Published in final edited form as:

Arch Biochem Biophys. 2011 September 1; 513(1): 19–26. doi:10.1016/j.abb.2011.06.002.

Structure of *Candida albicans* methionine synthase determined by employing surface residue mutagenesis

Devinder Ubhi, Kathryn L. Kavanagh, Arthur F. Monzingo, and Jon D. Robertus*

Institute for Cellular and Molecular Biology, Department of Chemistry and Biochemistry, University of Texas at Austin, Austin TX 78712

Abstract

Fungal methionine synthase, Met6p, transfers a methyl group from 5-methyl-tetrahydrofolate to homocysteine to generate methionine. The enzyme is essential to fungal growth and is a potential anti-fungal drug design target. We have characterized the enzyme from the pathogen *Candida albicans* but were unable to crystallize it in native form. We converted Lys103, Lys104, and Glu107 all to Tyr (Met6pY), Thr (Met6pT) and Ala (Met6pA). All variants showed wild-type kinetic activity and formed useful crystals, each with unique crystal packing. In each case the mutated residues participated in beneficial crystal contacts. We have solved the three structures at 2.0–2.8 Å resolution and analyzed crystal packing, active-site residues, and similarity to other known methionine synthase structures. *C. albicans* Met6p has a two domain structure with each of the domains having a (β_α)₈-barrel fold. The barrels are arranged face-to-face and the active site is located in a cleft between the two domains. Met6p utilizes a zinc ion for catalysis that is bound in the C-terminal domain and ligated by four conserved residues: His657, Cys659, Glu679 and Cys739.

Keywords

cobalamin-independent methionine synthase; surface entropy reduction; X-ray structure; zinc coordination; mutagenesis

1. Introduction

Methionine synthase enzymes catalyze the synthesis of methionine by transferring a methyl group from a donor to L-homocysteine (L-Hcy) [1]. There are two broad classes of methionine synthases: those that use a cobalamin cofactor, and those that transfer the methyl group directly from 5-methyl-tetrahydrofolate (5-methyl-THF) to the sulfur of L-Hcy, forming methionine and tetrahydrofolate. These cobalamin-independent enzymes are usually observed in organisms deficient in Vitamin B12, including fungi, plants, insects, and archaea. In contrast, vitamin B12-containing organisms, like humans, use cobalamin-dependent methionine synthase in which cobalamin acts as an intermediate methyl carrier. The physical and chemical properties of these enzyme groups have been reviewed [2–4].

© 2011 Elsevier Inc. All rights reserved.

*Corresponding author: Department of Chemistry and Biochemistry, University of Texas at Austin, Austin TX 78712, Tel. (512) 471-3175, jrobertus@mail.utexas.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The cobalamin-independent methionine synthases (referred to as MetE) are two-domain proteins of approximately 90 KDa [5]. Previously, the X-ray structures of the enzyme from plant *Arabidopsis thaliana* [6] and from bacteria *Thermotoga maritima* [5] and *Streptococcus mutans* (DOI:10.2210/pdb2nq5/pdb, unpublished) were solved and shown to be structurally homologous. Each domain is a $(\beta\alpha)_8$ barrel with extended loops at the C-terminal end of the β -strands. The barrels are arranged face to face, where the extended $\beta\alpha$ loops form the interface and a variable linker connects the two domains. Crystal structures containing L-Hcy and/or folate analogs place the methyl and L-Hcy thiolate ~ 7 Å apart [5, 6]. This large distance is inconsistent with the chemical reaction and suggests the structures are in an “open” conformation and that the reactive groups must be brought closer together by domain closure for catalysis to occur.

Although several of the reported structures have been co-crystallized with L-Hcy or methionine, this doesn't influence the overall open conformation of the protein and all X-ray structures with folate analogs have so far been produced by soaking the ligand into preformed open-state crystals. There may be a dynamic equilibrium between the open and closed forms in solution with the open form more stable for crystallization. Alternatively, a transition from the open to the closed form could be induced by ligand binding. If the latter is the case, folate is likely the ligand that triggers the conformational change since it contacts both domains. Ligand-induced domain movement has been described for a reference set of 203 proteins that have been crystallized in more than one conformation and in the majority of cases (150) the ligand that initiates domain closure has contact with both domains [7]. This suggests that a closed MetE structure will only be obtained by co-crystallizing with a folate analog or other ligand that elicits the closed conformation.

