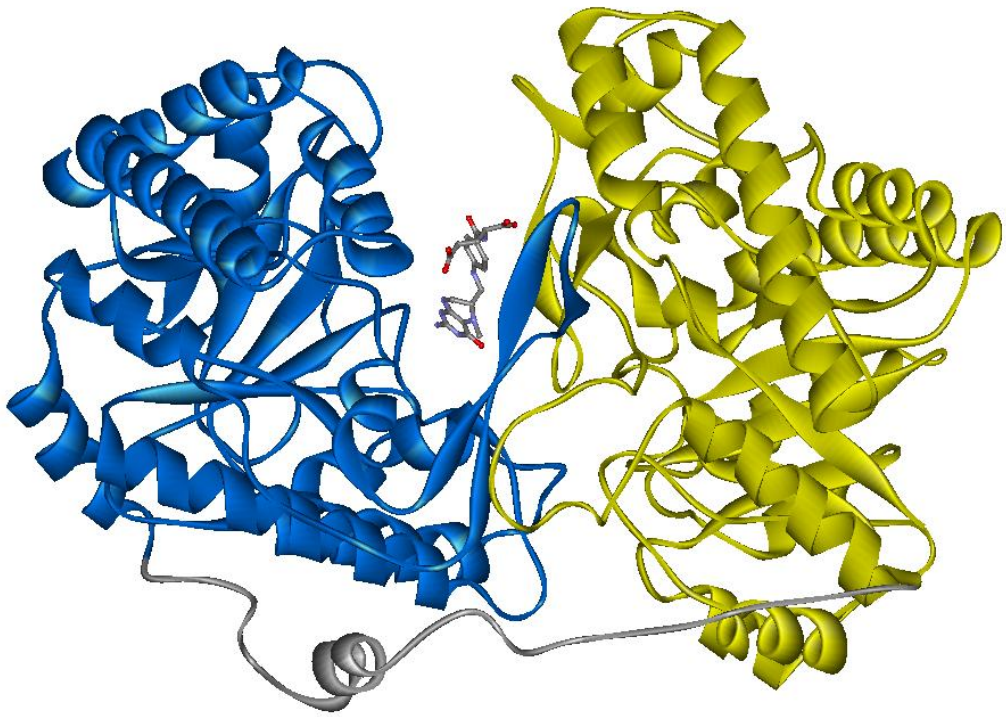


Figure 1.16 The structure of the *Thermotoga maritima* MetE protein (TmMetEp), in complex with 5-CH_3-H_4PteGlu$_3$. The N-terminal domain is in yellow, the C-terminal domain is in blue. Residues 1-351 form the N- terminal domain, and residues 387-734 form the C-terminal domain. The linker that joins the two domains is depicted in gray. (Pejchal *et al*, 2004).

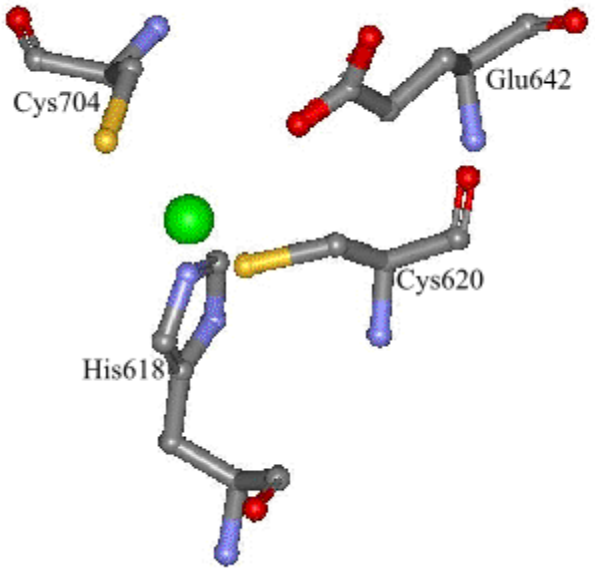


Figure 1.17 The active site zinc of TmMetEp is coordinated to four residues, His618, Cys620, Cys704, and Glu642. The zinc atom is represented as a green sphere. (Pejchal *et al*, 2004).

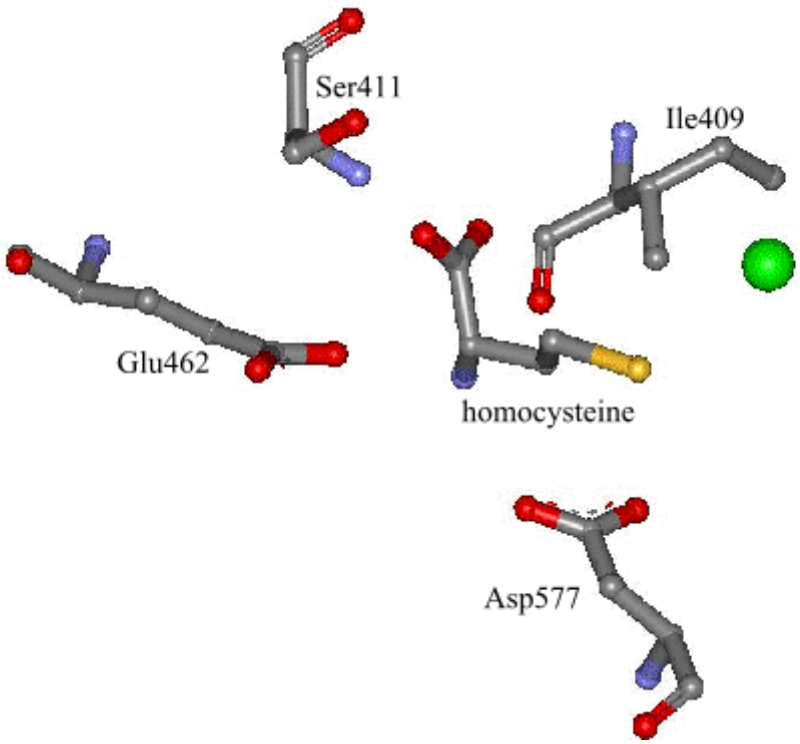


Figure 1.18 The homocysteine binding residues in the TmMetEp active site. The amino group of homocysteine makes hydrogen bonds with Asp577, Glu462, and Ile409. The carboxyl group of homocysteine binds to Ser411, and the sulfur coordinates to zinc (green sphere). (Pejchal *et al*, 2004).

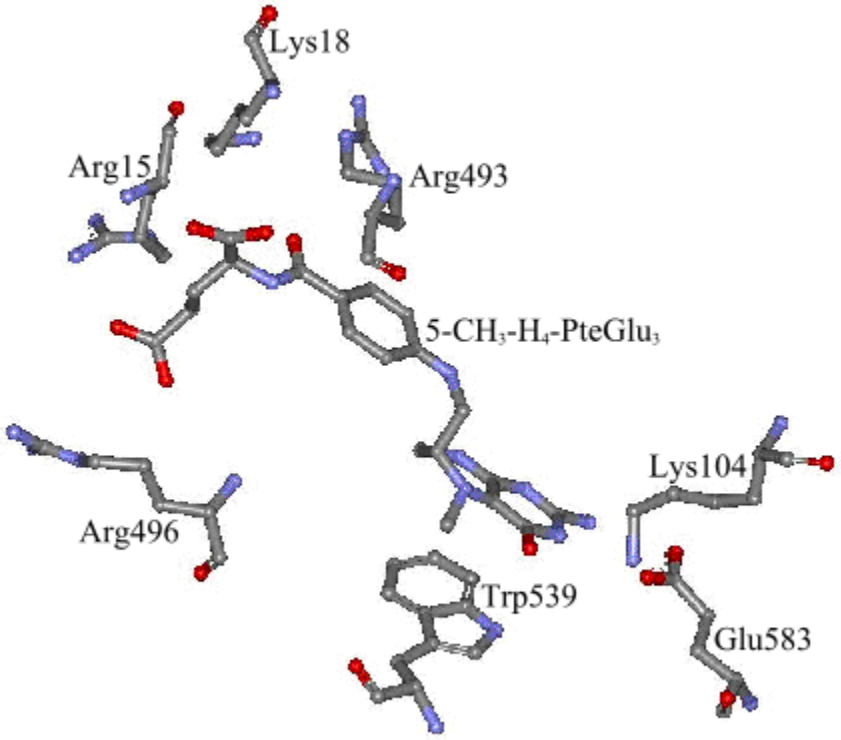


Figure 1.19 Binding of the folate substrate in the active site of TmMetEp. The pteridine ring of 5-CH_3-H_4PteGlu$_3$ stacks against a conserved Trp539, and makes hydrogen bond interactions with Lys104. Glu583 interacts with 2-NH2 and N3 groups of the pteridine ring. The first glutamyl residue, of the polyglutamate chain of 5-CH_3-H_4PteGlu$_3$, binds to Arg15, Lys18, Arg493, and Arg496. (Pejchal *et al*, 2004).

In both of the cobalamin-independent methionine synthase structures only one glutamyl residue is observed, even though the length of the polyglutamate chain is critical for enzyme activity (Ferrer *et al*., 2004; Pejchal *et al*., 2004). One reason for this is that the polyglutamate chain may not interact with specific groups in the active site (Fu *et al*., 2003). If the polyglutamate chain occupies several different conformations, the electron density will not be clearly resolved. Also, the high salt concentrations in the crystal conditions may interfere with the binding of the anionic polyglutamate chain to the active site (Fu *et al*., 2003).

Proposed Catalytic Mechanisms

Zinc is often a cofactor in proteins that use thiols as nucleophiles, including proteins that transfer methyl groups to thiols (Matthews *et al*., 1997). Both MetHp and MetEp activate the homocysteine upon binding it to zinc (Peariso *et al*., 2001). Both enzymes must catalyze the transfer of a poor leaving group, methyl from 5-CH_3-H_4PteGlu$_n$, to a poor nucleophile, the sulfur of homocysteine (Pejchal *et al*., 2004). MetHp utilizes cobalamin to facilitate this reaction. MetEp appears to catalyze the direct transfer of the methyl group, from 5-CH_3-H_4PteGlu$_n$, to the methyl acceptor, homocysteine (Pejchal *et al*., 2004). In order for the methyl to become a leaving group, 5-CH_3-H_4PteGlu$_n$ needs to be protonated. This only occurs when a ternary complex forms, when the MetEp binds to 5-CH_3-H_4PteGlu$_n$ and homocysteine (Taurog *et al*., 2006). Upon binding homocysteine alone, the active site zinc binds and converts the thiol to a thiolate,

activating homocysteine (Taurog *et al*., 2006). In contrast, when MetEp binds 5-CH_3-H_4PteGlu$_n$, the methyl is pointed away from the zinc and is not protonated. However, both these binary complexes are suitable for methyl transfer reactions (Taurog *et al*., 2006). The rate constants and equilibrium constants have been determined for MetEp, and these constants were used to explain the steps in substrate binding (Taurog *et al*., 2006). The two substrates were found to bind synergistically, where the binding of one substrate increases the affinity of the other by ~30 fold (Taurog *et al*., 2006). So, binding of either 5-CH_3-H_4PteGlu$_n$ or homocysteine appears to reorganize the active site in favor of binding the other substrate (Taurog *et al*., 2006).

Both MetHp and MetEp have similar strategies for catalyzing the transfer of the methyl group from 5-CH_3-H_4PteGlu$_n$ to homocysteine (Taurog *et al*., 2006), but the actual mechanisms by which this occurs is different. In MetHp, 5-CH_3-H_4PteGlu$_n$ and homocysteine bind to separate domains, and cobalamin mediates the transfer of the methyl group from 5-CH_3-H_4PteGlu$_n$ to homocysteine. MetEp catalyzes the direct transfer of the methyl group from 5-CH_3-H_4PteGlu$_n$ to homocysteine, which requires 5-CH_3-H_4PteGlu$_n$ to be in proximity to homocysteine. The significant mechanistic differences between MetHp and MetEp make the cobalamin independent methionine synthase an intriguing anti-fungal drug target. A compound consisting of a fusion of both substrates can be used as a specific inhibitor for cobalamin-independent

methionine synthases. In *Saccharomyces cerevisiae*, the cobalamin-independent methionine synthase gene is labeled *MET6* (Csaikl and Csaikl, 1986). I will refer to the *S. cerevisiae MET6* gene as *ScMET*6 in this thesis. The *ScMET6* open reading frame (*Saccharomyces* Genome Database ORF YER091c; http://www.yeastgenome.org/) is located on chromosome V of the *S. cerevisiae* genome, and encodes a protein of 767 amino acids. The *MET6* gene is conserved in many other fungi, including some pathogens. The *MET6* gene in *Candida albicans* (*CaMET6* ORF CA0653; http://genolist.pasteur.fr/CandidaDB/) encodes a homologous protein that is 75% identical to that encoded by the *ScMET6* gene (Fig. 1.11). Since humans use only cobalamin-dependent methionine synthase and all fungi investigated to date, including *C. albicans*, use only the cobalamin-independent enzyme, the Met6 protein could be a promising anti-fungal drug target.

Saccharomyces cerevisiae

Among the many different kinds of yeasts, *S. cerevisiae* is traditionally used for experimentation, and the complete DNA sequence of its genome has been determined. There are three types of *S. cerevisiae* cells, the **a**, the α, and the **a**/α. The **a** and the α are haploid, and the **a**/α is diploid. Both haploid and diploid cells can reproduce asexually by budding (Figure 1.20). When a bud forms and nearly reaches the size of the parent cell, the nucleus divides, and the two cells separate into the parent cell and the daughter cell (Pringle *et al.,* 1981). Budding

leaves behind a bud scar. The diploid cells can undergo meiosis and recover the haploid mating types.

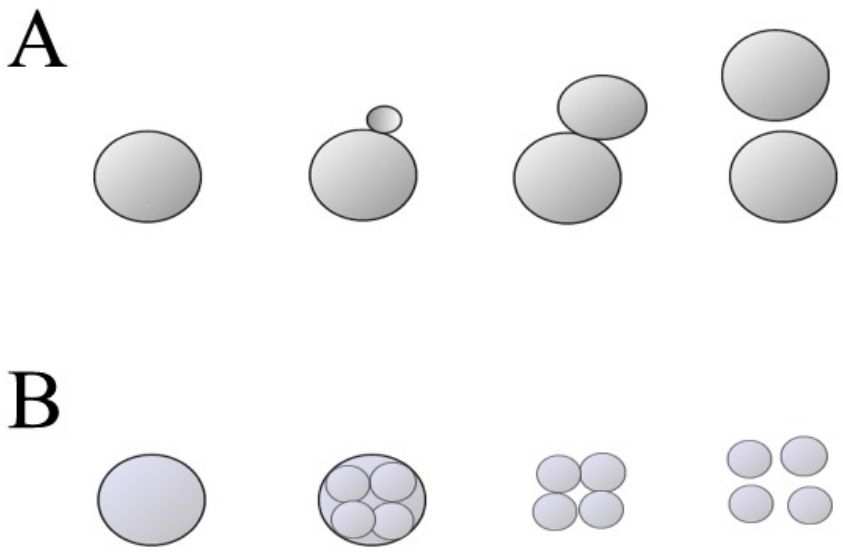


Figure 1.20 Asexual Reproduction in Yeast. **A.** Both haploid and diploid yeast cells can reproduce through budding. **B.** Under starvation conditions, diploid yeast cells can undergo meiosis and form haploid ascospores that give rise to yeast cells after they germinate.

Haploid *S. cerevisiae* cells can undergo sexual reproduction, which occurs when the **a** and the α cells mate (Figure 1.21). The sexual reproduction cycle alternates between haploid and diploid yeast cells. Sexual reproduction begins when pheromones are released, and bind to cell surface G-coupled protein receptors. Each cell type responds to the signals by forming a gamete. When this occurs, the cells stop dividing and they elongate to form shmoos (Figure 1.21). When shmoos of opposite mating type come into close proximity to each other, they fuse to form a zygote. This fusion results from two shmoos joining together at their small ends with a constriction in between. Each shmoo contains a haploid nucleus, and both nuclei come together and fuse to form a diploid **a**/α nucleus. Zygotes are easy to identify when they form the first bud, and they form a diploid colony. Cells of the diploid colony then undergo mitosis. The next step in the life cycle is the conversion of the diploid cell into the ascus, which contains the ascospores that are produced through meiosis. This step marks the transition from the diploid phase back to the haploid phase of the life cycle. The spores are then released from the ascus, and the spores germinate to form either **a** or α haploid cells.

Plasmid DNA containing a gene of interest can be easily transformed into yeast cells. Genes that complement specific auxotrophic strains are used as markers to select for the cells that have been successfully transformed. Some common selection markers used are the yeast *URA3* gene, *LEU2* gene, *HIS3* gene,

and *TRP1* gene, which complement *ura3*, *leu2*, *his3*, and *trp1* mutants respectively. Dominant genes are denoted using capitalized italics for all three letters, and recessive genes are denoted using lowercase italics for all three letters.

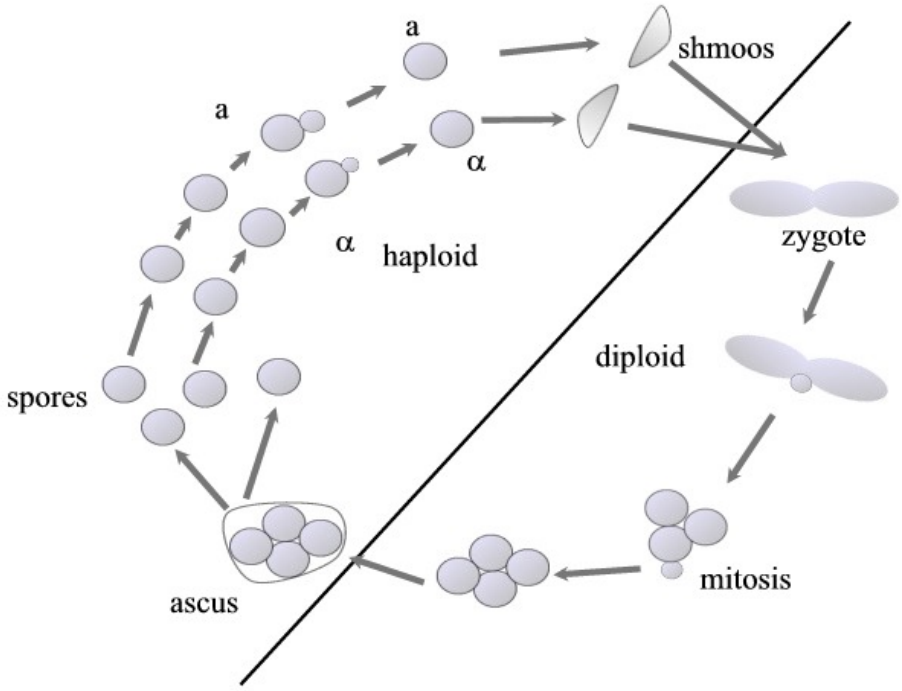


Figure 1.21 The yeast life cycle. The **a** and the α haploid mating types form shmoos, that fuse together to form a diploid zygote. The zygote forms a bud, and divides by mitosis, forming a diploid colony. An ascus is formed, and spores are the result of meiosis. The ascospores are released, and germinate to form haploid cells. (Adapted from http://www.phys.ksu.edu/gene)

Genes can be cloned into yeast vectors using the same approach used for *E. coli.* CEN plasmids are low copy, autonomously replicating, vectors maintained at 1-3 copies per cell. A CEN plasmid contains a centromere, which allows the attachment of the plasmid to the mitotic spindle. This ensures that the plasmid segregates to the mother and daughter cells during the cell cycle. These vectors can be used with any promoter.

YEp vectors are high copy autonomously replicating vectors and are present at 20-50 copies per cell (Burke *et al.*, 2000). These plasmids have a 2μ origin of replication, which allows the plasmids to segregate to the mother and daughter cells during mitosis (Figure 1.22). These plasmids can also be used with any promoter. A high copy vector coupled with a strong promoter can produce an abundant amount of protein. Many yeast vectors also contain an *E. coli* origin of replication, and an ampicillin resistance gene, for cloning and propagation in *E. coli*.

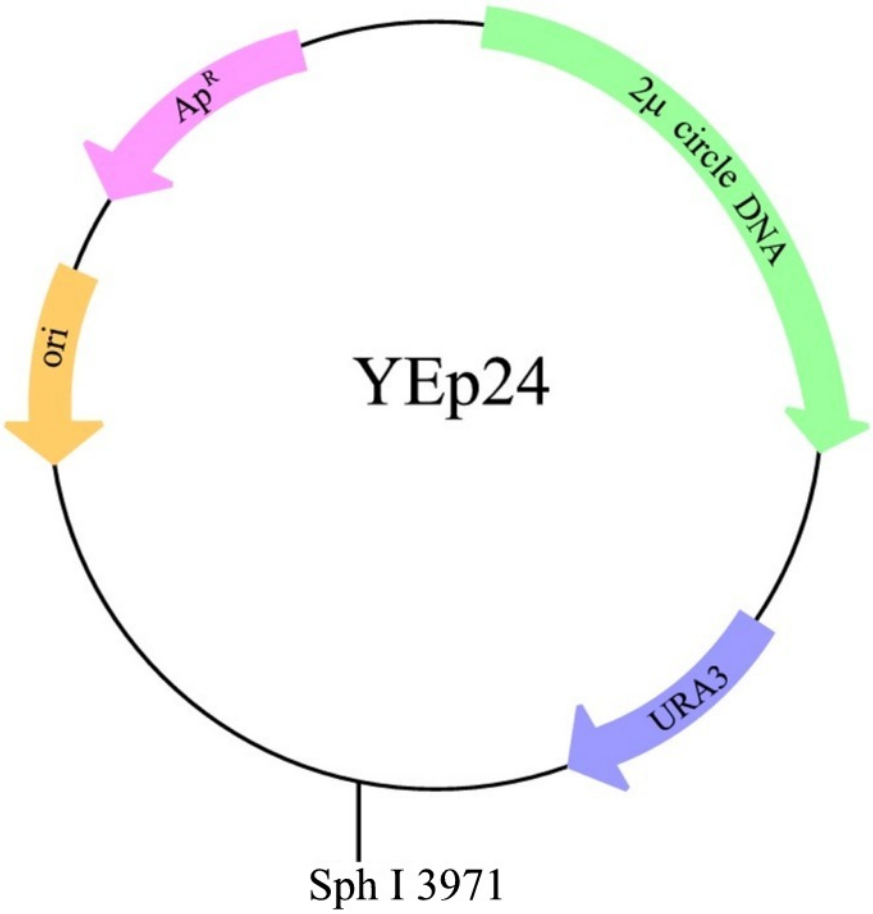


Figure 1.22 Map of the YEp24 yeast expression vector. This vector has a 2μ origin of replication, and *URA*3 auxotrophic marker. It also has the bacterial origin of replication (*ori*), and ampicillin resistance gene (Ap^R).

Candida albicans

C. albicans is genetically very similar to *Saccharomyces cerevisiae*, and is an important model for pathogenic yeast. However, *C. albicans* has distinct differences, which are thought to be the key to its pathogenicity. Possibly, the two important differences are that *C. albicans* is asexual and diploid (Whelan *et al*., 1981). Also, *C. albicans* has additional morphologies (Figure 1.23), which develop under different environmental conditions. For example, *C. albicans* is one of few *Candida* species that forms a morphologically distinct type of germ tube. Changes in temperature, changes in pH, immune responses, and an increase in *C. albicans* population trigger production of germ tubes. Germ-tube formation can be induced in-vitro by growing the cells in serum, and this is the traditional method for identifying *C. albicans* in clinical samples (Taschdjian *et al*., 1960). Furthermore, *C. albicans* can reproduce in two different ways. One way, is to bud from yeast cells, and the other way is to branch off into pseudohyphae, which eventually break off and form new individuals (Figure 1.23).

C. albicans is normally present in the natural flora of mammals including human beings. It inhabits mucosal surfaces in the oral cavity, esophagus, vaginal cavity, and gastrointestinal tract. In humans with healthy immune systems bacteria present in the normal flora, such as *Escherichia coli, Pseudomonas aeruginosa,* and *Lactobacillus acidophilus* (Fitzsimmons *et al*., 1994; Jack *et al*., 1990), keep *C. albicans* growth under control. In physiologically or medically

immunosuppressed patients, or immunocompromised patients *C. albicans* is able to overgrow and cause infection. In the past two decades, *C. albicans* has become one of the most common opportunistic microorganisms causing nosocomial infections. Patients susceptible to *Candia albicans* infections include those with autoimmune diseases, HIV/AIDS, catheters, pregnancy, antibiotic therapy, neutropenia, and solid organ transplants.

There are two types of *C. albicans* infections, superficial and invasive. Examples of superficial infections include oral and vaginal thrush. Under favorable conditions *Candida* cells multiply and cause a white build up of cells and secretions. Superficial *C. albicans* infections are easier to diagnose because those areas of the body are readily accessible for testing. Samples of the infected tissue can be taken and tested in the laboratory. Oral candidiasis can change from the superficial oral thrush into the more invasive oropharangeal candidiasis in AIDS patients. This occurs when the disease spreads from the oral cavity to the esophagus, creating a severe form of the infection.

Systemic infections are invasive, and the main source of *C. albicans* cells involved is the gastrointestinal tract. Overgrowths of cells in the intestine cause *C. albicans* to undergo a morphologic change from yeast cells to hyphae. If the infection is untreated, the hyphae can tunnel through the cellular tissue of the gastrointestinal tract and release secreted aspartic proteinases and *C. albicans* yeast cells into the blood stream. Once in the blood stream, the secreted aspartic

proteinases (SAPs) allow *C. albicans* to invade organs in other parts of the body. Some results of systemic infections include invasion of cardiac tissue, kidneys, and the brain.

Systemic infections can be induced in mice, which are very similar to those found in humans. In the mouse model, the main focus of infection is in the kidneys. After infecting immunocompromised mice, *C. albicans* is deposited in lungs, liver, spleen and kidney, but only the kidneys become infected. The kidneys are where it is possible for researchers to best follow the course of the disease (Molero *et al*., 1998). Systemic infections in humans most often occur in neutropenic patients. Neutropenic patients are those that lack the important neutrophil immune cells. Neutrophils are innate phagocytic cells involved in killing, release of chemokines and cytokines, and the costimulation in response to invading pathogens. Systemic *C. albicans* infections are very difficult to diagnose, because of the inability to distinguish between normal levels and overgrown levels of *C. albicans* in the body. This in turn delays treatment, which allows the infection to progress and become more severe.

Once a *C. albicans* infection has been identified there are drug treatments available. One class of drug used to treat various *C. albicans* infections is the polyene class of anti-fungal agents. One of the commonly used drugs from this class is Amphotericin B (Figure 1.24). Amphotericin B binds to sterols, preferentially ergosterol, and disrupts the osmotic integrity of the fungal

membrane (Terrell et. al., 1992). This disruption causes important components of the fungal cell to leak out and the organism dies. Amphotericin B is an effective fungicide, but resistance to this drug is growing rapidly (Nolte *et al*., 1997). *C. albicans* becomes resistant to Amphotericin B through a mechanism that allows for alteration of the membrane sterols (Nolte *et al*., 1997). Resistant *C. albicans* has been shown to have a diminished ergosterol content, and an increase in other types of sterols. For maximum effectiveness, Amphotericin B is used in combination with other anti-fungal drugs. One of these drugs is Fluconazole, which belongs to the largest family of anti-fungal agents, the azoles (Figure 1.24). Fluconazole binds to the heme iron of cytochrome P450 (Erg11p), and inhibits enzymatic reaction (Kelly *et al*., 1997). Another target for azoles is the inhibition of sterol 14α-demethylase, a cytochrome P450 enzyme that is involved in ergosterol biosynthesis (Kelly *et al*., 1997). Fluconazole, unlike most other azoles, is water soluble, and is a favorite anti-fungal agent. *C. albicans* can acquire resistance to fluconazole, which is often seen in HIV-patients (Rex *et al*., 1995). Increasing drug resistance and severe side effects results in the constant search for new drug targets to create better and more effective therapies.

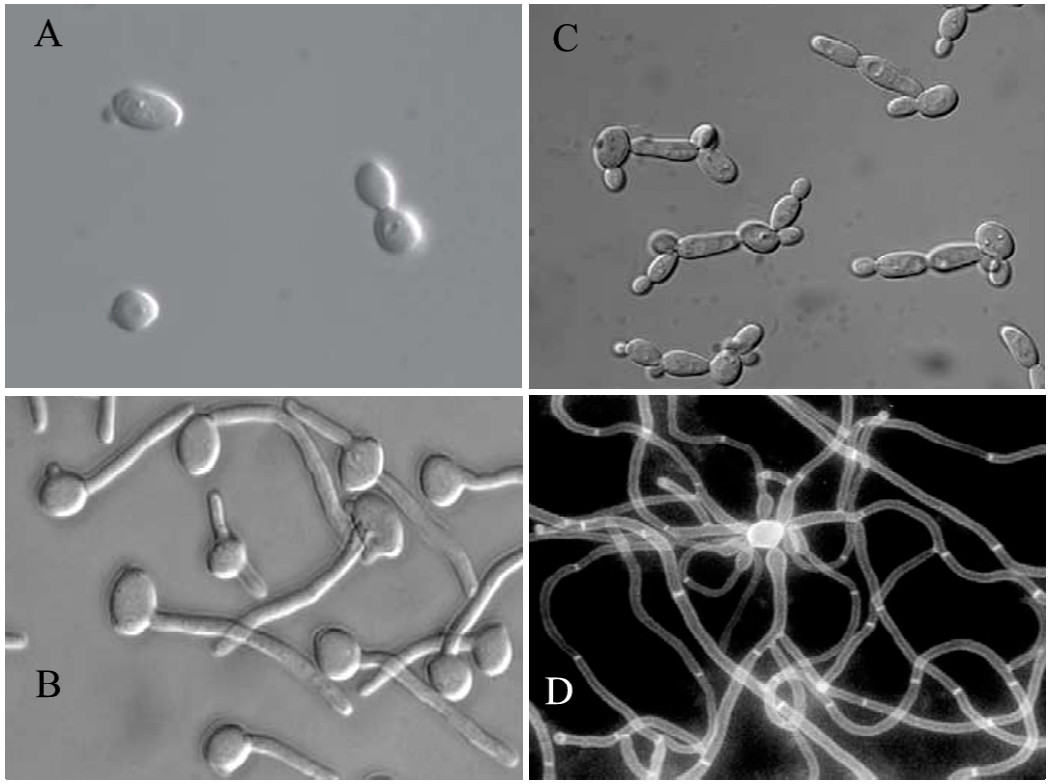


Figure 1.23: Different morphologies of *C. albicans.* (A) *C. albicans* yeast cells. They are similar to *S. cerevisiae* diploid cells. (B) Germ tube formations extend from the yeast cells, and are narrow and uniform. (C) Pseudohyphae are elongated, with constrictions at the septa. (D) The hyphal form has parallel walls. (Reprinted from *Trends in Microbiology*, Volume 12, Peter Sudbery, Neil Gow, and Judith Berman, The Distinct Morphogenic States of *Candida albicans*, Pages 317-324, July 2004, with permission from Elsevier.)

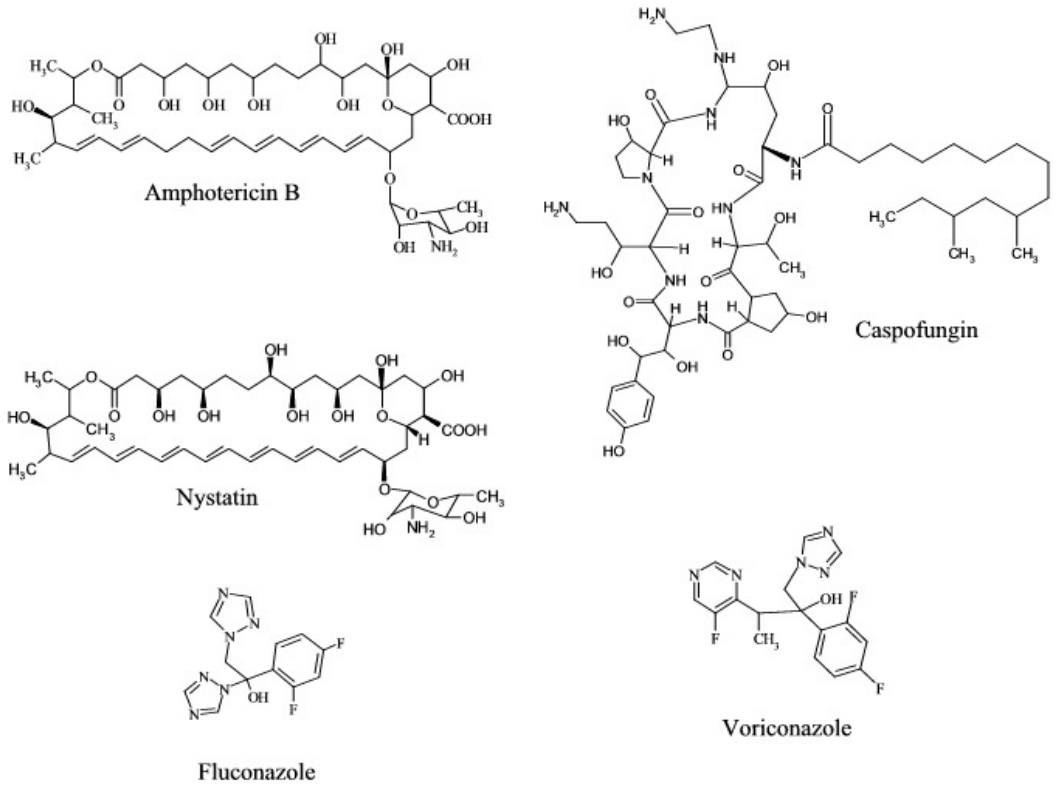


Figure 1.24: Structures of commonly used drugs for treatment of *C. albicans* infections.

Experimentation in *Candida albicans*

C. albicans is a major model for pathogenic yeast. *C. albicans* gene functions can be assessed by complementation of *S. cerevisiae* mutants, although study in *C. albicans* is preferable. The *C. albicans* genome is difficult to manipulate due to its diploid nature, and the lack of a complete sexual cycle for use in experimental manipulations (Molero *et al*., 1998). In recent years there have been many advancements in transformation, gene expression, and gene disruptions for *C. albicans*. These tools, along with the complete genome sequence of *C. albicans,* have allowed for rapid identification of essential genes. Gene disruption methods rely on the ability to transform with DNA, and the integration of that DNA into the *C. albicans* genome through homologous recombination, which occurs frequently in *C. albicans*. This allowed for auxotrophic mutants to be created and used as hosts for transformation experiments. These auxotrophs include strains with the following mutations: *ura3, ade2, leu2, his1, arg4, and arg5, 6* (Calderone *et al*., 2002). The ability to test for essential genes allows the validation of potential anti-fungal drug targets.

Project Goals

In this study, methionine synthases from *C. albicans* (*CaMET6p*) and *S. cerevisiae* (*ScMET6p*) were investigated. This ongoing project is a collaborative effort with Appling laboratory (The University of Texas at Austin). The initial experiments began when Bert Vick, a technician from the Appling laboratory,

cloned *ScMET6*, including the native *ScMET6* promoter into the YEp24 yeast shuttle vector (Suliman *et al*., 2005). The ScMet6p was expressed in the *S. cerevisiae* cell strain DAY4.1 (**a** *ser1 ura3-52 trp1 leu2*). Susan Dubrowski, an undergraduate student from the Appling laboratory, constructed the SDYα *Scmet6* mutant strain (Suliman *et al*., 2005). The pEGKT construct containing the *ScMET6* promoter was created in the Robertus laboratory (The University of Texas at Austin), in which I worked.

The focus of my study was to clone, express and purify CaMet6p, and to express and purify ScMet6p. The kinetic parameters and the requirement for polyglutamylated 5-methyltetrahydrofolate were explored using the purified protein. A goal of this work was to implement a strategy for the study of site-directed mutants. Apart from the recombinant protein, preliminary experiments to study *CaMET6* in *C. albicans* were performed. *A* conditional cobalamin-independent methionine synthase (*CaMET6*) mutant in *C. albicans* was constructed, using the PCR-based gene disruption method, to assess the viability of the resulting null mutant strain. The results from these experiments have provided insights into enzyme function, and support the study of CaMet6p as an anti-fungal drug target.

CHAPTER 2: MATERIALS AND METHODS

MATERIALS

The pre-packed heparin column and the DEAE Sepharose beads were obtained from Pharmacia Biotech (Piscataway, NJ). PteroylGlu$_n$ was obtained from Schirck's Laboratories (Jona, Switzerland). $Pb(NO_3)_2$ was obtained from Mallinkrodt. L-Homocysteine thiolactone was obtained from Sigma Chemical (St. Louis, MO). The yeast expression vector YEp24 was obtained from Dr. Makkuni Jayaram and genomic *C. albicans* DNA was obtained from Dr. Paul J. Szaniszlo (both at The University of Texas at Austin). All chemicals and reagents were of high quality and were stored as per the recommendations of the manufacturer. Other specific materials and their sources are provided in the methods section and in Appendix A.