Cobalamin-independent methionine synthases from fungi are referred to as Met6p proteins. We have previously cloned, purified, and carried out steady-state kinetic analysis of the Met6p enzymes from *Saccharomyces cerevisiae* and *Candida albicans* [8] and used site directed mutagenesis to identify key catalytic residues [9]. More importantly, we used homologous recombination techniques to delete MET6 and showed that expression of Met6p is essential to the diploid pathogen *Candida albicans* [10]. We also placed MET6 under a regulatable promoter to reduce enzyme activity and mimic the effect of Met6p being partially inhibited by a drug. This showed that in an environment mimicking human tissue, a strong inhibitor of Met6p could act as a fungistatic agent, retarding the growth of the pathogen and validating Met6p as a drug design target [10]. Because mammals utilize a cobalamin-dependent methionine synthase, it is likely that drugs that inhibit fungal Met6p would not interfere with methionine synthesis in humans. Earlier, we built a model of *C. albicans* Met6p based upon the *Arabidopsis* structure, which has 49% sequence identity. Using this model we were able to predict key mechanistic residues in the active site [9]. Our current goal is to design drugs against the *C. albicans* Met6p and we feel that the theoretical model is not accurate enough for these studies.

Fungal pathogens pose a significant health concern to immuno-compromised individuals, with the *Candida* species associated with the highest mortality rate. In particular *C. albicans* leads in the number of patients diagnosed with fungal nosocomial bloodstream infections [11]. In order to facilitate the discovery of antifungal agents targeting the Met6p enzyme, we have endeavored to crystallize and solve the X-ray structure of this key enzyme. We have been unable to crystallize the wild-type protein, but here we report the use of surface entropy reduction, SER, [12, 13] to produce three Met6p variants, all with full enzyme activity, that crystallized and allowed high resolution structures to be elucidated.

2.0 MATERIALS AND METHODS

2.1 Design of SER mutants

The SERp server [14](<http://nihserver.mbi.ucla.edu/SER/>) was used to identify potential residues for surface entropy reduction mutations. The top scoring cluster, ¹⁰³LysLysAlaThrGlu¹⁰⁷, was then compared to known bacterial and plant structures and found to be on a loop that is ~10 residues longer in the fungal protein (Figure 1). This loop region was chosen for mutation. Based upon a previous study that looked at the role of different amino acids in promoting crystal contacts [13], the two lysine and one glutamate residues were mutated either all to Ala, all to Thr, or all to Tyr in variants labeled Met6pA, Met6pT, and Met6pY, respectively.

2.2 Mutagenesis, expression and purification

The gene encoding *C. albicans* Met6p was amplified from Yep24-CaMET6 [10] and cloned into the *E. coli* expression vector pNIC28-Bsa4. This plasmid has an N-terminal histidine tag followed by a tev-protease cleavage site. The desired mutations were introduced using PCR-based site-directed mutagenesis by overlap primer extension and the gene mutations were verified by DNA sequencing. The SER proteins (Met6pA, Met6pT, Met6pY) were expressed in 2–4 L LB media supplemented with 500 μ M ZnSO₄ and 50 mg/L kanamycin. Cells were grown at 37 °C until the OD₆₀₀ reached 0.8. The cultures were shifted to 25 °C, and expression was induced with 500 μ M IPTG and allowed to continue for 4 hours. Cells were harvested by centrifugation and the pellets stored at –80 °C.

Cell pellets were thawed and resuspended in lysis buffer (1X PBS, 20 mM Imidazole, 10 mM β -mercaptoethanol, pH 8.0) with protease inhibitors (Roche Complete EDTA-free, 1 tablet/50 ml). The cells were homogenized using a French pressure cell (3 passes, 1,000 psi) and the cell debris removed by centrifugation for 1 hour at 25,000 $\times$ g and 4 °C. The cell lysate was added to 2 ml of Ni-NTA resin and both were gently mixed for 30 minutes at 4 °C. This mixture was applied to a gravity flow column, washed with 20 ml lysis buffer and eluted with 6 ml of lysis buffer containing 500 mM imidazole. The eluted protein was first buffer exchanged into 20 mM Tris-HCl, 150 mM NaCl, 10 mM β -mercaptoethanol, pH 7.4 using a PD10 column (GE Healthcare) and then further purified using a size exclusion column (S200 16/60, GE Healthcare) run at 1 ml/min on an Akta purification system. The fractions were analyzed by SDS-PAGE and those containing Met6p protein were pooled and concentrated using a Centricon filtration unit with 30 kDa MW cut-off. Protein purity was estimated to be >95% by SDS-PAGE gel. Protein concentration was measured using the absorbance at 280 nm and an extinction coefficient calculated from the protein sequence (ϵ =90,760 M⁻¹ cm⁻¹ for wild type Met6p, Met6pA and Met6pT and ϵ =95,230 M⁻¹ cm⁻¹ for Met6pY).