METHODS

EXPRESSION OF ScMET6p

YEp-*ScMET6* was transformed into the *Saccharomyces cerevisiae* strain DAY4.1 (**a** *ser1 ura3-52 trp1 leu2*) using the lithium acetate method (Gietz and Woods, 2002). The DAY4.1 cell strain is auxotrophic for serine, uracil, tryptophan, and leucine. The YEp24 expression vector has a *URA3* selection marker, selecting for the ability to grow in media without uracil supplementation.

Transformed cells were grown in synthetic minimal medium (YMD) containing 0.7% yeast nitrogen base without amino acids (Difco Bacto®) and 2% glucose, supplemented with the following amino acids: L-serine (375 mg/L), L-leucine (30 mg/L), L-tryptophan (20 mg/L). The media, and all glucose and amino acid stocks, were made with the highest purity of deionized, distilled water available, to allow the yeast cells to grow optimally. The cell cultures were batch-grown at 30°C in a rotary shaker. Three liters of culture were grown at a time, each liter grown in a 2.8 L pyrex flask. When the cultures reached late log phase, 3-4 $OD_{600,}$ they were harvested in 500 ml centrifuge tubes at 5000 x g for 10 minutes.

Cloning and Expression of the *C. albicans MET6* gene.

The *C. albicans MET6* gene (CandidaDB; http://genolist.pasteur.fr/CandidaDB/) was obtained from the *C. albicans* genome by high-fidelity PCR using primers with the sequences 5'-**ATATCAAAAATGGTTCA**ATCTTCCGTC-3' [CaMET65'; overlap with ScMET6SOE primer (below) is in **bold** and the start codon of *MET6* orf is underlined] and 5'-CGACGGCATGCGTCTCCAAATTAATCAC-3' (CaMET63'; SphI site is underlined). The amplified fragment started 1 bp upstream of the AUG start codon and ended 187 bp after the stop codon, to include the poly adenylation sequence. Splice-overlap extension (SOE) PCR (Horton *et al*., 1993) was used to precisely replace the *S. cerevisiae MET6* ORF in YEp-ScMET6 with the *C. albicans MET6* ORF (Figure 2.1). The *S. cerevisiae*

MET6 promoter region (592 bp) was amplified from YEp-*ScMET6* using primers *ScMET6*5' and *ScMET6*SOE (5'-**TGAACCATTTTTGATAT**GTACTTTGA-3'; overlap with *CaMET6*5' primer is in **bold**). This fragment was joined with the *C. albicans MET6* ORF amplified fragment (1:1 ratio) in a second PCR using primers *ScMET6*5' and *CaMET6*3'. The spliced amplified fragment was digested with *SphI* and cloned into YEp24 as before. The insert was completely sequenced to ensure no PCR-induced mutations had occurred. This construct, YEp-*CaMET6*, has the same orientation as YEp-*ScMET6*, and the *C. albicans MET6* ORF was transcribed using the *S. cerevisiae MET6* promoter. For expression and purification of the *C. albicans Met6* gene product in *S. cerevisiae*, YEp-*CaMET6* was transformed into the *met6* disruptant strain SDYα, which allowed selection for the ability of transformants to grow without methionine supplementation. Transformed cells were batch-grown in YMD supplemented with L-serine, L-leucine, L-tryptophan, and L-histidine (20 mg/L) at 30°C in a rotary shaker and were harvested at 3-4 OD_{600}.

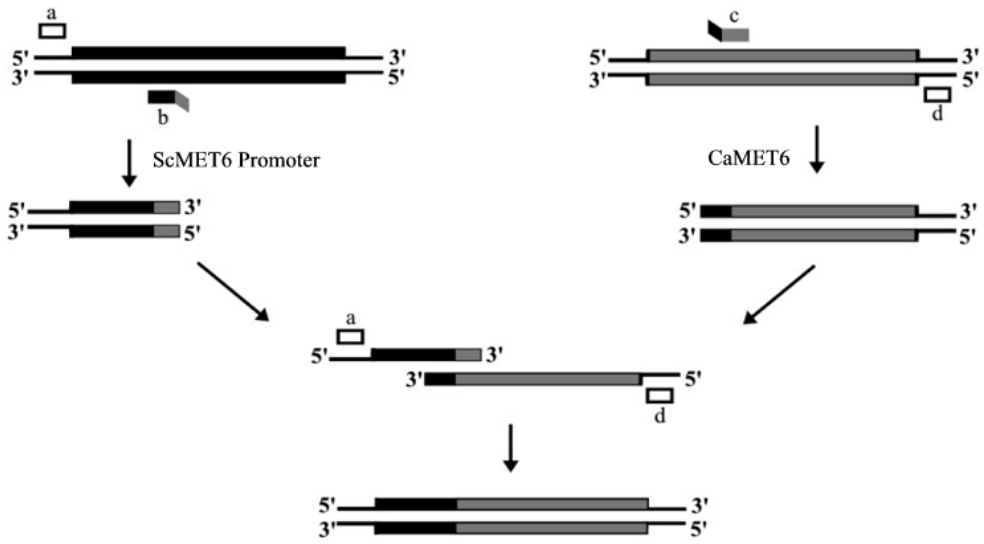


Figure 2.1: Splicing by overlap extension using polymerase chain reaction (PCR). In the first step, the *ScMET6* promoter is amplified from plasmid DNA using primers a and b, and the *CaMET6* gene is amplified from genomic DNA using primers c and d. Primers b and c contain sequences that will allow the two PCR products to overlap and bind to each other. In the Second step, the promoter and the gene are combined in a final PCR to produce the final product. (Adapted from Warrens *et al.*, 1997)

Purification of ScMet6p and CaMet6p

The purification protocols for ScMet6p and CaMet6p were modified from the purification reported by Burton and Sakami (1969). The following first three steps were used in purifying both ScMet6p and CaMet6p.

Step 1. Transformed *S. cerevisiae* cells were grown in three liters of minimal medium and were harvested at 3-4 OD_{600} by chilling and centrifuging at 4000 x g for 5 min. The cell pellet was suspended in 0.05 M KPO_4/pH 7 buffer (2 ml/gram wet weight of pellet). Four passes through a 20K French Press cell at 1000 psi disrupted the suspended cells. The broken cell suspension was centrifuged at 20,000 x g, at 4°C, for 1 hour to remove cell debris and unbroken cells.

Step 2. The supernatant was heated to 55°C, and kept at that temperature for 5 min. The solution was cooled rapidly to below 10°C in an ice bucket. The solution was then centrifuged at 8000 x g and 4°C for 10 min. to remove denatured protein. The supernatant solution was collected for further purification.

Step3. The solution pH was reduced to pH 4.5 by adding glacial acetic acid, and immediately centrifuged at 10,000 x g, at 4°C, for 10 min. The pellet was discarded, and the supernatant brought to pH 7.0 using 2M KOH.

Step4. ScMet6p was further purified using a 5 ml pre-packed HiTrap heparin column (Amersham Pharmacia Biotech.). The column was washed with 0.02 M KPO_4, pH 7.4, buffer. The enzyme solution was then applied to a heparin

column, and the column was washed with 10 ml 0.02 M KPO_4, pH 7.4 buffer. The enzyme was eluted using a linear gradient prepared from 25 ml 0.02 M KPO_4, pH7.4 buffer, and 25 ml 0.02 M KPO_4/1.5 M KCl buffer at pH 7.4.

CaMet6p was further purified using a 35 ml DEAE Sepharose column. The column was washed with 0.02 M KPO_4, pH 7.4 buffer. The enzyme solution was then applied to the column, and the column was washed with 70 ml of 0.02 M KPO_4, pH 7.4 buffer. The enzyme was eluted using a linear gradient prepared from 150 ml 0.02 M KPO_4, pH7.4 buffer and 150 ml 0.02M KPO_4/1.5 M KCl buffer at pH 7.4.

ENZYME ASSAY

The enzyme assay was performed using a modification of the procedure described by Drummond *et al*. (1995). Standard 400 µl reaction mixtures contained 50 mM potassium phosphate (pH 7.2), 50 mM Tris-Cl (pH 7.2), 100 µM $MgSO_4$, 10 mM dithiothreitol, 2 mM L-homocysteine, 66 µM (6*R,S*)5-CH_3-H_4PteGlu$_n$, and 9 µg (263 nM) of enzyme. The reactions were preincubated at 37°C for 5 minutes, then initiated by the addition of 50 µl of enzyme, and terminated after 10 min by the addition of 100 µl of 5N HCl/60% formic acid. The reactions were then incubated at 80°C for 10 min, followed by brief centrifugation to pellet any precipitated protein. The methenyl derivative of the H_4-PteGlu$_n$ product was quantified spectroscopically at an absorbance of 350 nm, using an extinction coefficient of 26,500 $M^{-1}cm^{-1}$. The K_m values for 5-CH_3-

$H_4PteGlu_n$ were determined from 6 different substrate concentrations within the range of 0-500 μM, at saturating L-homocysteine concentration (2 mM). The K_m values of L-homocysteine were determined from 6 different concentrations within the range of 0-200 μM, at saturating (6*R,S*)CH_3-$H_4PteGlu_n$ concentration (500 μM). Initial velocity data were fitted to the Michaelis-Menten equation using non-linear regression in the program SigmaPlot (Systat Software, Inc. Point Richmond, CA).

SYNTHESIS OF (6R,S)5-CH_3-H_4-PTEGLU$_N$ FROM PTEGLU$_N$

(6*R,S*)-5-CH_3-$H_4PteGlu_n$ was synthesized from $PteGlu_n$ (Schirck's Laboratories Jona, Switzerland) by a modification of the procedure of Yeo and Wagner (1992). 200 μl of 265 μM $Pb(NO_3)_2$ was added to 40 μmol of $PteGlu_n$, and then dissolved by adjusting the pH of the solution to 7.5 using a 5 M NaOH solution, and vortexing. $PteGlu_n$ was then reduced to $H_4PteGlu_n$ by adding 132 μmol $NaBH_4$, at 4°C while stirring. Once all of the $NaBH_4$ is added, the solution was moved to room temperature and stirred at maximum speed, in the dark and under N_2, for 2 hours. The excess $NaBH_4$ was destroyed by cooling the solution to 4°C, and lowering the pH to 5.0, by adding 5 M acetic acid. The pH was then adjusted to 7.8 using 5 M NaOH.

To synthesize CH_2-$H_4PteGlu_n$, $H_4PteGlu_n$ was condensed with 80 μmol of 37% formaldehyde at 45°C for 15 minutes, in the dark. CH_2-$H_4PteGlu_n$ was then

reduced to 5-CH_3-H_4PteGlu$_n$ using $NaBH_4$. Ten mg of $NaBH_4$ (264 μmol) was added to 50 μl of ddH_2O, and 10 μl of this solution was added to the tube 5 times over 20 minutes. The pH was maintained by adding 2-5 μl of 20% (w/v) citric acid per addition of $NaBH_4$. Once all of the $NaBH_4$ was added, the tube was flushed with N_2 and incubated at 45^{o}C for 60 minutes in the dark. After this mixture was cooled to 4^{o}C, 2-mercaptoethanol was added to a final concentration of 50 mM. The pH of the solution was then adjusted to 5.0 with 5 M acetic acid to destroy any excess NaBH4, and was vortexed until there was no more bubbling. The pH was finally brought back to 7.5 using 5 M NaOH. The final product, was confirmed to be 5-CH_3-H_4PteGlu$_n$, by checking the spectrum of a 10^{-4} dilution.

To determine the purity and yield of the 5-CH_3-H_4PteGlu$_n$, 2 μl of the product were diluted into 200 μl 10 mM Tris-Cl pH 7.5. 10 μl of the dilution was added into 400 μl of 10 mM Tris-Cl pH 7.5. 100 μl of 5 M HCl/60% formate was added, and mixture was heated 80^{o}C for 10 minutes. After cooling, the solution to room temperature, the spectrum was read. A peak at 350 nm is diagnostic of H_4-PteGlu$_n$, and the lack of a peak indicates 5-CH_3-H_4-PteGlu$_n$. The (6R,S)5-CH_3-H_4-PteGlu$_n$ was stored under N_2 in dark at –20^{o}C.

SYNTHESIS OF L-HOMOCYSTEINE FROM L-HOMOCYSTEINE THIOLACTONE

L-homocysteine was prepared from L-homocysteine thiolactone by alkaline hydrolysis (Drummond *et al*., 1995). Fifty mg of L-homocysteine thiolactone (Sigma) was dissolved in 1.7 ml ddH_2O that had been bubbled with nitrogen to removed dissolved oxygen. Alkaline hydrolysis was initiated by adding 0.83 ml of nitrogen-bubbled 800 mM NaOH solution. The reaction was flushed with nitrogen and incubated at 45^{o}C for 6 minutes. The reaction was terminated when the pH of the reaction solution was adjusted to pH 5.0 using 5.0 M acetic acid; L-homocysteine is stable at pH 5.0. The concentration of L-homocysteine was determined by titration with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and aliquots of 100 mM concentration were stored at -80^{o}C.

SITE-DIRECTED MUTAGENESIS

The *CaMET6* derived amino acid sequence was compared with the sequences of *T. maritima* and *A. thaliana*, for which x-ray structures exist, and a model of the CaMet6 protein was created (Figure 2.2). The comparison suggested residues that might participate in homocysteine binding. Two carboxylates were likely to pair with the amine group of homocysteine, Glu 499 and Asp614 (Figure 2.3). Asp614 in CaMet6p is homologous to Asp577 from *T. maritima* and Asp605 from *A. thaliana*. Therefore the following point mutations were made in the *CaMET6* gene sequence:

D614N Asp614 to Asn614

D614A Asp614 to Ala614

Site-directed mutagenesis was performed using a modification of the method based on the Quik Change® Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Using this protocol two complimentary, overlapping oligonucleotides were synthesized. These oligonucleotides were about 30 bp long and contain the desired mutation flanked by unmodified nucleotide sequence. A 50 µl PCR reaction was assembled using 250 ng of each oligonucleotide, combined with either 40 or 80 ng of methylated, dsDNA template, and the appropriate amount of dNTPs, Pfu polymerase, and 10X reaction buffer. After the reaction, the mixture was then digested with Dpn I, to remove all the non-mutated template plasmid. The mutated DNA was transformed into *E. coli* competent cells. To determine if the desired mutations were obtained, the plasmid DNA was purified from the *E. coli* and sequenced.

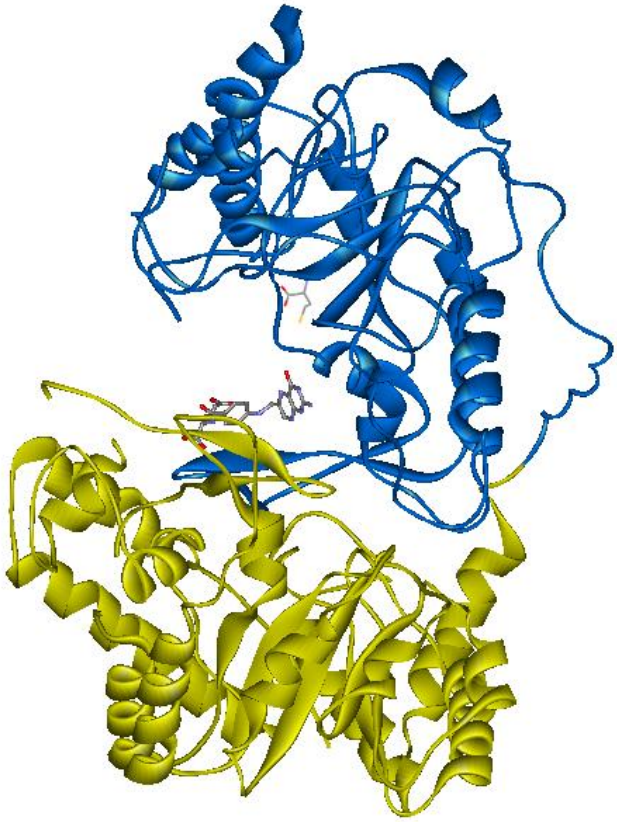


Figure 2.2 The sequence of CaMet6p was modeled on the structure of AtMetEp. The N-terminal domain is shown in yellow, and the C-terminal domain is shown in blue.

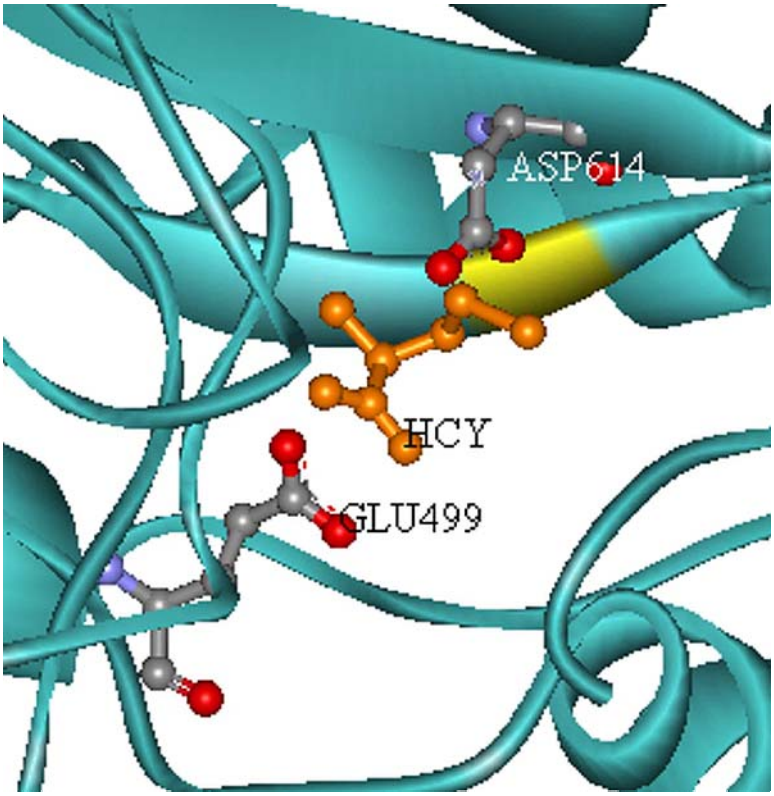


Figure 2.3 Modeled structure of the CaMet6 protein, and the bound homocysteine (HCY) is shown in orange. The Glu499 and the Asp614 are potentially important homocysteine binding residues.