2.3 Activity assay

The enzyme activity of each SER mutant was evaluated using a modified methionine synthase assay described by Drummond *et al.* [15]. The (6R,S)- 5-methyl-tetrahydrofolate-Glu3 substrate (5-methyl-THF) was synthesized from pterin with a three glutamate tail, PteGlu3, using a modified procedure of Yeo and Wagner [16], and the L-Hcy was made by alkaline hydrolysis of L-homocysteine thiolactone [8, 15, 16]. Initially a 380 μ L reaction containing 50 mM potassium phosphate (pH 7.0), 50 mM Tris-HCl (pH 7.4), 100 μ M MgSO₄, 10 mM dithiothreitol (DTT), 140 μ M 5-methyl-THF, and 400 μ M L-Hcy was prepared and preheated to 37 °C. The reaction was initiated by adding 9 μ g of enzyme (in a 20 μ L volume) and allowed to proceed for 20 minutes at 37 °C. The reaction was terminated by adding 100 μ L of 5 N HCl/60% formic acid and incubating for 10 minutes at 80 °C. During this high temperature step unmethylated THF is converted to methenyl-THF by the

formic acid. The reaction mixture was incubated on ice for 3 minutes and centrifuged to remove any precipitate. Methenyl-THF was quantified spectroscopically by measuring absorbance at 350 nm ($\epsilon=26,500 \text{ M}^{-1} \text{ cm}^{-1}$). All reactions were performed in triplicate.

2.4 Crystallization, Data Collection, Structure Determination

The SER variant proteins were concentrated to ~20 mg/ml in 20 mM Tris, 150 mM NaCl, 10 mM β -mercaptoethanol, pH 7.4. Crystallization trials were set up with both apo protein solutions and protein solutions containing 10 mM L-Hcy using the JCSG⁺ Suite (Qiagen) and PEG/Ion HT (Hampton Research) 96-well screening kits. The plates were set up using a Phoenix crystallization robot (Art Robbins Instruments) and were incubated at 20 °C. Several conditions producing initial crystals were identified, and two were chosen for optimization: JCSG⁺ G9 (30% [w/v] PEG-MME 2000, 0.1 M KSCN) and PEG/Ion A10 (20% [w/v] PEG3350, 0.2 M NaI). Crystal optimization experiments were set up with all three mutants in 96-well and 24-well formats.

Optimized crystals of the Met6pA mutant were rod-shaped with a maximum length of 800 μm . They were grown in hanging drops at 20 °C by mixing 1 μl of 20 mg/ml apo protein and 2 μl of a well solution consisting of 27% (w/v) PEG 3350, 50 mM NaI and equilibrated against 500 μl of the well solution. Prior to data collection, a single crystal was cryo-protected in 30% (w/v) PEG 3350, 50 mM NaI and flash-cooled in liquid nitrogen.

Optimized crystals of the Met6pT mutant were large plates with dimensions 800 $\times$ 300 $\times$ 50 μm . They were grown in hanging drops at 20 °C by mixing 1 μl of 20 mg/ml apo protein with 2 μl of a well solution composed of 25% (w/v) PEG-MME 2000, 75 mM KSCN and equilibrated against 500 μl of well solution. Prior to data collection, a single crystal was cryo-protected in 30% (w/v) PEG-MME 2000, 75 mM KSCN, and plunged into liquid nitrogen.

Optimized crystals of the Met6pY mutant were rod-shaped with a maximum length of 200 μm . They were grown in hanging drops at 20 °C by mixing 0.7 μl of 20 mg/ml apo protein and 1.3 μl of a well solution consisting of 20% (w/v) PEG 3350, 250 mM NaI and equilibrated against 500 μl of the well solution. Prior to data collection, a single crystal was cryo-protected in 20:80 (v/v) ratio of PEG 400 to well solution and flash-cooled in liquid nitrogen.

Among the three mutant proteins, only Met6pY crystallized with zinc at the active site while Met6pT and Met6pA both lost the zinc during crystallization. While Met6pT crystals were fragile and difficult to reproduce, Met6pA formed large well-diffracting crystals and were soaked in a zinc containing solution in order to obtain a zinc-replete crystal. Met6pA crystals were first soaked in reservoir solution supplemented with 250 μM zinc sulfate and 500 μM DTT for 3 hours followed by an 8–12 hour soak in reservoir solution including 500 μM DTT. Met6pA crystals plus zinc were then cryo-protected in 30% (w/v) PEG 3350, 50 mM NaI and flash-cooled in liquid nitrogen.

Diffraction data from the three variant crystals were collected at 100 K at the Advanced Light Source beam line 8.3.1 at the Lawrence Berkeley National Laboratory. The Met6pY and Met6pT data were integrated and scaled with Mosfilm and Scala [17]. HKL2000 [18] was used to process the Met6pA data. A molecular replacement solution for the Met6pY crystal was determined with Phaser [19] using the structure of *Arabidopsis* methionine synthase (PDB ID: 1U1H) [6] as the search model. A molecular replacement solution for Met6pT was found with Phaser, and a solution for Met6pA with and without zinc was found with MOLREP using the Met6pY structure as the search model. The models were improved

through iterative rounds of model building with COOT [20] and refinement using Phenix [21] and Refmac [22, 23].