5' FLUOROOROTIC ACID

The SDYα cell strain contains the *ScMET6* gene disrupted by a *URA3* cassette (Suliman, 2005). In order to use the *URA3* selection marker for other yeast expression vectors, while maintaining the *met6* mutation in the SDYα cell strain, the *URA3* cassette needed to be evicted. This was accomplished using 5-fluoroorotic acid (5-FOA) as a selective agent (Boeke *et al*., 1984; Boeke *et al*., 1987). The *URA3* gene codes for the enzyme, orotidine-5'-monophosphate decarboxylase, and acts in the last step of uracil biosynthesis. Orotidine-5'-monophosphate decarboxylase cannot distinguish between 5-FOA and its natural substrate, orotic acid, and therefore uses 5-FOA to make the toxic product 5-fluorouracil. Only cells lacking a functional *URA3* gene will grow on 5-FOA plates, because they will not be able to make the toxic product, and the cells will not die.

5-FOA agar plates were made by using the following recipe for minimal media: 6.7% yeast nitrogen base without amino acids, 2% glucose, 5-FOA (1.0 g/L), uracil (50 mg/L), L-serine (375 mg/L), L-leucine (30 mg/L), L-tryptophan (20 mg/L), L-methionine (20 mg/L), and L-histidine (20 mg/L). The uracil was needed to supplement the mutated strains. One hundred µl of SDYα cells from an overnight culture grown in YPD liquid media were plated on the 5-FOA plates. Colonies appeared within 4-7 days at 30^{o}C. The resulting colonies were tested for the lack of growth in the absence of uracil, and they were also tested for lack of

growth in the absence of methionine and in the presence of uracil. The resulting strain was labeled HSYα (α *ser1 ura3-52 trp1 leu2 his4 met6*).

Construction of the GST fusion CaMet6p

The pEGKT vector was constructed for the galactose inducible overexpression of glutathione S-transferase (GST) fusion proteins in yeast (Mitchell *et al*., 1993). Because the GAL1-10 upstream activation sequence (UAS) was not successfully removed from the pEGKT vector, it consequently repressed the *ScMET6* promoter (Figure 2.4). Therefore, the *ScMET6* promoter-GST fragment was digested from pEGKT using XhoI and XmaI. XmaI is an isoschizomer of SmaI, meaning both restriction enzymes recognize the same sequence. XmaI was used instead of SmaI because it produces a 5' extension, whereas SmaI produces blunt-ended fragments. The *ScMET6* promoter-GST fragment was then ligated into the XhoI and XmaI restriction sites on the pRS424 vector, which was obtained from the Arlen Johnson laboratory (University of Texas at Austin). The *CaMET6* gene was cloned into the XmaI restriction site (Figure 2.5). The following primers were used for amplification of the *CaMET6* gene: 5'-ATTACCCGGGATGGTTCAATCTTCCGTC-3' and 5'-CGACGCCCGGGGTCTCCAAATTAATCAC-3' (the XmaI restriction site is underlined). The PCR was carried out as described earlier for cloning of *CaMET6* into YEp24. This construct was sequenced to ensure no mutations

occurred, and to ensure that the *CaMET6* gene was in frame with the GST. The fusion protein was expressed in the SDYα cell strain, and purified using 10 ml of glutathione agarose beads. The supernatant of the lysed cells was incubated at 4^{o}C with shaking overnight, in a 50 ml conical tube. The mixture is then loaded on a column, and washed for three hours with 20 mM KPO_4/10 mM BME pH 7.4. Then the fusion protein was eluted using 200 ml of 10 mM reduced glutathione in 20 mM KPO_4/10 mM BME pH 7.4.

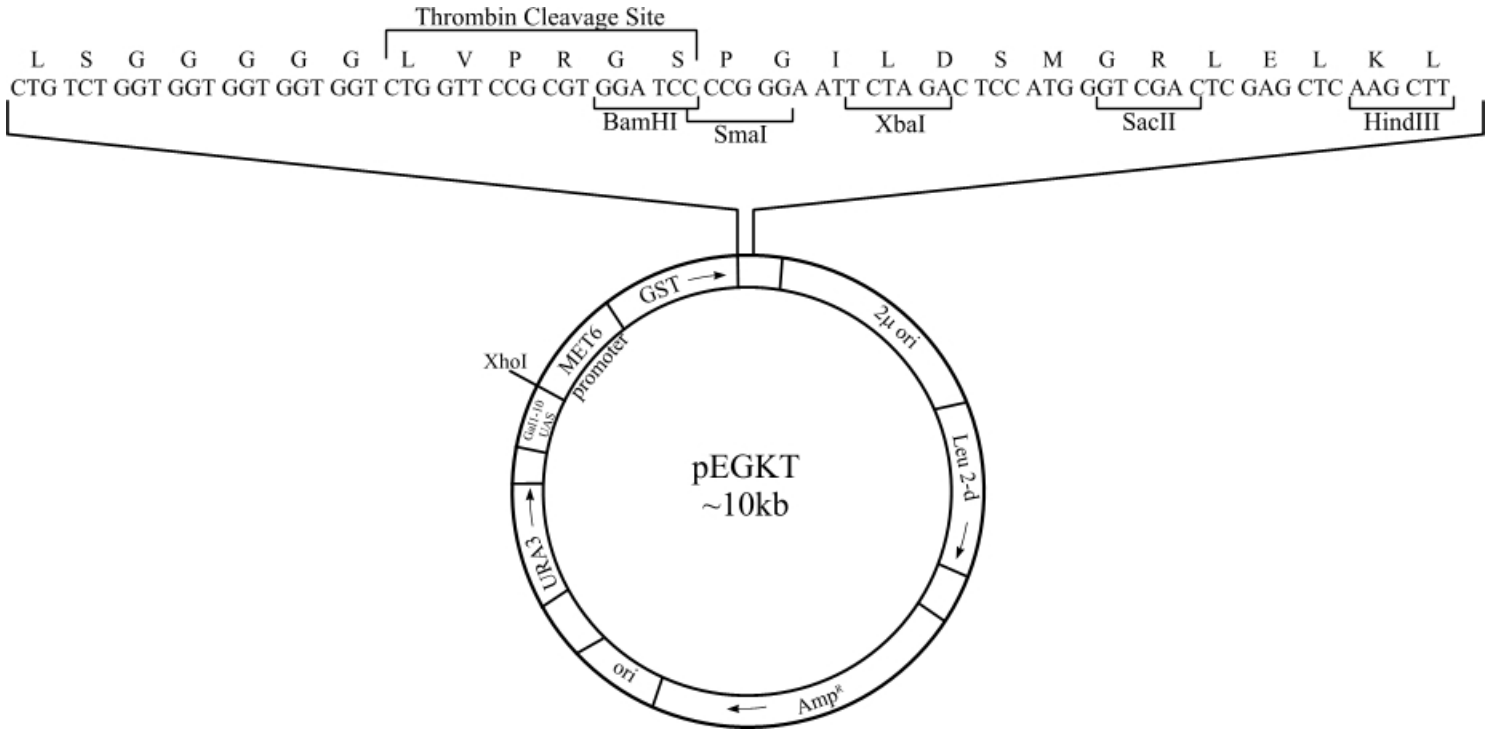


Figure 2.4 A map showing the *ScMET6* promoter in the pEGKT vector.

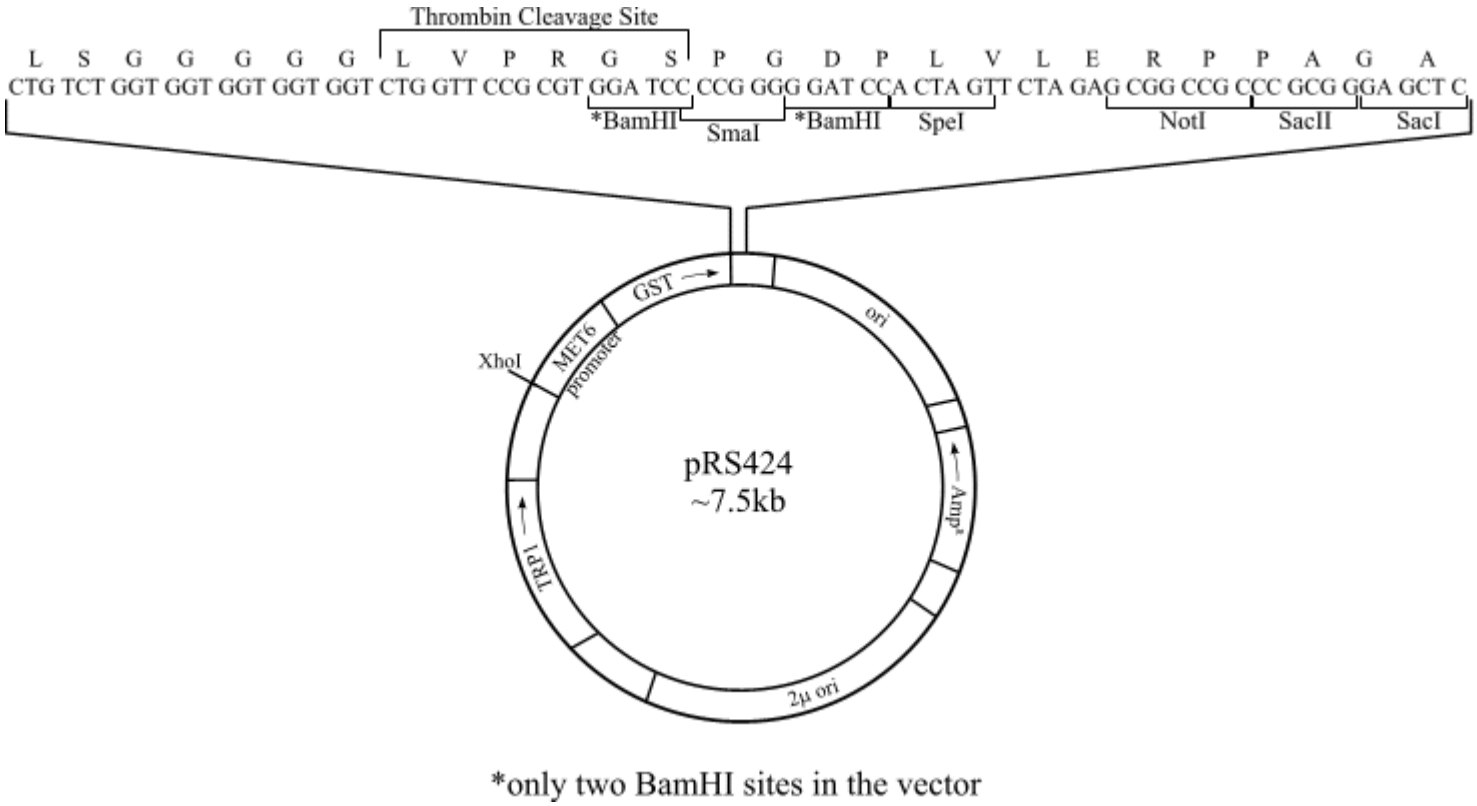


Figure 2.5 A map of the *ScMET6* promoter and GST construct.

Gene Deletion of *CaMET6*

The *C. albicans* strain BWP17 (*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*), and the plasmids pGEM-URA3 and pRS-ARG4 were generously provided by Dr. Aaron Mitchell (Wilson, 1999). The plasmid pURA3-PGAL1-GFP was obtained from Dr. Judith Berman (Gerami-Nejad, 2004). Cultures were grown at 30°C in YPD + Uri for routine nonselective growth, or YM medium for selective growth. YPD + Uri medium consisted of 10 g yeast extract, 20 g peptone, 20 g glucose, and 80 mg of uridine per liter. YM medium consisted of 6.7 g yeast nitrogen base without amino acids and 20 g glucose, and was supplemented with the necessary auxotrophic requirements including: 80 mg/L uridine, 20 mg/L L-histidine, 20 mg/L L-methionine, and 40 mg/L L-arginine. To induce the GAL1 promoter, 20 g glucose was replaced with 20 g raffinose and 20 g galactose. Agar plates contained 20 g/L agar.

To assess the importance of the cobalamin-independent methionine synthase gene in a *C. albicans* strain, gene deletion experiments were performed using the BWP17 strain. This strain is auxotrophic for uridine, histidine, and arginine. The PCR-based gene disruption method (Wilson, 1999) was used to attempt to create a homozygous *CaMET6* deletion mutant strain. Different selectable markers are used to disrupt each allele, so that each deletion could be tracked (Kurtz *et al*., 1989; Negredo *et al*., 1997; Wilson *et al.,* 1999). The 80-

mer primers used are listed in Table 2.1. The CaMET6-5DR and CaMET6-3DR primers were designed so that 20 nucleotides from each bind to both pRS-ARG4 and pGEM-URA3 plasmids, amplifying an *ARG4* and a *URA3* cassette respectively. The remaining 60 nucleotides are homologous to the sequences that flank the *CaMET6* open reading frame (ORF). The deletion cassettes were amplified in PCR reactions containing 4 µl of 10^{-4} diluted plasmid stock (Qiagen) of pGEM-URA3 or pRS-*ARG4*ΔspeI, 4 µl of a 10 µM stock of each primer (CaMET6-5DR and CaMET6-3DR), 10 µl of KOD polymerase 10X PCR buffer (Novagen), 4 µl of a mixture of 10 mM dNTPs, 6 µl of 25 mM $MgCl_2$, 67 µl deionized H_2O, and 1 µl KOD Hot Start DNA polymerase (Novagen). The mixture was incubated at 94˚C for 5 min, followed by 30 cycles of 94˚C for 45 sec, 50˚C for 1 min, and 72˚C for 3 min. After a final extension at 72˚C for 8 min, the samples were incubated at 4˚C until the samples were retrieved. The PCRs were checked by fractionating 5 µl of each sample on a 0.8% agarose gel. The remainder of each reaction was ethanol precipitated and resuspended in 5 µl of TE buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA pH 8.0), without further purification. To prepare the samples for transformation, 10 µl of 10 mg/ml herring-testes DNA (Sigma) is added to the PCR products.

Overnight cultures of BWP17 were diluted 100 fold in 50 ml of YPD + uridine media, and incubated at 30^{o}C, with shaking for four generations (approximately 6 hours). Cells were pelleted and washed with 5 ml sterile water,

and suspended in 0.5 ml TELiOAc (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, and 100 mM lithium acetate). 100 µl aliquots of cell suspension were dispensed into microcentrifuge tubes containing PCR product and herring-testes DNA, and incubated at room temperature for 30 minutes. Then, 0.7 ml of PLATE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 100 mM lithium acetate, 40% polyethylene glycol 3350) was added to each tube, mixed by inversion, and incubated at room temperature overnight (approximately 16 hours). The next day, the mixtures were heat shocked for 1 hour at 42˚C. Cells were pelleted in a microcentrifuge for 30 seconds, suspended in 1 ml of sterile water, and plated on selective media. The plates were incubated at 30˚C until transformants appeared, 3-5 days.

Table 2.1 Primer sequences

Primer	Sequence 5'-3'	Purpose
CaMET6-5DR[a]	TTGTTAAGATTTTATCTACAAATCAAAAACTTTTTATATCCCCCCCCCATTATAATCAACA**T GTGGAATTGTGAGCGGATA**	Deletion of CaMET6.
CaMET6-3DR[a]	TCAACATTTCAAGAATTGAAAATGCAAAATAAATCAATGAATGCTTTGAATCATAAAAG A**TTTCCCAGTCACGACGTT**	Deletion of CaMET6.
GAL1-5DR[b]	CCTTTTTCTTATTCTTCTTCTAATTTTGTTAAGATTTTATCTACAAATCAAAAACTTTTTAT ATCCCCCC**TCTAGAAGGACCACCTTTGATTG**	GAL1 promoter introduction.
GAL1-3DR[b]	GTAGGCCTCAGTGATTTTTTTTAATTCTCTTTGACCACCAATACGTGGGAAACCTAAGACG GAAGATTGAAC***CAT*TTTAATAAACGCGGATCC**	GAL1 promoter introduction.
FL-5DR	TATCCCCCCCTATTCTGAAA	Detection of disrupted alleles.
FL-3DR	GTCGTCATAAAACATAAAAAAGAAA	Detection of disrupted alleles.
ARG4-1012-5DR	CCATTATTTGATGCATTGAC	Detection of disrupted alleles.
URA3-517-5DR	GTTGAAATTGCTAAATCCG	Detection of disrupted alleles.

[a] Boldface sequences in CaMET6-5DR and CaMET6-3DR primers are segments that anneal to plasmids pGEM-URA3 and pRS-ARG4ΔSpeI for amplification of the *URA3* and *ARG4* deletion cassettes. [b] Boldface sequences in GAL1-5DR and GAL1-3DR primers are segments that anneal to plasmid pURA3-PGAL1-GFP for amplification of the P_{GAL1} cassette.

GENOMIC DNA ISOLATION AND IDENTIFICATION OF INTEGRATION EVENTS

To determine if the integration events were successful, genomic DNA was isolated from the transformants. Overnight cultures in 3 ml selective media, were grown and the cells were spun down in a microcentrifuge, for 5 minutes. The cells are washed in 1.0 ml sterile water, and the cells were suspended vigorously in 0.4 ml TENTS (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 100 mM NaCl, 2% Triton X-100, 1% Sodium Dodecyl Sulfate). The cells were vortexed for 2 minutes, after 0.2 ml of 0.45 mm glass beads and 0.4 mL phenol-chloroform were added. The tubes were then spun for ten minutes at maximum speed. The upper aqueous layer was pippetted into a new tube, and 1 ml of ethanol was added. All of the nucleic acid was precipitated by inverting the tube 5-10 times, and was pelleted in a microcentrifuge at 4˚C for 5 minutes. The pellet was dissolved in 200 µl of TE containing 10 µg/ml RNAse A, and incubated at room temperature for 30 minutes. The DNA was then ethanol-precipitated, and dissolved in 100 µl TE on ice for 1 hour. The genomic DNA samples were then screened using PCR. The genomic DNA from the *URA3* cassette transformations was screened using the FL-5DR and FL-3DR primers, which annealed to the target gene locus outside of the altered region. The genomic DNA from the *ARG4* cassette transformations was screened using the ARG-4-1020-5DR primer, which annealed within the *ARG4* cassette, and FL-3DR primer, which annealed outside of the altered region. Each PCR mixture contained 2 µl of genomic DNA, 5 µl 10X PCR buffer

(Novagen), 3 µl 25 mM $MgCl_2$ (Novagen), 2 µl 10 mM dNTPs, 1 µl 10 µM of each primer, and 1 µl KOD Hot Start DNA Polymerase (Novagen). The reactions were incubated at 94˚C for 5 minutes, followed by 30 cycles of 94˚C for 1 minute, 50˚C for 1 minute, and 72˚C for 3 minutes. After a final extension at 72˚C for 8 minutes, the samples were fractionated on a 0.8% agarose gel.