2.5 Atomic coordinates

Coordinates and structure factors of the refined models of Met6pA with and without Zn in the active site, Met6pT(−Zn), and Met6pY(+Zn) have been deposited in the Protein Data Bank with entry codes 3PPG, 3PPF, 3PPH, and 3PPC, respectively.

3.0 RESULTS AND DISCUSSION

3.1 Surface entropy reduction mutants

We undertook the SER engineering of *C. albicans* Met6p because we were unable to crystallize the wild-type protein. Our engineering focused on an elongated surface loop, initially identified by the SERp server. Residues 103–112 in the fungal enzyme lengthen a surface loop common to all members of the family (Figure 1). Three variants were constructed by site-directed mutagenesis: Met6pA, Met6pT and Met6pY. Protein was purified for all three and tested for activity using a kinetic assay. The catalytic activity of the mutant proteins was indistinguishable from the wild-type protein, showing that the surface mutations had not affected enzyme function (data are summarized in Table 1). All three proteins formed well-diffracting crystals which allowed us to collect data at 2.0 – 2.8 Å resolution. Crystallographic statistics are given in Table 2.

3.1.1 Met6pA—The Met6pA protein crystallized in spacegroup $P2_12_12_1$ with one monomer in the asymmetric unit. The N-terminally His-tagged Met6pA consists of 789 amino acids, of which 740 were modeled into the electron density. The histidine tag, residues within the engineered loop (106–110; see Figure 1) and four loops connecting α -helices and β -strands in the C-terminal domain were disordered and left un-modeled. The final model includes Met6p residues 1–105, 111–659, 663–679, 689–702, 710–740, 744–767, one Zn ion and 501 water molecules.

Analysis of crystal packing supports an important role of the mutated residues in forming crystal contacts. The engineered loop is arranged as an extended β -hairpin with the main-chain atoms of residues at the base of the loop (101–103 and 112–114) forming two short antiparallel strands. Although the tip of the loop is disordered, it projects towards the interface of the two symmetry-related molecules and directly facilitates crystal packing (Figures 2A, 2B). Ala103 and Ala104 have van der Waals contacts with Glu511, Gln512, Leu513 and Asn514 of a symmetry-related molecule. Analysis by PISA [24] shows that this interaction buries 88 Å² of surface area for Ala103–Ala104 and 75 Å² for amino acids 511–514. This contact would not be possible for the wild-type loop since the elongated and highly mobile Lys103 and Lys104 would sterically interfere with this packing. Although residues 106–110 are disordered, there is plenty of space at the interface to accommodate different conformations of the loop.

The zinc-depleted Met6pA crystal structure closely resembles its zinc-containing counterpart with an RMSD value of 0.45 Å over 739 equivalent C α (calculated with SSM[25]). The loss of zinc allows a disulfide bridge to form between two of the zinc-ligating residues, Cys659 and Cys739. Both Met6pA structures, with and without zinc, have the same unit cell dimensions as well as identical crystal packing.

3.1.2 Met6pT—The Met6pT mutant protein crystallized in spacegroup C2 with two molecules in the asymmetric unit. As observed in Met6pA, the protein also lost zinc during crystallization and a disulfide bond is formed between Cys659 and Cys739. As can be seen

from figures 2A and 2C, the crystal packing of Met6pT is similar to the packing for the Met6pA variant. When molecule A of Met6pT is superimposed onto Met6pA, the orientation of the Met6pT B molecule is related to a Met6pA symmetry molecule (denoted Mol A') by a $\sim 7^\circ$ rotation and ~ 11 Å translation. This rearrangement is enough to shift the spacegroup from $P2_12_12_1$ to $C2$. Disordered residues in molecules A and B include the N-terminal His tag and loops connecting $\beta\alpha$ motifs in the C-terminal domain. In particular, residues 661-662, 705-712 and 767 could not be modeled in molecule A and molecule B is missing electron density for residues 103-113, 661-662 and 705-711. An important difference between the monomers is that the SER loop for molecule A is well ordered with all residues defined by electron density, while residues 103-113 are mobile and disordered in molecule B. The final model for Met6pT includes 1502 protein residues and 50 water molecules.

The introduced Thr residues participate in crystal packing in only one of the monomers in the asymmetric unit. The SER loop from molecule A is again in an extended β -hairpin conformation and interacts with a cavity on molecule B (Figure 2D). Thr103^A and Thr104^A are not directly involved in crystal packing although Thr103^A does stabilize the antiparallel β structure at the base of the loop. Engineered Thr107^A donates a hydrogen bond to the carbonyl of Phe516^B and accepts one from the amide nitrogen of Gly515^B. This Gly515-Phe516 region is remarkably close to the loop-interaction area for the Met6pA mutant (residues 511-514).