CONSTRUCTION OF THE CONDITIONAL *CaMET6* MUTANT

The Berman Laboratory created plasmids containing cassettes for PCR-mediated construction of regulatable genes (Gerami-Nejad, 2004). The plasmid pURA3-PGAL1-GFP contains a cassette composed of a *URA3* selection marker, a GAL1 regulatable promoter, and a GFP gene. For our experiments the GFP tag was not needed, and only the portion of the cassette that contains the *URA3* selection marker and the GAL1 regulatable promoter was used; this cassette was labeled P_{GAL1}. To integrate P_{GAL1} upstream of *CaMET6*, a forward primer was designed to include 70 nucleotides from the sequence immediately upstream of –15 from the *CaMET6* start codon, and 23 nucleotides that bind on the P_{GAL1} cassette (GAL1-5DR, Table 2.1). The reverse primer was designed to include the reverse complement of nucleotides 1-74 of the *CaMET6* ORF, and the reverse compliment of 18 nucleotides that bind on the P_{GAL1} cassette (GAL1-3DR, Table 2.1). The P_{GAL1} cassette was amplified in PCRs containing 1 µl of 200 ng/µl stock of plasmid pURA3-PGAL1-GFP, 4 µl of 10 µM stock of each primer (GAL1-5DR and GAL1-3DR), 10 µl of KOD polymerase 10X PCR buffer (Novagen), 4

µl of a mixture of 10 mM dNTPs, 12 µl of 25 mM $MgCl_2$, 20 µl of 1 mg/ml BSA stock, 44 µL deionized H_2O, and 1 µL KOD Hot Start DNA polymerase (Novagen). The mixture was incubated at 94˚C for 5 minutes, followed by 30 cycles of 94˚C for 45 seconds, 50˚C for 1 minute, and 68˚C for 3 minutes. After a final extension at 68˚C for 8 minutes, the samples were incubated at 4˚C until the samples were retrieved. The PCRs were checked by fractionating 5 µl of each sample on a 0.8% agarose gel. The remainder of each reaction was ethanol-precipitated and resuspended in 5 µl of TE buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA pH 8.0), without further purification. Ten of the samples were combined and transformed into the heterozygous *CaMET6/Camet6* mutant that was disrupted with the *ARG4* selection marker. The genomic DNA from the transformants was screened using the ARG4-1020-5' primer, which annealed within the *ARG4* cassette, and the FL-3DR primer, which annealed outside of the altered region, to test for the presence of the *ARG4* cassette. The transformants were also screened using the URA3-517-5DR, which annealed within the *URA3* portion of the P_{GAL1} cassette, and the FL-5DR primer, which annealed outside of the altered region, to test for the presence of the P_{GAL1} cassette. Further screening reactions were set up with the FL-5DR and FL-3DR, which both anneal outside the altered region.

CaMet6p POLYCLONAL ANTIBODIES

The CaMet6 protein was purified as previously described in the materials and methods section. A 5 ml sample of CaMet6p, at a concentration of 2 mg/ml, was sent to The University of Texas MD Anderson Cancer Center Department of Veterinary Sciences (Bastrop, TX). Polyclonal antibodies were raised against CaMet6p in two male rabbits. The immune serum was used in Western Blot experiments.

SDS-PAGE AND WESTERN BLOTTING

C. albicans strains were grown under inducing and repressing conditions to an A_{600} of 1, and spun down in a centrifuge for 5 minutes at 5000 x g. The cell pellets were resuspended in 2 ml/gram wet weight of 50 mM KPO4/ 10 mM BME/1 mM phenylmethylsulfonyl fluoride, pH 7.0. The cells were broken open using 450 μm glass beads (Sigma), and the cell debris was pelleted in a tabletop centrifuge. The protein concentrations in the whole cell lysates were estimated using absorbance at 280 nm. Equal amounts of protein from each of the samples were loaded on an 8% SDS-polyacrylamide gel, and SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (Laemmli, 1970). The protein samples were transferred to a PVDF membrane (Millipore) using the Bio-Rad semi-dry blotter apparatus at 100 mA for 20 minutes, and the membrane was blocked with 5% non-fat milk. The primary antibody, polyclonal anti-CaMet6p,

was diluted to 1:1000, and the secondary antibody, goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad), was diluted to 1:2000. CaMet6p was detected by chemiluminescence, using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

GROWTH ASSAYS

Overnight cultures of BWP17 were grown to logarithmic phase at 30˚C in YPD + uridine media, and inoculated in YM media containing 2 % glucose, 20 mg/L L-histidine, 40 mg/L L-arginine, and 80 mg/L uridine. Overnight cultures of the conditional *CaMET6* mutant were grown to logarithmic phase at 30 ˚C in YM media containing 2% glucose, 20 mg/L L-histidine and 20 mg/L L-methionine, and inoculated in YM media containing 2% glucose and 20 mg/L L-histidine. Both strains had a starting OD_{600} of 0.09, and were assayed under three concentrations of L-methionine; 10 μM, 25 μM, and 50 μM. These concentrations mimic serum levels in humans. The cultures were grown in triplicate at 30˚C, with shaking. Samples were taken at frequent intervals to measure OD_{600}.

CHAPTER 3: RESULTS

EXPRESSION OF THE *MET6* GENES

The YEp24-*ScMET6* construct was expressed in the Day4.1 *Saccharomyces cerevisiae* cell strain. YEp-*CaMET6* was expressed in the *met6* mutant of *S. cerevisiae* cell strain, SDYα. The SDYα cell strain is a strict methionine auxotroph, and is not viable without the addition of methionine (Figure 3.1). The SDYα strain has no detectable methionine synthase activity, and was complemented by the YEp24 vector expressing the *ScMET6* gene under the control the *ScMET6* native promoter. The *CaMET6* gene also complemented the *Scmet6* deletion strain (Figure 3.1).

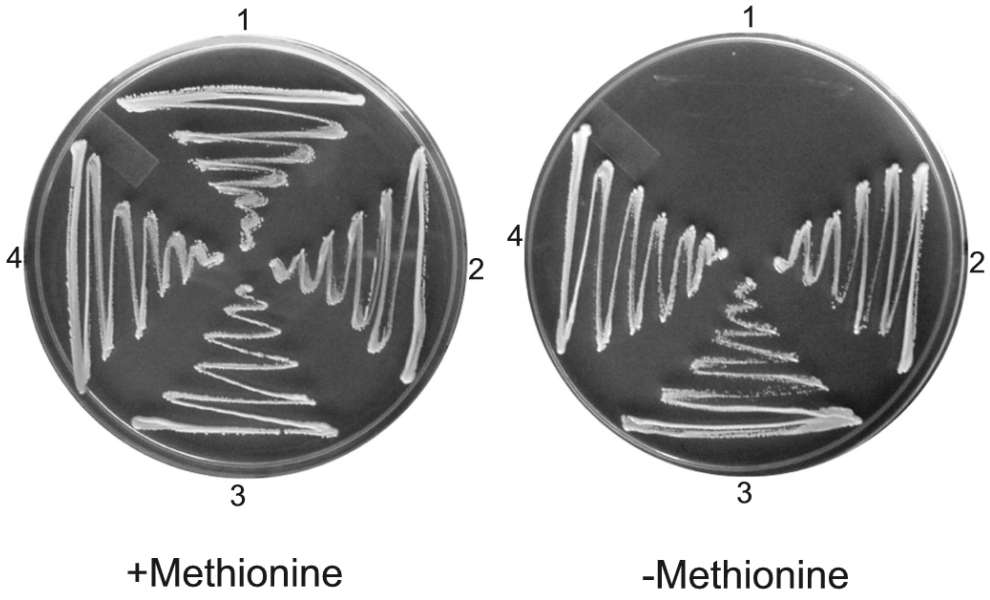


Figure 3.1: Complementation of *S. cerevisiae* Δ*met6* mutant. SDYα (Δ*met6*) (1), Day4.1 transformed with YEp24 (wt control) (2), SDYα transformed with YEp24-*ScMET6* (3), and SDYα transformed with YEp24-*CaMET6* (4). Plates contain yeast minimal medium supplemented with histidine, leucine, tryptophan, and serine, plus or minus methionine.

PURIFICATION OF *CaMET6* AND *ScMET6* PROTEINS

The *ScMET6* promoter is among the strongest of the *S. cerevisiae* promoters (Ohtake *et al*., 1988). The YEp24-*ScMET6* construct overexpressed methionine synthase activity approximately 10-fold when compared to the wild-type methionine synthase activity in DAY4.1 cells. The *CaMET6* gene was expressed in the *S. cerevisiae* null mutant SDYα strain, a strain with no inherent methionine synthase. The purification protocols for ScMet6p and CaMet6p were modified from the purification protocol reported in the literature (Burton and Sakami, 1969). Table 3.1 shows the purification data obtained for CaMet6p and ScMet6p at each step of the purification procedure, and Figure 3.2 shows the Coomassie Brilliant Blue Stained SDS gels of CaMet6p and ScMet6p preparation at the different purification steps. ScMet6p and CaMet6p were stable at high temperatures and at low pH, and in those purification steps both Met6 proteins remain stable in solution while other proteins were denatured and formed a pellet. The specific activity of CaMet6p and ScMet6p increased 6-10-fold during purification. One liter of SDYα cells produced 5.5 mg of CaMet6p, which was purified to about 80% homogeneity (Figure 3.2A). One liter of DAY4.1 cells produced 7 mg of ScMet6p, which was purified to about 90% homogeneity (Figure 3.2B).

Table 3.1: Purification of CaMet6p and ScMet6p from 3 liters of culture.

Enzyme	Total Volume (ml)	Total Protein (mg)	Specific Activity (5-CH_3-$H_4PteGlu_3$) (U/mg)	Purification Factor
CaMet6p				
1. Crude Extract	30	255	3.60	-
2. Heat Treatment	20	182	8.55	2.4
3. Acid Treatment	17.5	70	10.63	3.0
4. Deae Sepharose Column	12	17	42.10	11.7
ScMet6p				
1. Crude Extract	35	521	5.20	-
2. Heat Treatment	34	374	11.40	2.2
3. Acid Treatment	34	255	13.10	2.5
4. Heparin Column	15	20	29.30	5.6

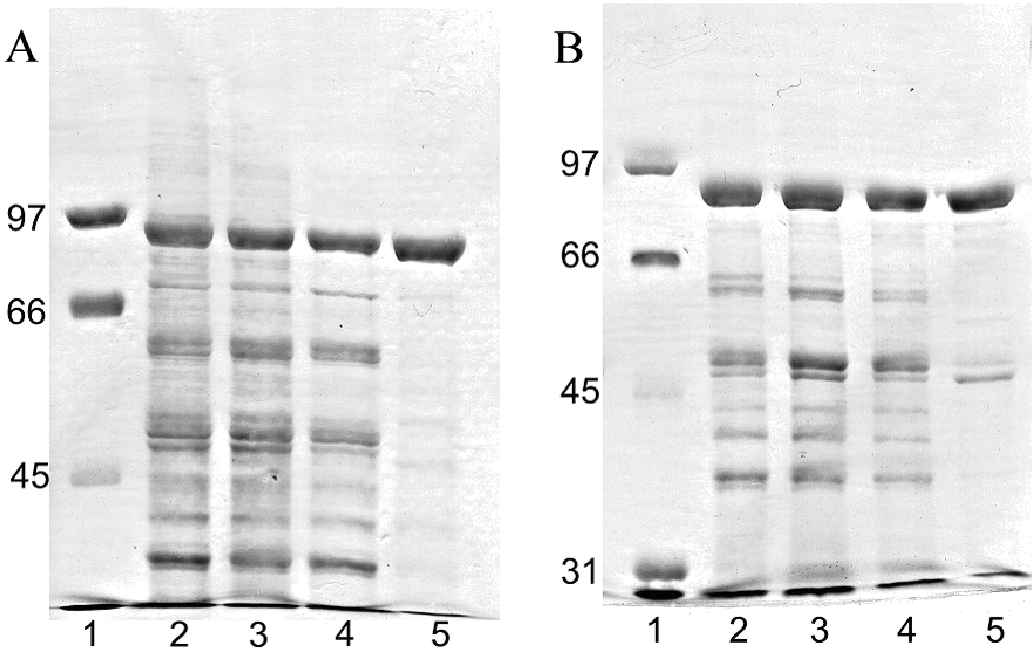


Figure 3.2: A. Purification of CaMet6p. Lanes: 1, Marker; 2, crude extract; 3, heat treatment; 4, acid treatment; 5, DEAE Sepharose column. B. Purification of ScMet6p. Lanes: 1, Marker; 2, crude extract; 3, heat treatment; 4, acid treatment; 5, Heparin column.

KINETIC ANALYSIS

A non-radioactive enzyme assay was performed, using a modification of the procedure described by Drummond *et al*. (1995). Kinetic experiments were performed to determine the substrate specificity of CaMet6p and ScMet6p. ScMet6p was believed to accept only polyglutamated substrates along with L-homocysteine for catalysis (Burton *et al*., 1969). The substrates (6R,S)5-methyltetrahydropteroyl(mono-, di-. tri-, tetra-)glutamate and L-homocysteine, were each assayed with ScMet6p and CaMet6p. The assays were performed in duplicate and fitted to the Michaelis Menten Equation. The K_m and k_{cat} values calculated from the assays for CaMet6p and ScMet6p are listed in Table 3.2. Figure 3.3 shows the steady-state kinetic curves for CaMet6p, and Figure 3.4 shows the steady-state kinetic curves for ScMet6p.

Table 3.2: Kinetic data for CaMet6p and ScMet6p.

Substrate	CaMet6p *C. albicans*	ScMet6p *S. cerevisiae*
*K*m (6*R*,*S*)-5-CH_3–H_4PteGlu$_1$ (μM)	No activity	No activity
*k*cat (1/min)		
*K*m (6*R*,*S*)-5-CH_3–H_4PteGlu$_2$ (μM)	113 ± 19	109 ± 10
*k*cat (1/min)	21 ± 1.1	17 ± 0.4
*K*m (6*R*,*S*)-5-CH_3–H_4PteGlu$_3$ (μM)	129 ± 25	84 ± 8
*k*cat (1/min)	24 ± 1.4	32 ± 0.8
*K*m (6*R*,*S*)-5-CH_3–H_4PteGlu$_4$ (μM)	120 ± 14	94 ± 22
*k*cat (1/min)	25 ± 0.9	36 ± 2.4
*K*m L-homocysteine (μM)	14 ± 2.3	13 ± 2.6
*k*cat (1/min)	25 ± 0.9	20 ± 0.9

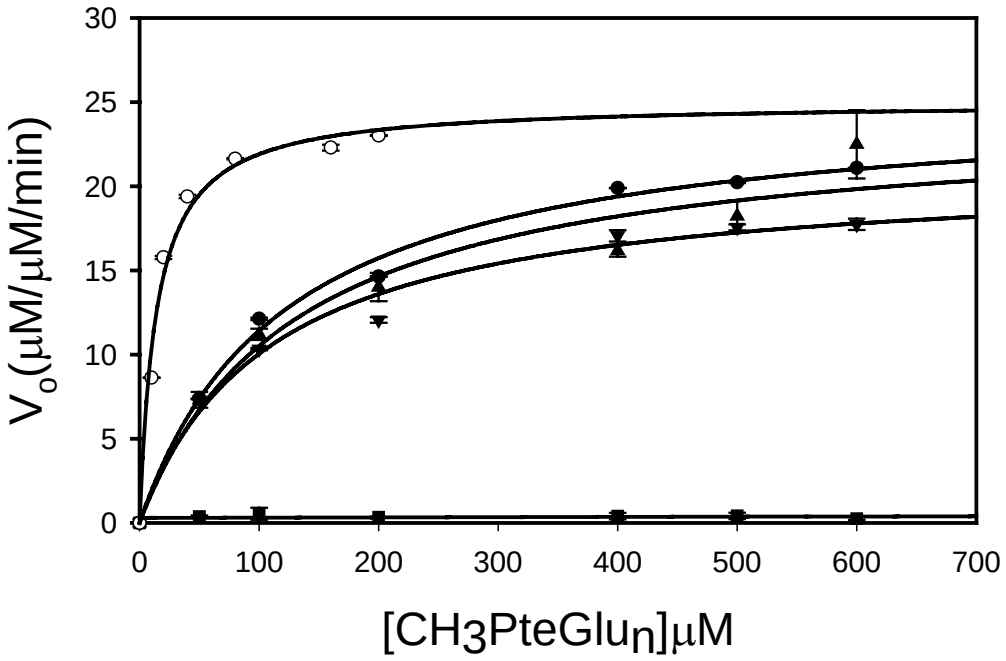


Figure 3.3: Steady State Kinetic Curves for CaMet6p. Assays were performed in duplicate and averaged. (6*R*,*S*)5-CH_3–H_4PteGlu$_1$ (■), (6*R*,*S*)5-CH_3–H_4PteGlu$_2$ (▼), (6*R*,*S*)5-CH_3–H_4PteGlu$_3$ (▲), (6*R*,*S*)5-CH_3–H_4PteGlu$_4$ (●),L-homocysteine (○).

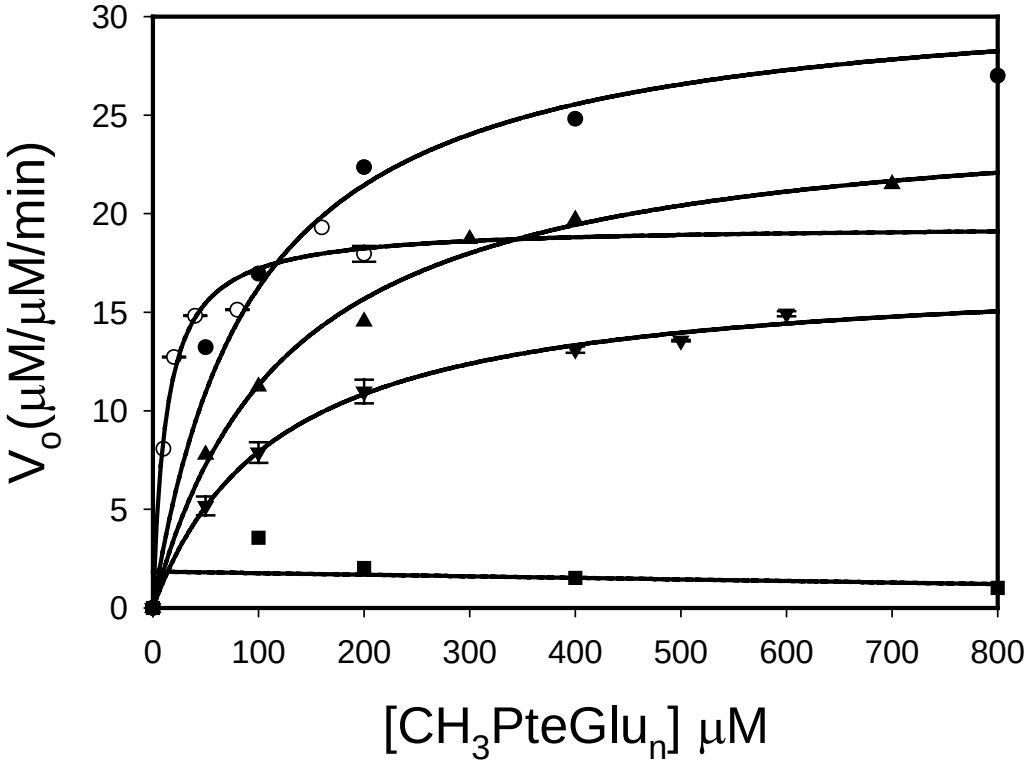


Figure 3.4: Steady State Kinetic Curves for ScMet6p. Assays were performed in duplicate and averaged. (6*R*,*S*)5-CH_3–H_4PteGlu$_1$ (■), (6*R*,*S*)5-CH_3–H_4Pte-Glu$_2$ (▼), (6*R*,*S*)5-CH_3–H_4PteGlu$_3$ (▲), (6*R*,*S*)5-CH_3–H_4PteGlu$_4$ (●), L-homocysteine (○).

Kinetic analyses using 5-CH_3-H_4PteGlu$_n$ of different number of glutamates were performed over 6 different concentrations of (6R,S)-5-CH_3-H_4PteGlu$_n$ and in the presence of saturating L-homocysteine, 2 mM. It was proven that neither protein could accept a (6R,S)5-methyltetrahydropteroyl substrate containing only one glutamate. Although the monoglutamated substrate did not bind, there was no significant difference among the K_m or k_{cat} values for the di-, tri-, or tetraglutamated substrates. All the K_m values were approximately 100 μM and all the k_{cat} values were around 25/min.

The steady-state kinetic parameters for L-homocysteine were also measured. These kinetic experiments were performed in the presence of 500 μM (6R,S)-5-CH_3-H_4PteGlu$_3$, which was approaching saturation and sufficiently high to measure the effect of homocysteine independently. Six increasing concentrations of L-homocysteine were assayed, giving apparent K_m values for CaMet6p and ScMet6p of 13μM and 14 μM respectively.

SITE-DIRECTED MUTAGENESIS

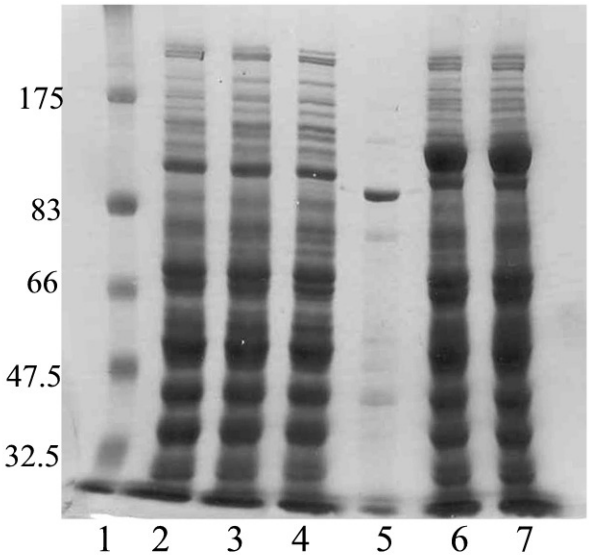
The goal of this experiment was to explore the enzyme recognition site for homocysteine, using site-directed mutagenesis. As described earlier, homocysteine is thought to be oriented in its binding pocket by ionic interaction between the substrate and amino acids Glu499 and Asp614. To initiate mutagenesis studies, Asp614 was altered. The mutations D614N and D614A were successfully created in the YEp24-*CaMET6* plasmid. The two plasmids, YEp24-*CaMET6*-D614N and YEp24-*CaMET6*-D614A, were transformed in the *Scmet6* mutant cell strain, SDYα. The only selection for this cell strain is the complementation of the *Scmet6* mutation by the recombinant CaMet6p. Neither plasmid with the mutation was able to compliment the *Scmet6* mutant strain.

To be able to express the mutated enzymes in the *Scmet6* mutant strain, using a different selection marker, the HSYα strain was constructed. This strain allowed for the use of the *URA3* selection marker on the YEp24 plasmid, because the strain was rendered *ura3*-. The mutated plasmids were then transformed into the HSYα strain, and plated on media supplemented with methionine. A 0.5 liter liquid cell culture was grown, and samples were probed with CaMet6p polyclonal antibodies on a Western Blot (Figure 3.5). The blot revealed that there was no mutant CaMet6p in the soluble fraction.

In order to bypass the apparent insolubility of the *CaMet6* mutant protein, a vector expressing wild type CaMet6p as a GST fusion under the control of the

ScMET6 constitutive promoter was constructed in the pRS424 vector. The GST fusion was successfully expressed (Figure 3.5) and complemented the SDYα *Scmet6* mutant strain. The fusion protein was successfully purified over glutathione agarose (Figure 3.6). The fusion protein bound to the glutathione agarose column with a decreased binding capacity, and most of the fusion protein was in the flow-through (Figure 3.6). The diminished binding capacity is due to the large size of the fusion protein, which is approximately 112 kDa. The same site directed mutants, D614A and D614N, were created in the GST fusion construct, expressed in SDYα, and analyzed on an 8% SDS gel (Figure 3.7). This gel revealed that GST fusions of the site directed mutants expressed, and this was confirmed with a Western Blot (Figure 3.7). The GST fusions of D614A and D614N did not complement the SDYα strain.

A.



B.

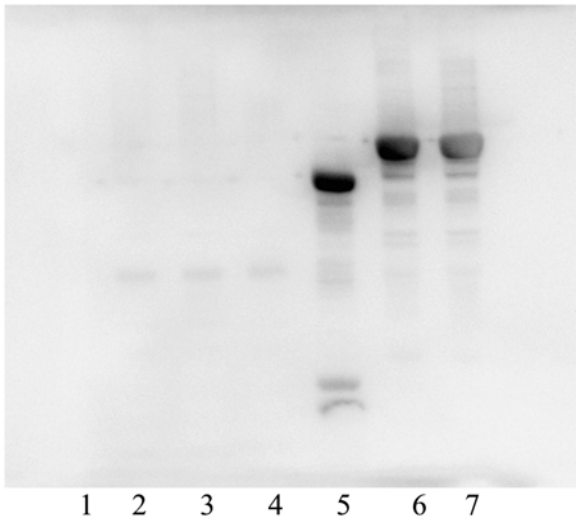


Figure 3.5 8% SDS PAGE (A.) and Western Blot (B.) analysis of site-directed mutants, and wild-type GST-CaMet6p fusion probed with the polyclonal CaMet6p antibody. Lanes: 1, protein ladder; 2, HSYα cell strain (negative control); 3, YEp24-*CaMET6*-D614N; 4, YEp24-*CaMET6*-D614A; 5, wild-type CaMet6p; 6 + 7, GST-CaMet6p fusion.

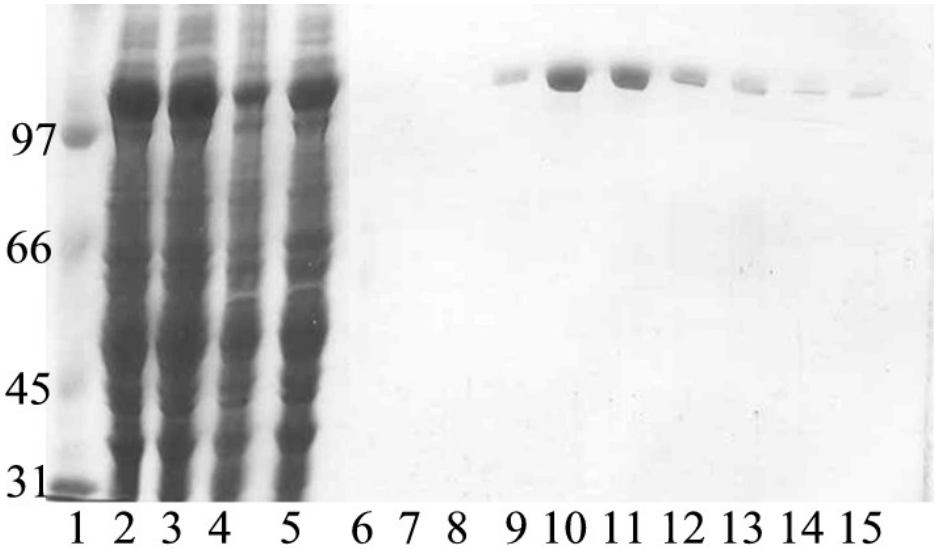
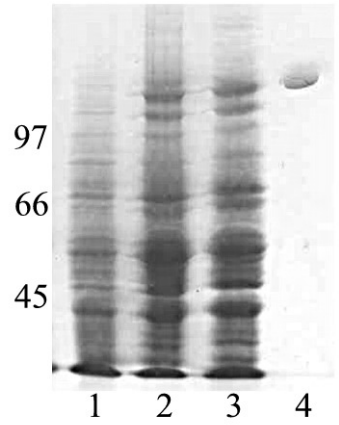


Figure 3.6 8% SDS PAGE of the purification of the wild-type GST-CaMet6p fusion. Lanes: 1, protein ladder; 2 + 3, crude extract; 4, pellet; 5 flow through; 6-15, eluted fractions of GST-CaMet6p fusion.

A.



B.

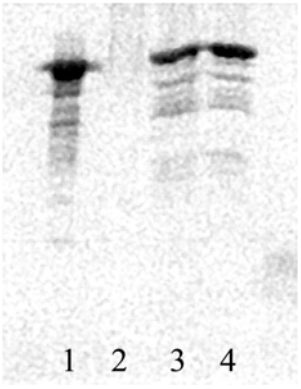


Figure 3.7 **A.** 8% SDS PAGE GST-fusion site-directed mutants. Lanes: 1, SDYα (negative control); 2, GST-D614N; 3, GST-D614A; 4, purified wild-type GST-CaMet6p fusion (positive control). **B**. Western blot analysis of the GST-fusion mutants. Lanes: 1, wild-type GST-CaMet6p fusion (positive control); 2, SDYα (negative control); 3, GST-D614N; 4, GST-D614A.

CAMET6 GENE DELETION

Experiments in *C. albicans* were carried out to create *CaMET6* deletion mutants, and to assess the viability of the resulting null mutant *C. albicans* strain. The parental *C. albicans* strain used was BWP17 *(ura3/ura3 arg4/arg4 his1/his1)*, which is auxotrophic for uridine, arginine, and histidine (Wilson, 1999). The first gene deletion experiments were performed using the *URA3* cassette and the *ARG4* cassette separately. Each cassette had 60 nucleotides at each end that complemented the sequences flanking the DNA coding for the *CaMET6* ORF; through homologous recombination these cassettes should replace the *CaMET6* gene, as illustrated in Figures 3.8A and 3.9A. The PCR amplified cassettes were transformed into the BWP17 strain, and the transformed cells were plated on media that either lacked uridine or lacked arginine. Using this strategy, only the *C. albicans* cells that successfully integrated the *URA3* or *ARG4* cassettes should grow, but because *C. albicans* is diploid only one chromosomal allele is altered. After colonies appeared within 3-5 days, the genomic DNA purified from a number of colonies was tested for the deletion using PCR. Figure 3.8B shows a 0.8% agarose gel of the PCR of one transfomant performed using primers that flank the deleted region in the *URA3* cassette transformants (The primer locations are indicated in Figure 3.8A). The undeleted allele resulted in a 2800 bp PCR product, and the allele deleted with the *URA3* cassette resulted in an 1800 bp PCR product. The two bands proved that one copy of *CaMET6* was effectively

removed from the genome, creating a heterozygous *CaMET6/Camet6* mutant. Figure 3.9B shows a 0.8% agarose gel of the PCR tests performed using one *ARG4* transformant. The *ARG4* cassette is the same size as the *CaMET6* gene, and when using the primers that flank the deleted region, only one band appears. To test whether the *ARG4* cassette had successfully replaced the *CaMET6* gene, additional PCRs were performed using a 5' primer that bound within the *ARG4* cassette and a 3' primer that bound outside of the deleted region. A 650 bp band corresponding to this amplification was obtained (Figure 3.9B), confirming that the *ARG4* cassette replaced one copy of the *CaMET6* gene, creating a heterozygous *CaMET6/Camet6* mutant.

C. albicans is diploid, and a second experiment is needed in order to delete the second copy of *CaMET6*. The *URA3* heterozygous strain was therefore transformed with the *ARG4* cassette, and the *ARG4* heterozygous strain was transformed with the *URA3* cassette. All of the transformations were plated on agar plates that lacked uridine and arginine, and contained histidine and methionine. No colonies resulted from the second round of deletion experiments, consistent with the notion that the transformations were successful, but the double knockout was lethal. Multiple attempts failed to produce viable double-deleted colonies.

Figure 3.8 A.

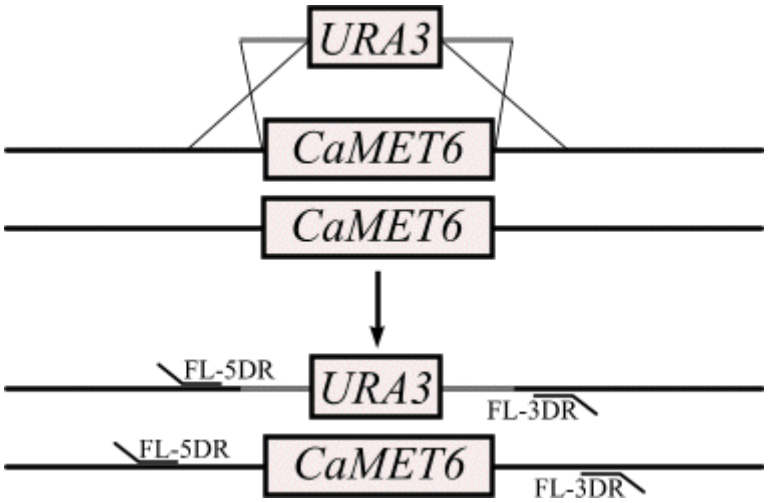


Figure 3.8 B.

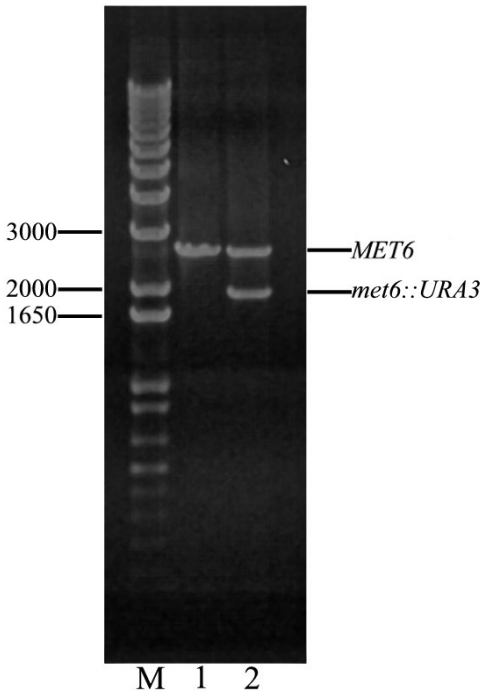


Figure 3.8 (continued) A. A schematic of homologous recombination occurring after transformation with the *URA3* cassette, and primer binding locations. **B.** Analysis of *URA3* transformant strains. Low range DNA marker, lane M. PCR analysis of genomic DNA from the BWP17 strain amplified with FL-5DR and FL-3DR, lane 1. PCR analysis genomic DNA from BWP17 *URA3* transformant amplified with FL-5DR and FL-3DR, lane 2. PCR amplification of the *CaMET6* gene with FL-5DR and FL-3DR results in a 2800 bp product, and amplification of the *URA3* cassette results in a 1800 bp product.

Figure 3.9 A.

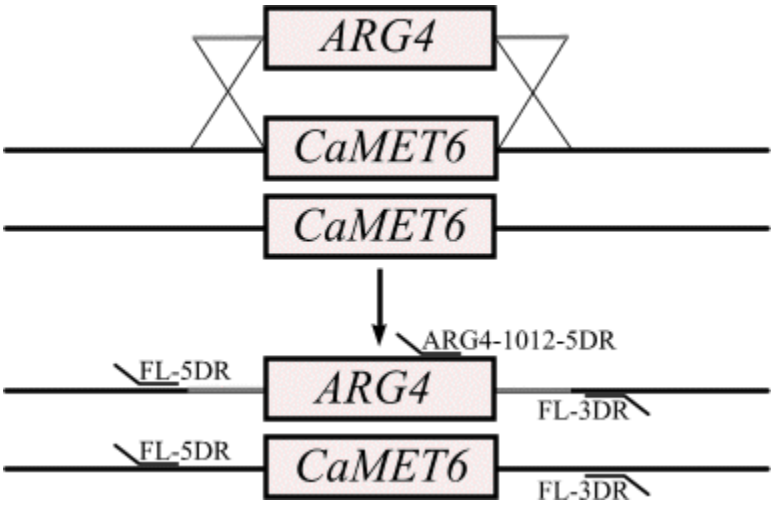


Figure 3.9 B.

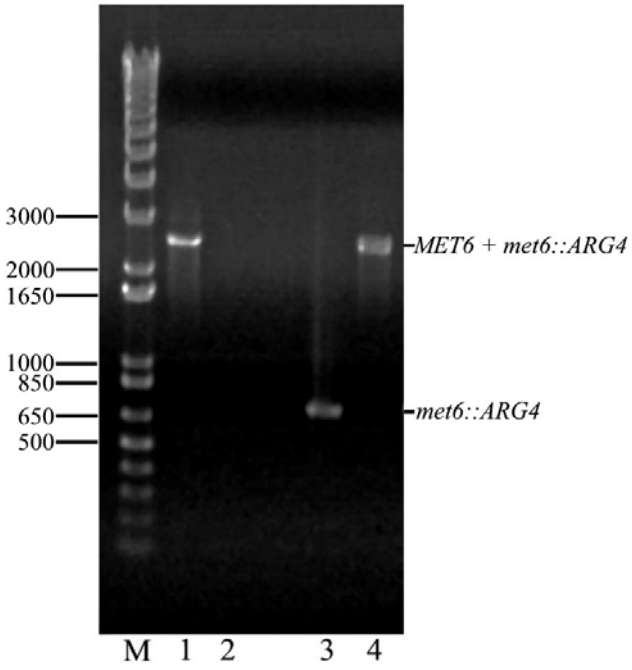


Figure 3.9 (continued) A. A schematic of homologous recombination occurring after transformation with the *ARG4* cassette. The detection primers are listed in Table 2.1. **B.** Analysis of *ARG4* transformants. Low range DNA marker, lane M. PCR analysis of genomic DNA from the BWP17 strain amplified with FL-5DR and FL-3DR, lane 1; ARG4-1012-5DR and FL-3DR, lane2. PCR analysis genomic DNA from BWP17 *ARG4* transformants amplified with ARG4-1012-5DR and FL-3DR, lane 3; FL-5DR and FL-3DR, lane 4. PCR amplification of the *CaMET6* gene with FL-5DR and FL-3DR results in a 2800 bp product, and amplification of the *ARG4* cassette also results in a 2800 bp product. PCR amplification of the *ARG4* cassette with ARG4-1012-5DR and FL-3DR results in a 650 bp product, and amplification of *CaMET6* gene with the same primers shows no product.