3.1.3 Met6pY—The Met6pY variant crystallized in spacegroup $P2_12_12_1$ with two monomers in the asymmetric unit. Like Met6pA and Met6pT, the disordered regions include the N-terminal His tag, portions of the SER loop and loops between the $\beta\alpha$ motifs in the C-terminal domain. In molecule A residues 107-114, 413, 452-454 and 682-685 are disordered while molecule B lacks density for residues 105-114, 453-468 and 682-688. The final model includes 1485 protein residues, two Zn ions, eight chloride ions (located by their anomalous signal), and 432 waters.

The Met6pY crystal lattice is distinct from the other two lattices in that the engineered SER loop forms a more compact structure and participates in crystal contacts which bury more surface area. As shown in Figure 2E, the engineered Tyr103 and Tyr104 at the base of the loop form a helical structure. The introduced tyrosines interact with other residues within the monomer to create a surface for intermolecular contacts. Some of these interactions are shown in Figure 2F whereby Tyr103 joins an aromatic stack with Arg99 and Trp26. The side chain of Tyr104 sits roughly perpendicular to that stack, making non-polar contacts, while the hydroxyl of Tyr104 accepts a hydrogen bond from Arg99. This patch within each molecule is then utilized to interact with an adjacent molecule. Formation of this intermolecular dimer buries ~ 1300 Å² and packing of the engineered loops contributes 415 Å² of buried surface area.

3.1.4 Met6p Structural Comparison—All three SER variants crystallized using the loop to create new packing interfaces. Of the three variant proteins, Met6pY crystals have the lowest solvent content with a Matthews coefficient (V_m) of 1.94 and 37% solvent content. Crystal contacts bury 4792 Å² of the surface area for the two molecules in the asymmetric unit. The Met6pA and Met6pT crystals are not as densely packed. The Met6pA crystal form has $V_m = 2.17$ and 43% solvent content with 1108 Å² buried surface area. Met6pT has the highest solvent content with $V_m = 2.77$ and 56% solvent content and only 2082 Å² of buried surface area for the two molecules in the asymmetric unit.

C. albicans Met6p has a distinct two-domain structure that is similar to other cobalamin-independent methionine synthases for which structures have been determined. In *C. albicans*

Met6p the N-terminal domain contains residues 1-398 and a linker of 5 residues separates it from the C-terminal domain comprised of residues 405-767. Using the program SSM[25], the Ca positions for 648 aligned residues between Met6pA and *A. thaliana* MetE superimpose with an RMSD of 1.75 Å. The resemblance can be clearly seen in Figure 3 which shows the superposition of the backbone of Met6pA (PDB ID: 3PPG) with that of methionine synthase from *A. thaliana* (PDB ID: 1U1H), *S. mutans* (PDB ID: 3L7S) and *T. maritima* (PDB ID: 3BQ5).

3.2 Met6p Active Site

Methionine synthase transfers a methyl group from a folate cofactor to L-Hcy. The pterin ring of folate stacks with the indole of Trp576 and is oriented by hydrogen bonds with Asp504 (*Candida* enzyme numbering is used unless otherwise specified). The role these residues play in substrate binding and catalysis has been confirmed for the *C. albicans* enzyme by site-directed mutagenesis [9]. Substrate-bound structures of methionine synthase from *A. thaliana* and *T. maritima* were aligned with our substrate-free Met6pA in order to compare the active sites. Figure 1 shows that despite an overall sequence homology of <50%, the 5-methyl-THF and L-Hcy binding residues are highly conserved.

To activate L-Hcy for catalysis Met6p uses a Zn ion that is bound in the C-terminal domain and is ligated by three conserved residues; His657, Cys659, and Cys739 (Figure 4). Zinc typically has tetrahedral coordination and analysis of our structures shows that Glu679 is the forth ligand for Met6pA while Met6pY has trigonal planar geometry with a distant axial water. Variations in zinc coordination are also seen in other methionine synthases: in addition to the His and two Cys ligands, Ferrer *et al.* observed water to be the fourth ligand in the *A. thaliana* enzyme [6], while in the *S. mutans* structure (PDB ID: 3L7S, unpublished) and in the substrate free *T. maritima* enzyme [5] glutamate is the forth ligand. A second *T. maritima* structure determined at low pH with L-Hcy bound exhibits trigonal bipyramidal geometry with relatively long distances for the axial ligands [5]. Compared to the ligating glutamate residue, L-Hcy approaches the zinc from the opposite face of the plane formed by the three conserved zinc ligands (Figure 4). This would mean that, during L-Hcy binding or methionine release, shifting the forth zinc ligand from Glu to L-Hcy (or from product methionine to Glu) would essentially invert the tetrahedral Zn geometry as has been observed for the *T. maritima* enzyme [26]. Although the implications of these zinc coordination differences for methionine synthase activity are unclear at the moment, structural reorganization of ligands surrounding a metal ion are also observed in zinc-dependent alcohol dehydrogenases and are proposed to be a necessary component of catalysis [27]. Regardless of the differences in zinc coordination observed in crystal structures, EXAFS experiments demonstrate that the thiol of L-Hcy is activated for catalysis by displacing the fourth ligand and assuming that role itself [28].