ANALYSIS OF A CONDITIONAL *CaMET6* MUTANT

Since I was unable to obtain a double null mutant *C. albicans* cell strain, I attempted to construct a conditional *CaMET6* mutant using the P_{GAL1} cassette (Gerami-Nejad, 2004). This cassette contains a *URA3* selection marker and a *GAL1* promoter, which was amplified from the plasmid pURA3-PGAL1-GFP. The P_{GAL1} cassette was then transformed into the *ARG4* heterozygous *CaMET6/Camet6* mutant strain where, through homologous recombination, the *GAL1* promoter was inserted in front of the remaining copy of *CaMET6* (Figure 3.10A). The selection of transformants was on media containing raffinose, galactose, and histidine, where only those that integrated the P_{GAL1} cassette would grow. Colonies appeared after 7 days. The resulting transformants were tested using PCR to confirm that the cassette had integrated in front of the *CaMET6* ORF (Figure 3.10B). When amplified with the FL-5DR and FL-3DR primers there should be two PCR products, one at 2800 bp, which represents the *ARG4* cassette, and one at ~6000 bp which represents the P_{GAL1} cassette plus the *CaMET6* ORF. Only the 2800 bp product was observed (Figure 3.10B, lane 9), because the ~6000 bp region was too large to amplify. The presence of the *ARG4* cassette was tested the same way as in the heterozygous *CaMET6/Camet6::ARG4* mutant, with the same results. However, amplification with FL-5DR and URA3-517-5DR primers confirmed the presence of the P_{GAL1} cassette in front of the remaining *CaMET6* ORF (Figure 3.10B, lane 11). An attempt was made to

amplify the P_{GAL1} cassette, and the *CaMET6* ORF, using primers GAL1-5DR and FL-3DR, but this product was also large and, again, did not amplify (Figure 3.10B, lane 12).

The *GAL1* promoter is induced by galactose and repressed by glucose (Berman, 2002; Srikantha, 1996). Under inducing conditions, in the presence of galactose, the conditional *CaMET6* mutant strain grows in a manner similar to that of the heterozygous *C. albicans* mutants. Under repressive conditions, in media containing glucose and histidine, the conditional *CaMET6* mutant strain did not grow (Figure 3.11A). However, it did grow on media containing glucose, histidine, and methionine. A Western Blot revealed that even under repressing conditions (glucose), *CaMET6* was still expressed at low amounts (Figure 3.11B).

To estimate the effect of the conditional mutation on *C. albicans* metabolism, growth curves were measured for the wild type and the conditional mutant in the presence of three concentrations of methionine: the results are shown in Figure 3.12. Growth rates for the wild type, which can synthesize the amino acid, is independent of exogenous methionine. The culture reaches half its stationary concentration in 9 hours. The conditional mutant grown under repressing conditions, in glucose, and does not grow in the absence of exogenous methionine. However its time to half stationary levels are 56, 42, and 34 hours when supplemented with 10, 25, and 50 μM methionine respectively.

Figure 3.10 A.

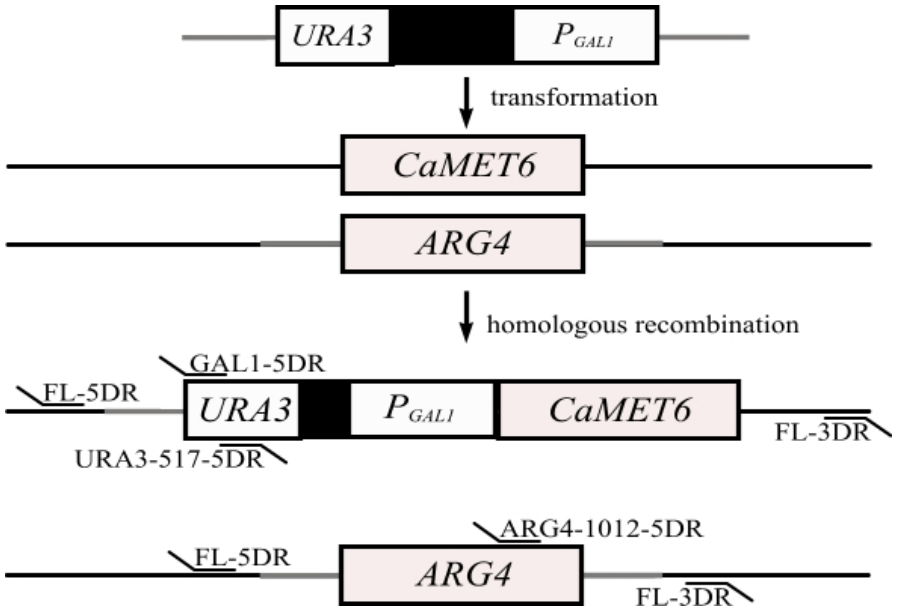


Figure 3.10 B.

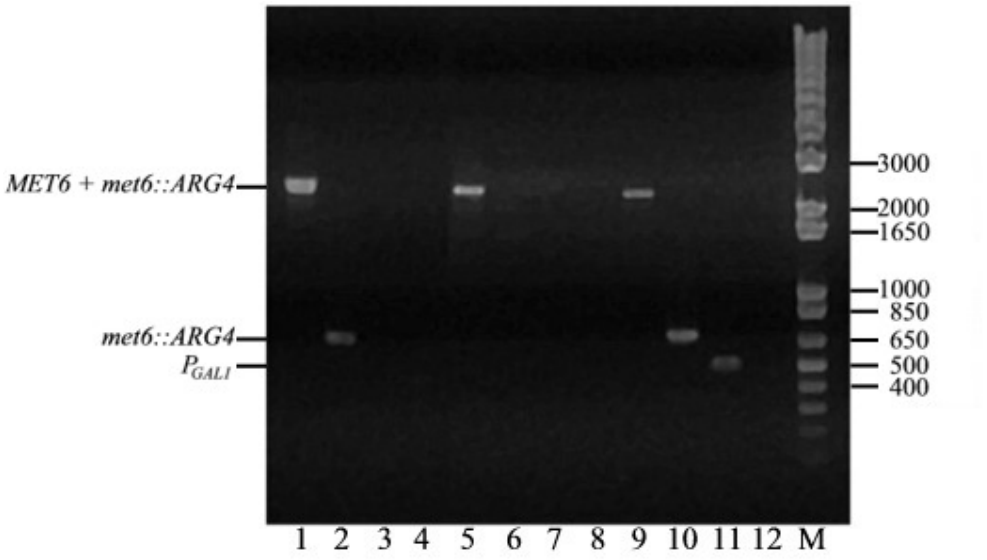
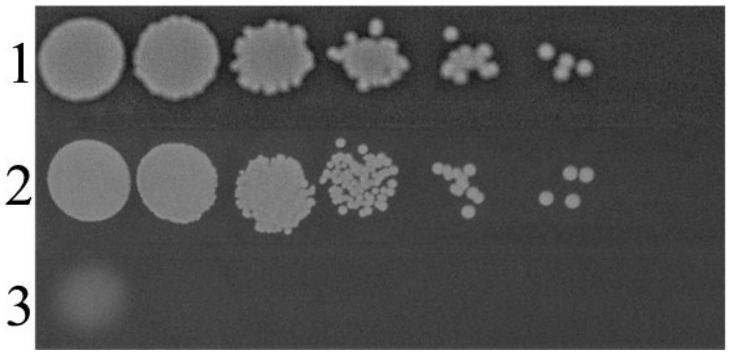


Figure 3.10 (continued) A. A schematic of homologous recombination occurring after transformation with the P_{GAL1} cassette into the heterozygous *ARG4* mutant, and primer binding. **B.** Analysis of *ARG4* transformants. PCR Analysis of P_{GAL1} transformants. Genomic DNA from *met6::ARG4* heterozygous mutant amplified with FL-5DR and FL-3DR, lane 1; ARG4-1012-5DR and FL-3DR, lane2; URA3-517-5DR and FL-5DR, lane 3; GAL1-5DR and FL-3DR, lane 4. PCR analysis of genomic DNA from the BWP17 strain amplified with FL-5DR and FL-3DR, lane 5; ARG4-1012-5DR and FL-3DR, lane 6; URA3-517-5DR and FL-5DR, lane 7; GAL1-5DR and FL-3DR, lane 8. Genomic DNA from the conditional mutant amplified with FL-5DR and FL-3DR, lane 9; ARG4-1012-5DR and FL-3DR, lane 10; URA3-517-5DR and FL-5DR, lane 11; GAL1-5DR and FL-3DR, lane 12. Low range DNA marker, lane M.

A.



B.

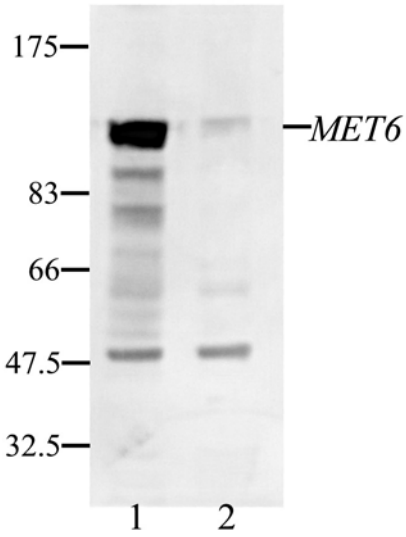


Figure 3.11 A. Serial 10-fold dilutions of a liquid culture containing the conditional mutant. Media: 1, medium containing galactose and histidine; 2, medium containing glucose, histidine, and methionine; 3, medium containing glucose and histidine. **B.** Western Blot analysis of cellular extracts probed with the polyclonal CaMet6p antibody. Conditional mutant induced with galactose, lane 1. Conditional mutant repressed with glucose, and supplemented with methionine, lane 2.

Figure 3.12 A.

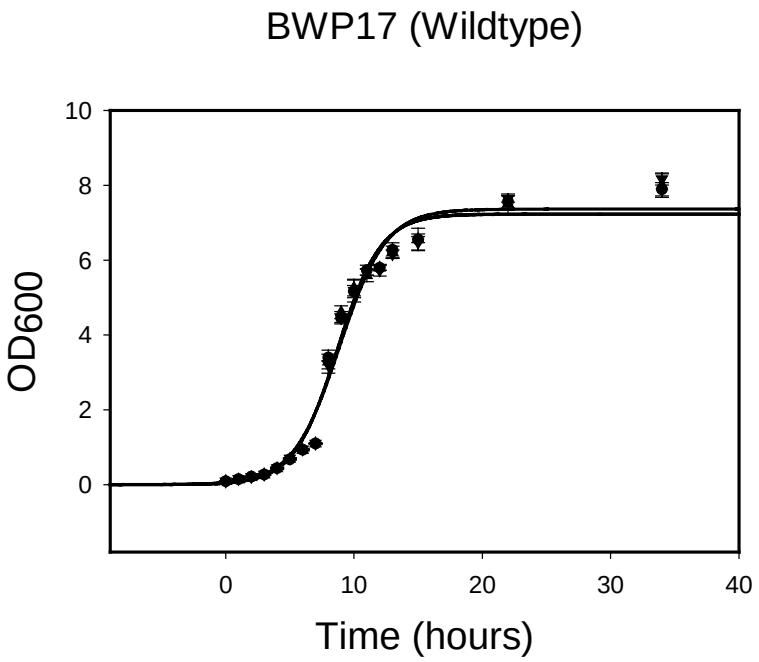


Figure 3.12 B.

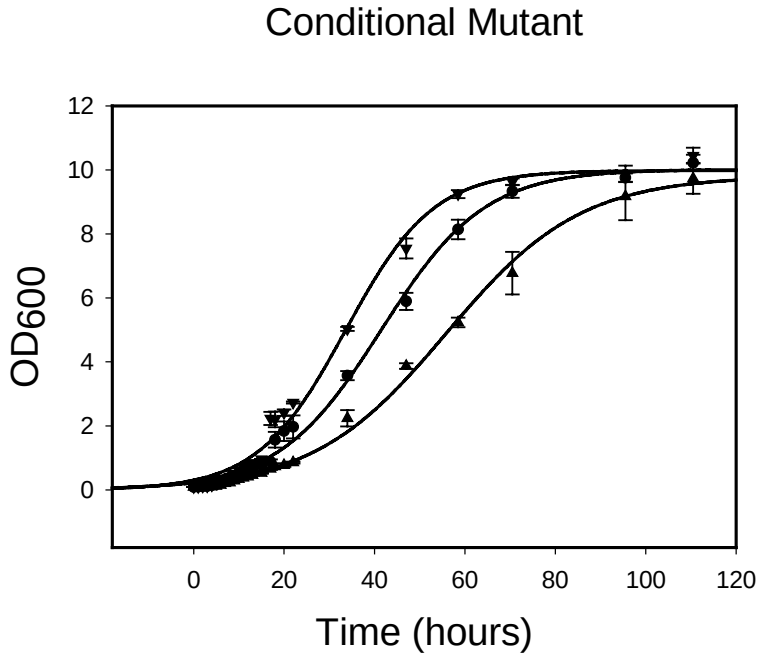


Figure 3.12 (continued). Comparison of growth rates for wild type and conditional mutant. Cultures were grown in triplicate and averaged. **A.** The growth rate for wild-type C. albicans in the presence of 10 (▲), 25 (●), and 50 (▼) μM Methionine. **B.** Growth curves for the conditional *Camet6* mutant in the presence of 10 (▲), 25 (●), and 50 (▼) μM Methionine.

CHAPTER 4: DISCUSSION

MEASURING KINETIC PARAMETERS

Complementation of the *Scmet6* mutant strain by both *C. albicans* and *S. cerevisiae* Met6 proteins, demonstrated that the two enzymes have similar activities, and function similarly under *in vivo* conditions. The specific activity of CaMet6p and ScMet6p during purification increased only 6-10 fold due to the overexpression of the enzymes in the crude extract. The purified enzymes were used for kinetic analysis. The behavior of CaMet6p and ScMet6p are very similar, consistent with their high sequence homology. Both enzymes require at least two glutamates for 5-CH_3-H_4PteGlu$_n$ to be a substrate. These results are consistent with a previous study that showed the ScMet6 enzyme requires at least a diglutamated substrate (Burton *et al*., 1969). That same study reported a K_m value for (6R,S)5-CH_3-H_4PteGlu$_2$ of 430 μM and a K_m value for (6R,S)5-CH_3-H_4PteGlu$_3$ of 380 μM, which are approximately four times higher than the values observed in the present experiments. The use of recombinant protein, a different method of synthesizing substrate, and a modified assay can account for these differences. Similar K_m values for the (6R,S)5-CH_3-H_4PteGlu$_2$ and the (6R,S)5-CH_3-H_4PteGlu$_3$ substrates were reported in another study by McClurg, 410 μM and 350 μM respectively (McClurg, 1996), but the *kcat* values for the di- and triglutamated substrates was reported to be about 25/min, identical to our values.

McClurg also assayed a heptaglutamated substrate, which resulted in a much lower K_m value, 18 μM.

Previous studies, and my work, show that both CaMet6p and ScMet6p were not able to use the monoglutamated form of (6R,S)-5-CH_3-H_4PteGlu$_n$ as a measurable substrate. This observation might mean that the enzymes did not bind to the substrate, or that the substrate bound to the enzymes at a site or in an orientation not contributing to catalysis. Experiments were therefore performed measuring the rate of activity for the (6R,S)-5-CH_3-H_4PteGlu$_3$ substrate in the presence of increasing concentration of the (6R,S)-5-CH_3-H_4PteGlu$_1$ as a potential inhibitor. In these experiments, there was no evidence that the monoglutamated derivative was able to compete with polyglutamated substrate. This observation is consistent with the concept that the monoglutamated derivative does not bind to the active site of the enzymes at any reasonable concentration.

SITE-DIRECTED MUTAGENESIS

The D614N and D614A site-directed mutant forms of CaMet6p were constructed to implement a strategy for the study of important active site residues. These mutant forms of the *CaMET6* gene were not detected in the soluble fraction of cell extracts. This suggested that these mutants failed to fold properly. To solve this problem, a GST fusion wild type CaMet6p was expressed. The same site-directed mutants were created as GST fusions, and these constructs were successfully expressed. GST is known to enhance protein solubility, and facilitate

protein purification (Busso *et al*., 2005). This strategy will allow for the isolation of various site-directed mutant proteins, and the kinetic characterization of those mutants.

CaMET6 GENE DELETION

Initial attempts to create a viable *CaMET6* double null mutant using the PCR-based gene disruption method were unsuccessful, most likely because the double disruption was lethal. After the first round of transformations, one copy of *CaMET6* was completely replaced by a selection cassette. This was successful with both the *URA3* and the *ARG4* cassettes. These heterozygous mutants grew normally. However, subsequent attempts to delete the second copy of *CaMET6* in the heterozygous mutants, using a different selection cassette, failed to produce viable homozygous *Camet6* mutants. Transformations from these experiments were plated, multiple times, on media supplemented with histidine and methionine, but no colonies appeared on the plates even when allowed to incubate for up to 10 days. The results from these experiments indicate that *CaMET6* is essential, and the double deletions are probably lethal since colonies did not grow even when supplemented with exogenous methionine.

To further test this hypothesis, I created a conditional *met6* mutant in *C. albicans*. One copy of the *CaMET6* gene was replaced with an *ARG4* cassette, and a *GAL1* promoter was inserted in front of the second copy. Clearly, the conditional *CaMET6* gene was being expressed and the protein was functional.