Much of the work to characterize cobalamin-independent methionine synthase has focused on the *E. coli* enzyme. *E. coli* MetE activity is dependent on phosphate and stimulated by Mg^{+2} (or Mn^{+2})[29]. While phosphate is required for *C. albicans* Met6p activity, addition of Mg^{+2} had no effect (data not shown). The role of phosphate is unknown though it has been hypothesized to increase reactivity of the Zn-bound L-Hcy thiolate by acting as the 5th Zn ligand [28]. While there is no obvious phosphate binding site in the MetE structures, analysis of crystal structures where sulfate was used as a co-precipitant [5, 6] may yield some clues. The sulfate molecules in these structures cluster in three distinct locations: they interact with Ser661 ~10Å from the Zn ion; they associate with Gln15, Arg16 and Lys19 that are presumed to be involved in binding the 5-methyl-THF polyglutamate tail; and they are found near the backbone of Gly585 on the surface of the protein remote from the active-site cleft. The first location may support the role of phosphate as a potential Zn ligand while the second site would argue for phosphate involvement in product release. Release of the

tetrahydrofolate product is slow and may be partially rate limiting [30, 31], which would also support a role for phosphate in product release. However, more experiments are necessary to verify the specific role of phosphate in the catalytic cycle. *E. coli* MetE is also contains a highly reactive cysteine, Cys645, that is glutathionylated in response to oxidative stress, inactivating the enzyme [32]. The residue that aligns with *E. coli* Cys645 is Ser661 in *C. albicans* Met6p so glutathionylation is not possible; in fact the only cysteine residues in the *C. albicans* enzyme are the two Zn-ligating residues.

3.3 Conclusion

This study provides another positive example of using surface mutations to crystallize recalcitrant targets. Although the crystal packing of each variant was unique, in each case the mutated loop formed at least one crystal contact. It is difficult to predict if either of the loop structures we observed, extended β -hairpin or α -helical, reflect the structure of the native loop but the mutations we made did not affect enzyme activity. Crystals of the Met6pT variant had the highest solvent content and only diffracted to 2.8 Å resolution while Met6pY and Met6pA crystals diffracted to 2.1 and 2.0 Å resolution, respectively. The Met6pA crystals have been easier to reproduce than Met6pY crystals so we are focusing on the Met6pA mutant for further crystallographic studies. One other factor that favors pursuing the Met6pA crystals over the Met6pY crystals is the number of residues to be modeled per asymmetric unit (767 for Met6pA versus >1500 for Met6pY).

As seen for all other structures of cobalamin-independent methionine synthases, the enzymes were all in an “open” configuration where the binding sites of L-Hcy and folate are too far apart for methyl group transfer. Crystallization of a closed methionine synthase structure would yield valuable information for mechanistic studies and drug design but will likely require co-crystallization with folate or other ligand that can induce the closed conformation. Ligand-bound structures will help to fine tune synthesis of small molecule drug leads. The crystals obtained by surface residue mutagenesis will be used in an ongoing study to find selective inhibitors for *C. albicans* Met6p.

Acknowledgments

This work was supported by NIH grant AI 074497, by Robert A. Welch Foundation grant F1225, and by the College of Natural Sciences support to the Center for Structural Biology.

Portions of this research were conducted at the Advanced Light Source, a national user facility operated by Lawrence Berkeley National Laboratory on behalf of the U.S. Department of Energy, Office of Basic Energy Sciences.

Abbreviations

5-methyl-THF	5-methyl-tetrahydrofolate
DTT	dithiothreitol
L-Hcy	L-homocysteine
Met6p	fungal cobalamin-independent methionine synthase
Met6pA	Met6pLys103AlaLys104AlaGlu107Ala
Met6pT	Met6pLys103ThrLys104ThrGlu107Thr
Met6pY	Met6pLys103TyrLys104TyrGlu107Tyr
MetE	cobalamin-independent methionine synthase

SER

surface entropy reduction

References

1. Matthews RG, Smith AE, Zhou ZS, Taurog RE, Bandarian V, Evans JC, Ludwig ML. *Helvetica Chim Acta*. 2003; 86:3939–3954.
2. Drummond JT, Huang S, Blumenthal RM, Matthews RG. *Biochemistry*. 1993; 32:9290–9295. [PubMed: 8369297]
3. Matthews RG, Goulding CW. *Curr Opin Chem Biol*. 1997; 1:332–339. [PubMed: 9667865]
4. Gonzalez JC, Banerjee RV, Huang S, Sumner JS, Matthews RG. *Biochemistry*. 1992; 31:6045–6056. [PubMed: 1339288]
5. Pejchal R, Ludwig ML. *PLoS Biol*. 2005; 3:e31. [PubMed: 15630480]
6. Ferrer JL, Ravanel S, Robert M, Dumas R. *J Biol Chem*. 2004; 279:44235–44238. [PubMed: 15326182]
7. Qi G, Hayward S. *BMC Struct Biol*. 2009; 9:13. [PubMed: 19267915]
8. Suliman HS, Sawyer GM, Appling DR, Robertus JD. *Arch Biochem Biophys*. 2005; 441:56–63. [PubMed: 16083849]
9. Prasannan P, Suliman HS, Robertus JD. *Biochem Biophys Res Commun*. 2009; 382:730–734. [PubMed: 19324024]
10. Suliman HS, Appling DR, Robertus JD. *Arch Biochem Biophys*. 2007; 467:218–226. [PubMed: 17935688]
11. Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RP. *Clin Infect Dis*. 1999; 29:239–244. [PubMed: 10476719]
12. Longenecker KL, Garrard SM, Sheffield PJ, Derewenda ZS. *Acta Crystallogr D Biol Crystallogr*. 2001; 57:679–688. [PubMed: 11320308]
13. Cooper DR, Boczek T, Grelewski K, Pinkowska M, Sikorska M, Zawadzki M, Derewenda Z. *Acta Crystallogr D Biol Crystallogr*. 2007; 63:636–645. [PubMed: 17452789]
14. Goldschmidt L, Cooper DR, Derewenda ZS, Eisenberg D. *Protein Sci*. 2007; 16:1569–1576. [PubMed: 17656576]
15. Drummond JT, Jarrett J, Gonzalez JC, Huang S, Matthews RG. *Anal Biochem*. 1995; 228:323–329. [PubMed: 8572314]
16. Yeo EJ, Wagner C. *J Biol Chem*. 1992; 267:24669–24674. [PubMed: 1332963]
17. Leslie AGW. Joint CCP4 and ESF-EACMB Newsletter on Protein Crystallography. 1992; 26:27–33.
18. Otwinowski Z, Minor W. *Methods Enzymol*. 1997; 27:307–326.
19. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. *J Appl Crystallogr*. 2007; 40:658–674. [PubMed: 19461840]
20. Emsley P, Cowtan K. *Acta Crystallogr D Biol Crystallogr*. 2004; 60:2126–2132. [PubMed: 15572765]
21. Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH. *Acta Crystallogr D Biol Crystallogr*. 2010; 66:213–221. [PubMed: 20124702]
22. Murshudov GN, Vagin AA, Dodson EJ. *Acta Crystallogr D Biol Crystallogr*. 1997; 53:240–255. [PubMed: 15299926]
23. CCP4. *Acta Cryst*. 1994; D5:760–763.
24. Krissinel E, Henrick K. *J Mol Biol*. 2007; 372:774–797. [PubMed: 17681537]
25. Krissinel E, Henrick K. *Acta Crystallogr D Biol Crystallogr*. 2004; 60:2256–2268. [PubMed: 15572779]
26. Koutmos M, Pejchal R, Bomer TM, Matthews RG, Smith JL, Ludwig ML. *Proc Natl Acad Sci U S A*. 2008; 105:3286–3291. [PubMed: 18296644]

27. Baker PJ, Britton KL, Fisher M, Esclapez J, Pire C, Bonete MJ, Ferrer J, Rice DW. *Proc Natl Acad Sci U S A*. 2009; 106:779–784. [PubMed: 19131516]
28. Peariso K, Goulding CW, Huang S, Matthews RG, Penner-Hahn JE. *J Am Chem Soc*. 1998; 120:8410–8416.
29. Whitfield CD, Steers EJ Jr, Weissbach H. *J Biol Chem*. 1970; 245:390–401. [PubMed: 4904482]
30. Taurog RE, Jakubowski H, Matthews RG. *Biochemistry*. 2006; 45:5083–5091. [PubMed: 16618097]
31. Taurog RE, Matthews RG. *Biochemistry*. 2006; 45:5092–5102. [PubMed: 16618098]
32. Hondorp ER, Matthews RG. *J Bacteriol*. 2009; 191:3407–3410. [PubMed: 19286805]

- *C. albicans* methionine synthase is a potential target for fungistatic agents.
- We were unable to crystallize the wild-type protein.
- We have crystallized the protein by mutating residues on a surface loop.
- The mutated residues are involved in crystal contacts.
- These crystal forms can be used in an ongoing program to study methionine synthase - small molecule interactions.