The *GAL1* promoter was induced with galactose, and the conditional mutant grew well on media that contained galactose and histidine. In contrast, when the *GAL1* promoter was repressed with glucose, the conditional mutant did not grow when supplemented only with histidine, but did grow when supplemented with both histidine and methionine. These results suggested that *C. albicans* could import enough methionine to meet its metabolic needs. However, this seems inconsistent with the observation that a double knockout cannot grow even when supplied with exogenous methionine. It may be that the enzyme has an additional function that is essential to cell growth.

To help elucidate the essential nature of the *met6* gene, the conditional mutant was further analyzed using a Western Blot. The CaMet6 protein levels were compared between the induced conditional mutant and the repressed conditional mutant, revealing that even though the *GAL1* promoter was repressed, the CaMet6 protein was still expressed, although at much a lower level than occurred under inducing conditions. This confirms previous reports that the *GAL1* promoter is not completely repressed in glucose (Berman, 2002; Srikantha, 1996). These findings also indicate that whereas *C. albicans* is able to import sufficient methionine from the media to survive, the *CaMET6* gene probably has some other important role or roles in addition to the synthesis of methionine. For example, even when expressed at low levels, the *CaMET6* protein may overcome homocysteine buildup, and thereby avoid the affects of homocysteine toxicity

(Pascon *et al*., 2004; Jakubowski *et al*., 1991). An accumulation of intracellular homocysteine is observed in both *S. cerevisiae* and *S. pombe* (Fujita *et al*., 2006). Thus, the additional growth defects, not rescued by methionine, could be caused by toxicity of intracellular homocysteine. Homocysteine has been reported to interfere with purine biosynthesis and sterol biosynthesis (Hatanaka *et al*., 1974; McCammon *et al*., 1981).

Disruption mutants of methionine synthase in *S. cerevisiae* and *Aspergillus nidulans* can be fully rescued by methionine, which contrasts with my results (Kacprzak *et al*., 2003; Suliman *et al*., 2005). Pascon *et al*. showed that a *met6* mutant strain of *Cryptococcus neoformans* is not viable without methionine, but does grow at a slow rate when supplemented with methionine (Pascon, 2004). This is similar to the situation we have observed for *C. albicans*, where the repressed conditional mutant grows about six times more slowly than wild type under conditions that mimic intercellular fluids (10 μM methionine).

The *C. neoformans met6* mutant was also avirulent in a mouse inhalation model, and is more susceptible to current anti-fungal drugs (Pascon *et al*., 2004). Seong *et al*. observed that a methionine synthase gene (*msy1*) deletion mutant of *Fusarium graminearum* is defective in aerial hyphal grown even in presence of methionine (Seong *et al*., 2005). Likewise, Fujita *et al*. reported that disruption of the methionine synthase gene (*met26*) in *Schizosaccharomyces pombe* leads to a requirement for both methionine and adenine (Fujita *et al*., 2006). It is reasonable

to assume that *C. albicans* conditional mutant would behave like these cases, and also that a drug repressed wild-type strain would be similarly avirulent.

CONCLUSION

The work presented here provides a strong foundation for further study of cobalamin-independent methionine synthase as anti-fungal drug target. There is still a lot to learn about the mechanism of action of the cobalamin-independent enzymes. For example, no known intermediate or catalytic residues have been specifically identified. Understanding how these enzymes catalyze this important reaction may aid in the design of potent inhibitors. The gene disruption experiments in *C. albicans* show the good possibility that cobalamin-independent methionine synthase can be an effective anti-fungal drug target. The repressed conditional mutant does express a low amount of methionine synthase, and may be a good model for the activity of the enzyme in the presence of an inhibitory drug. The phenotype of a methionine synthase mutant seems to be more complicated than just methionine auxotrophy. The growth defects observed in *C. albicans* could be due to the toxic effect of homocysteine buildup or some other function, perhaps nonenzymatic, of methionine synthase.

FUTURE WORK

Structure Determination

Numerous attempts have been made to crystallize both ScMet6p and CaMet6p for the purpose of structure determination, but with no success. Efforts are underway to use other Met6 proteins from different species of pathogenic fungi for this purpose. Other pathogenic fungi of interest include *Cryptococcus neoformans* and *Wangiella dermatitidis*. The *C. neoformans* genome has been sequenced, and cDNA is available through http://www.genome.ou.edu/cneo.html. *Wangiella dermatitidis* will be a challenge, because its genome is not sequenced, and its genome contains introns. The *MET6* gene will be identified through sequence homology with other *MET6* genes, and cDNA will need to be obtained, or the gene altered to remove introns.

Inhibitor Design

Anti-folates are a major class of known drugs with wide utility. Since methionine synthase is a folate enzyme, it is reasonable to test some as inhibitors. Methotrexate, folinic acid, spermine, and sperimidine, were in fact tested against ScMet6p, but none inhibited enzyme activity. My kinetic work has shown that Met6 proteins need at least a diglutamated substrate to bind in the active site, and

the same charge requirement may be true for inhibitors. The potential inhibitors that were tested were all monoglutamated, and may not have bound to the enzymes. In order to find an effective inhibitor, folate substrate analogues would need to be polyglutamated. Then dose responses, and kinetic analyses can be performed to determine the effectiveness of the potential inhibitors.

Inhibitors can be designed by analyzing the structures of cobalamin-independent methionine synthase from *Arabidopsis thaliana,* and *Thermotoga maritima.* These structures have substrate bound, and give insight into how the two substrates bind in the active site. From this, an inhibitor consisting of a homocysteine-5-CH_3-H_4-$PteGlu_n$ fusion may be developed. Further study may also suggest the chemistry and geometry for a transition state analog. Similar to what was done with betaine homocysteine methyltransferase, this potential transition state analog can be used to further study binding contacts between the substrates and the enzyme (Evans *et al*., 2002; Castro *et al*., 2004).

Site Directed Mutagenesis

Once purified mutant forms of CaMet6p are obtained, they can be kinetically characterized, and compared to the wild-type data. Pursuing these experiments will give insight into the active site of the enzymes, and possibly shed light on the mechanism of action during catalysis.

APPENDIX A

MATERIALS

Material	Manufacturer
Acrylamide	J.T. Baker, Phillipsburg, NJ
Agarose	Life Technologies,
Ampicillin	Fischer Scientific, Pittsburgh, PA
Ammonium persulfate	Fischer Scientific, Pittsburgh, PA
B-mercaptoethanol	Sigma, St. Louis, MO
Bacto agar	Difco Laboratories, Detroit, MI
Bis-acrylamide	Sigma, St. Louis, MO
Brilliant blue (Coomassie)	Sigma, St. Louis, MO
Bromophenol blue	Difco Laboratories, Detroit, MI
BSA	Sigma, St. Louis, MO
Calcium Chloride	Sigma, St. Louis, MO
Dithiothreitol (DTT)	Sigma, St. Louis, MO
Ethidium bromide	Sigma, St. Louis, MO
Glucose	Mallinkrodt, Paris, Kentucky

Glycerol	EM Science, Cherry Hill, NJ
Herring Testes DNA	Sigma, St. Louis, MO
Histidine	Sigma, St. Louis, MO
KOD polymerase	Invitrogen,
Leucine	Sigma, St. Louis, MO
Magnesium chloride	Sigma, St. Louis, MO
Methionine	Sigma, St. Louis, MO
Pfu polymerase	New England Biolabs,
Polyethyleneglycol (PEG) 3350	Sigma, St. Louis, MO
Potassium chloride	Sigma, St. Louis, MO
Serine	Sigma, St. Louis, MO
Sodium dodecyl sulfate	New England Biolabs, , MA
Taq DNA polymerase	Sigma, St. Louis, MO
Trizma® (Tris base)	Sigma, St. Louis, MO
Tryptone peptone	Difco Laboratories, Detroit, MI
Yeast Nitrogen Base	Difco Laboratories, Detroit, MI

APPENDIX B

PROTOCOLS

YM (minimal media)

7 g Yeast Nitrogen Base w/o amino acids

20 g agar (only when making plates)

ddH_2O

autoclave

Then add:

100 ml of 20% glucose

10 ml of 100X amino acids as needed up to 1 Liter total

YPD (rich media)

20 g tryptone

10 g yeast extract

900 ml ddH_2O

20 g agar (only when making plates)

autoclave

Then add:

100 ml of 20% glucose

RbCl competent cells (*e. coli*)

1) Streak desired E. coli strain on fresh LB plate. Grow overnight at 37°C.

2) Inoculate a single colony into 10 ml LB. Grow overnight, shaking at 37° C.

3) Inoculate about 1 ml into 200 ml LB in a 2 L flask. Shake at 37°C until OD_{600} = 0.5.

4) Chill flask in ice-water 5 minutes.

5) Spin 5 minutes at 6,000 rpm in GS3 rotor.

6) Resuspend pellet in 80 ml ice cold Buffer 1.

7) Chill in ice-water 5 minutes.

8) Spin 5 minutes at 6,000 rpm in GS3 rotor.

9) Resuspend pellet in 8 ml ice cold Buffer 2.

10) Chill in ice-water 15 minutes.

11) Make 100 µl aliquots in 1.5 ml Eppendorf tubes, and store at –70°C.

Buffer 1(500 ml)	**Buffer 2 (100 ml)**
30 mM KOAc (1.47 g)	10 mM MOPS (0.21 g)
100 mM $RbC1_2$ (6.04 g)	75 mM $CaCl_2$ (1.10 g)
10 mM $CaC1_2$ (0.74 g)	10 mM $RbCl_2$ (0.12g)
50 mM $MnC1_2$-$4H_20$ (4.94 g)	15% glycerol (15 ml)
15% glycerol (75 ml)	
pH to 5.8 with dilute acetic acid	pH to 6.5 with 1 M KOH
sterile filter	sterile filter

LURIA BERTANI (LB) MEDIA

10 g peptone

5 g yeast extract

5 g NaCl

ddH2O up to 1 liter

***E. coli* transformation**

1) Thaw competent cells on ice.

2) Add 1 µl of DNA to one tube of competent cells, and incubate on ice 40-60 minutes.

3) Heat shock at 42^{o}C for 90 seconds.

4) Add an equal volume of LB (no antibiotics) to the tube, and incubate on ice for 2 minutes.

5) Incubate the tube at 37^{o}C for 30-60 minutes.

6) Plate 50 µl of the transformation mix on selective media.

YEAST TRANSFORMATION ("THE BEST METHOD", GIETZ LAB)

1) Start a 5 ml overnight culture of the yeast cells to be transformed, in YPD.

2) Next morning, inoculate 25 ml of YPD with 1 ml of the overnight culture. Allow cells to grow to A_{600} = 1.0.

3) Centrifuge at 5000 rpm for 5 minutes.

4) Resuspend the cells in 3 ml of ddH_2O, and centrifuge for 5 minutes.

5) Resuspend the cell pellet in 1 ml of 100 mM LiAc and incubate for 10 min at 30° C. (you can keep the cells overnight at 4°C if necessary)

6) Place 100 µl of cells from the previous step into a 1.5 ml tube for each transformation reaction.

7) Spin the suspension at top speed in a microcentrifuge for 5 sec. Remove the supernatant with a micropipette.

8) Add the following components into the tube on top of the cell pellet in this order:

- 240 µL of PEG 3,350 (50% w/v)
- 36 µL 1.0 M. LiAc
- 15 µL SS-DNA (10.0 mg/ml)
- 5.0 µL of plasmid or linear DNA
- 64 µL of sterile ddH_2O.

9) Vortex the cell pellet for at least 1 min to resuspend the cell pellet in the transformation mix.

10) Incubate the cells for 30 minutes at 30°C.

11) Incubate the cells for 20 minutes at 42°C.

12) Pellet the cells at top speed in a microcentrifuge for 10 sec. Remove the supernatant using a micropipette.

13) Add 200-400 µL of sterile ddH_2O water and leave at room temperature for 5 minutes, and resuspend by slowly pipetting up and down.

14) Plate the cell suspension onto 1 or 2 plates of omission medium that selects for the presence of the plasmid. If necessary, let the plates sit a little to absorb the mixture. Colonies should be visible in 2 -4 days at 30° C.

SYNTHESIS OF (6R,S)-5-CH_3-H_4-PTEGLU$_N$ FROM H_4PTEGLU$_N$

(6*R,S*)-5-CH_3-H_4PteGlu$_n$ was synthesized from H_4PteGlu$_n$ (Schirck's Laboratories Jona, Switzerland) by a modification of the procedure of Yeo and Wagner (1992). 40 µmol of PteGlu$_n$, 20-40 mg depending on the number of glutamates, is weighed in a 2 ml free-standing screwcap tube with a round bottom. Make a 265 µM $Pb(NO_3)_2$ solution by first making 26.5 mM $Pb(NO_3)_2$ solution in ddH_2O, then diluting 100 times with 10 mM Tris-Cl, pH 7.5. Add 200 µl of the 265 µM $Pb(NO_3)_2$ to the weighed PteGlu$_n$. To dissolve the PteGlu$_n$, adjust the pH of the solution to 7.5 using a 5 M NaOH solution, then vortex. Spot small amounts on pH paper to check the pH. Add 5 M NaOH as needed, and

continue until all the $PteGlu_n$ is dissolved resulting in a clear, dark yellow solution. $PteGlu_n$ with higher numbers of glutamates will require considerable vortexing and more NaOH to dissolve completely. Check the spectrum of a 10^{-4} dilution in 10 mM Tris-Cl, pH 7.5; it should show peaks at 282nm and at 350nm.

$PteGlu_n$ is then reduced to $H_4PteGlu_n$ using $NaBH_4$. In this reaction, $Pb(NO_3)_2$ is used as a catalyst to reduce the amount of $NaBH_4$ used. It is important to use fresh active NaBH4, especially since it is hygroscopic. Add a stir bar to the tube containing the $PteGlu_n$ and move it to the cold room. Use an eppendorf tube rack to hold the tube, while stirring at top speed. Add 5 mg NaBH4 (132 μmol) to 25 μl ddH_2O, and then add 5 μl of this solution to the $PteGlu_n$ 5 times over 20 minutes. The pH of this solution is maintained by adding 2-5 μl of 20%(w/v) citric acid per addition of $NaBH_4$. Once all of the $NaBH_4$ is added, move to room temperature, and flush the tube with argon or N_2, and stir at maximum speed, in the dark, for 2 hours.

Next, cool the tube to 4^oC, and lower the pH to 5.0 by adding 5 M Acetic Acid, some bubbling will occur, to destroy the excess $NaBH_4$. Then bring the pH back to 7.8 using 5 M NaOH. The product should be $H_4PteGlu_n$. Check the spectrum of a 10^{-4} dilution in 10mM Tris-Cl, pH 7.5. There should be a peak at 298nm and a trough at 244nm.

The $H_4PteGlu_n$ is then condensed with formaldehyde to produce CH_2-$H_4PteGlu_n$. 6.4 μl (80 μmol) of 37% formaldehyde is added, and the tube is

flushed with argon (N_2). The tube is incubated at 45°C for 15 minutes, in the dark to convert $H_4PteGlu_n$ to CH_2-$H_4PteGlu_n$.

CH_2-$H_4PteGlu_n$ is then reduced to 5-CH_3-$H_4PteGlu_n$ using $NaBH_4$. Add 10 mg of $NaBH_4$ (264 μmol) to 50 μl of ddH2O. Add 10 μl of this solution to the tube 5 times over 20 minutes. Maintain the pH by adding 2-5 μl of 20%(w/v) citric acid per addition of $NaBH_4$. Once all of the $NaBH_4$ is added, flush the tube with argon (N_2) and incubate at 45°C for 60 minutes in the dark.

Remove the stir bar from the tube, and cool to 4°C. Add 2-mercaptoethanol to 50 mM, and adjust the pH to 5.0 with 5 M Acetic Acid to destroy any excess NaBH4. Vortex the tube until there is no more bubbling. Bring the pH back to 7.5 using 5 M NaOH. The product should be 5-CH_3-$H_4PteGlu_n$. Check the spectrum of a 10^{-4} dilution. There should be a peak at 290-292 nm, and a trough at 248 nm.

To test the yield, dilute 2 μl of the product into 200 μl 10 mM Tris-Cl pH 7.5. Then add 10 μl of this dilution into 400 μl of 10 mM Tris-Cl pH 7.5. Add 100 μl 5 M HCl/60% formate, and heat the solution at 80°C for 10 minutes. Cool the solution to room temperature and read the spectrum. A peak at 350 nm is diagnostic of H_4-$PteGlu_n$, and the lack of a peak indicates CH_3-H_4-$PteGlu_n$. The (6R,S)5-CH_3-H_4-$PteGlu_n$ should be stored under argon(N_2) in dark at –20°C. This procedure gives ~93% yield, and the final product has less than 3% $H_4PteGlu_n$

based on its reaction with 5 M HCl/60% formate. For long term storage, can be stored at –80oC for at least a year (Drummond *et al.*, 1995).

SYNTHESIS OF L-HOMOCYSTEINE FROM L-HOMOCYSTEINE THIOLACTONE

L-homocysteine was prepared from L-homocysteine thiolactone by alkaline hydrolysis (Drummond *et al*., 1995). Fifty mg of L-homocysteine thiolactone (Sigma) is weighed and dissolved in 1.7 ml ddH_2O. Bubble an 800 mM NaOH solution with argon (N_2) for 5 minutes, then 830 ml of the NaOH solution is added to the L-homocysteine thiolactone solution. Incubate this solution at 45^oC for 6 minutes. Adjust the pH to 5.0 using 5.0 M acetic acid, checking the pH with pH paper. Dilute the solution to 3.3 ml using argon (N_2) bubbled ddH_2O, resulting in a ~100 mM solution. Store L-homocysteine in 1 ml aliquots at –80^oC.

Titrate the L-homocysteine solution with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to check the actual concentration. Make a 10 mM DTNB stock solution in 50 mM potassium phosphate buffer pH 7.0. Dilute the L-homocysteine solution 100 fold, and add 50 µl to 900 µl of 100 mM sodium phosphate buffer pH 8.0. To this solution add 50 ml of the DTNB stock, and let it stand for 2-3 minutes. Then read the spectrum of the solution at 412 nm. Subtract the buffer blank, and divide the corrected absorbance at 412 nm by the extinction coefficient for the liberated thionitrobenzoate anion (13.6 mM^{-1}) to calculate the actual L-homocysteine concentration.

5X LOADING BUFFER FOR DNA SAMPLES

3.0 ml Glycerol

0.025 g Bromophenol Blue

0.025 g Xylene Cyanol FF

Autoclaved ddH_2O up to 10 ml

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VITA

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Suliman, H. S., Sawyer, G. M., Appling, D. R., and Robertus, J. D. (2005) Purification and properties of cobalamin-independent methionine synthase from *Candida albicans* and *Saccharomyces cerevisiae. Archives of Biochemistry and Biophysics*. 441:56-63.

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