Figure 1. Sequence alignment of *C. albicans* Met6p with homologs from *A. thaliana*, *S. mutans*, *T. maritima* and *E. coli*. Identical residues are shown with white lettering on red background and similar residues are shown with red lettering on white background. Secondary structural elements from Met6pA are shown above the alignment and the disordered regions are designated by red dashes. The elongated loop is indicated by a blue line and residues that were mutated in this study are identified by blue asterisks. Zinc-ligating residues are indicated by a green ‘Z’, and residues in the L-Hcy and folate binding pockets are labeled with a green ‘H’ and ‘F’, respectively. The cysteine that gets glutathionylated in the *E. coli* enzyme is marked by a black asterisk.

Figure 2.

Comparison of crystal packing and loop interactions. The zinc is shown in cyan and the N and C termini are marked by blue and red spheres, respectively. The loop that was mutated is colored orange. (A) The Met6pA asymmetric unit contains a monomer, represented in blue. Two symmetry-related molecules are shown in grey and labeled Mol A' and Mol A". (B) Ala103 and Ala104 of Mol A form van der Waals interactions with residues 511-514 of Mol A'. Residues 106-110 are disordered and were left un-modeled. (C) The Met6pT asymmetric unit contains two monomers shown in gold and yellow and labeled Mol A and B. Two symmetry-related molecules are shown in grey. (D) Thr107 in Mol A forms hydrogen bonds with the backbone of Gly515 and Phe516 in Mol B. Thr108A further

stabilizes this crystal contact by hydrogen bonding to Asn514 of Mol B. (E) The Met6pY asymmetric unit contains two molecules shown in light and dark green. (F) Within each monomer Tyr103 joins an aromatic stack with Arg99 and Trp26; Tyr104 sits perpendicular to this aromatic stack and forms a hydrogen bond to Arg99. In the crystal contact, these two stabilized helical elements form van der Waals interactions with each other (3.5–4 Å).

Figure 3.

Overlay of Met6pA with structural homologs. A secondary-structural representation (stereo image) of Met6pA (blue) superimposed with MetE from *A. thaliana* (1U1H, black), *S. mutans* (3L7S, red) and *T. maritima* (3BQ5, grey). The N and C termini are marked by blue and red spheres, respectively, and the zinc from Met6pA is displayed in cyan.

Figure 4. Met6p zinc binding site. A superposition of the zinc binding site in Met6pA (blue carbons, cyan zinc) and L-Hcy bound MetE from *T. maritima* (3BQ5, grey carbons and zinc). In L-Hcy free Met6pA, zinc is coordinated by His557, Cys659, Glu679 and Cys739. In the presence of L-Hcy, the zinc moves ~3 Å away from the glutamate ligand and is coordinated by the histidine, two cysteines and L-Hcy.

Table 1

Enzyme activity. The activity of wild-type Met6p and engineered loop mutants is shown as μM product formed (see section 2.3 for reaction conditions).

Protein Sample	[5, 10-Methenyl THF] (μM)
Met6p wildtype ^a	13.81 ± 0.45
Met6pA ^a	13.38 ± 0.54
Met6pY ^a	13.34 ± 0.58
Met6pT ^b	14.49 ± 0.71

^aProteins were stored at $-80\text{ }^{\circ}\text{C}$ and thawed prior to use.

^bProtein was freshly purified.

Table 2

X-ray data collection and refinement statistics

	Met6pY+Zn	Met6pT	Met6pA+Zn	Met6pA
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>C</i> 2	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Cell dimensions				
a, b, c (Å)	77.7,92.7,191.0	163.6,98.1,162.6	77.2,99.2,100.8	76.9,98.8,100.8
α, β, γ (°)	90, 90, 90	90, 131.1, 90	90, 90, 90	90, 90, 90
Resolution (Å) ^a	50-2.2 (2.32-2.20)	50-2.8 (2.95-2.80)	50-2.0 (2.01-1.98)	50-2.3 (2.34-2.30)
R _{merge}	0.097(0.43)	0.109(0.659)	0.084(0.629)	0.087(0.545)
<I/σ _I >	13.2 (3.2)	9.2 (1.9)	10.1 (3.4)	9.9 (3.8)
Completeness (%)	96.5 (82.7)	99.8 (99.9)	100 (100)	99.9 (99.3)
Unique reflections	68,226	47,736	54,885	35,447
Redundancy	6.4	3.6	10	6.4
R _{work}	0.182	0.217	0.178	0.181
R _{free}	0.231	0.278	0.220	0.253
Average B factor for protein atoms (Å ²)	19.0	57.8	37.5	33.7
Average B factor for solvent atoms (Å ²)	17.2	38.2	37.5	33.4
Rms deviation from ideality				
Bond length (Å)	0.018	0.008	0.007	0.007
Bond angle (°)	1.506	1.156	0.982	1.047

^aValues in parentheses are for data in highest resolution shell.