The Cobalamin-Independent Methionine Synthase Enzyme Captured in a Substrate Induced Closed Conformation

Authors: Devinder K. Ubhi, Jon D. Robertus*

Affiliations:

Department of Molecular Biosciences, University of Texas at Austin, Austin, TX 78712, USA.

*Corresponding author. Department of Molecular Biosciences, University of Texas at Austin, Austin, TX 78712, USA. Phone: (512) 471-3175 Email: jrobertus@cm.utexas.edu

Abstract: The cobalamin-independent methionine synthase enzyme catalyzes a challenging reaction; the direct transfer of a methyl from 5-methyl-tetrahydrofolate-glutamate₃ (5-Me-THF-Glu₃) to the L-homocysteine (Hcy) thiol. The enzyme has a dual ($\beta\alpha$)₈ TIM barrel structure that binds, activates and brings the reactants into reaction proximity by conformational movements. In the previously observed open structures the substrates bind too far apart to react, but we have captured a ternary complex with both substrates bound in a closed form of the enzyme. The closing is described in terms of a hinge between the N and C terminal TIM barrels, and a rearrangement of key loops within the C domain. The substrate specificity can now be rationalized and the structure reveals His707 as the acid that protonates the THF leaving group through a water molecule trapped in the closed active site. The substrates are correctly oriented for an in line attack by Hcy on the N⁵-methyl.

Key Words: Fungal enzyme, X-ray structure, Zinc, Tetrahydrofolate activation, Homocysteine

Abbreviations: 5-Me-THF-Glu₃, 5-methyl-tetrahydrofolate-glutamate₃; pGlu_n, polyglutamate; Hcy, L-homocysteine; Met6p, fungal cobalamin-independent methionine synthase; Met6pA, Met6pLys103AlaLys104AlaGlu107Ala; BME, β-mercaptoethanol

Introduction:

Methionine synthesis is essential for all living systems, producing methionine for proteins and as part of the one-carbon metabolic cycle. Plants, fungi, and bacteria use a cobalamin-independent methionine synthase (Met6p) to catalyze direct methyl transfer from 5-methyl-tetrahydrofolate-glutamate_n (5-Me-THF-Glu_n) to L-homocysteine (Hcy) creating L-methionine (Met). The *Candida albicans* Met6p requires a folate substrate with at least two glutamates in the tail ¹, while physiological forms of folate contain 3-7 glutamates ^{2;3}. Homologous protein structures from *Arabidopsis thaliana*, *Thermatoga maritima*, *Streptococcus mutans* and *Candida albicans* have been reported ^{4;5;6;7}. In contrast, mammals employ a cobalamin-dependent methionine synthase which is not structurally or mechanistically related ^{8;9}. Since these two classes of methionine synthases share no sequence or structural similarity, the cobalamin-independent methionine synthase enzyme (Met6p) is a potential broad-spectrum antifungal drug target ¹⁰.

The 90 kDa Met6p folds into two (βα)₈ barrels which are connected by a linker and arranged face to face through non-covalent interactions between extended surface loops. Hcy and a catalytic zinc bind in a pocket of the C-terminal barrel while the folate is loosely secured at the solvent exposed domain interface. To date, all Met6p structures and complexes have been observed in an “open” conformation; the two domains are separated by a wide cleft with the N⁵ methyl ~7 Å away from the Hcy thiol. This suggests that during catalysis the enzyme must

1
2
3
4 undergo conformational rearrangements in which the two domains “close” around the active site
5
6 to stabilize the transition state for methyl transfer.
7
8
9

10
11 The chemistry of the cobalamin-independent reaction has been of considerable biological and
12 chemical interest ^{10; 11; 12}. The transfer of the methyl group from 5-Me-THF to Hcy is very difficult
13
14 because at physiologic pH the THF is a very poor leaving group and the Hcy thiol, ligated to the
15
16 active site zinc, is not a good nucleophile. It is clear that at a minimum, the THF must be
17
18 protonated at N⁵ to facilitate leaving, and to date it has been unclear how this is accomplished.
19
20 Folate activation by protonation also remains unresolved in other methyltransferases like the
21
22 cobalamin-dependent methionine synthase and 5-methyltetrahydrofolate:corrinoid/iron-sulfur
23
24 protein ¹³.
25
26
27
28
29
30
31
32

33 We have previously shown that the *MET6* gene is essential for fungal growth ¹⁴, and have
34
35 studied the enzyme using steady state kinetics ¹, site-directed mutagenesis ¹⁵ and X-ray
36
37 crystallography ^{7; 16}. We have been unable to crystallize wild type Met6p from *C. albicans*, but
38
39 we have solved the structures of several fully active mutants using surface entropy reduction ⁷.
40
41 Substitution of three surface polar residues to Ala created a form called Met6pA which was used
42
43 to describe substrate complexes in the open form ¹⁶ and which is now described in the closed
44
45 form.
46
47
48
49
50
51

52 **Results and Discussion:**

53
54

55 In this report we present the first closed structure of any Met6p bound with Hcy and THF-Glu₃.
56
57 Comparison of this new structure with that of previous open forms allows us to describe the
58
59 dynamics of Met6p as it binds substrates. More importantly, we see a ternary complex with
60
61
62
63
64
65

1
2
3
4 geometry suggestive of the transition state of the reaction. We identify the catalytic acid and
5
6 propose a plausible scenario for the methyl transfer reaction.
7
8
9

10 11 12 **Structural dynamics** 13

14
15 It is convenient to describe the substrate induced dynamics of Met6p closure into two motions: a
16
17 rotation movement of the N terminal domain (residues 1-400) with respect to the C terminal
18
19 domain (residues 401-767), and a rearrangement of loops within the C terminal domain. In
20
21 Figure 1a, the C terminal domains of both forms are superposed. Superposition of 361 Ca
22
23 atoms of the C terminal domains showed an rms deviation of 1.31 Å, consistent with the
24
25 observation that several surface loops in this substrate binding domain experienced significant
26
27 rearrangements upon closure. The N terminal domain of the closed form is nearly identical to
28
29 that seen previously in the open forms. A least squares superposition of 390 Ca atoms has an
30
31 rms deviation of 0.52 Å. In the closed form, the N terminal barrel appears to collapse as a rigid
32
33 body onto the C terminal barrel. Analysis using the program DynDom, shows this motion can
34
35 be described as a rigid body rotation of 15.7° about the axis shown in Figure 1a ¹⁷.
36
37
38

39 Crystallographic data for the new structure is provided in Table 1.
40
41
42
43
44
45
46

47
48 The rigid rotation of the N terminal domain “pushes” the C domain loop composed of residues
49
50 518-534 deeper into the active site pocket as the structure closes. In both open and closed
51
52 forms this extended loop wraps around THF-Glu₃. However, in the transition from open to
53
54 closed the loop Ca positions move 2.4-6.4 Å and the bound folate migrates ~5 Å (Figure 1b).
55
56 Analysis using DynDom shows these loop residues form a twisted anti-parallel β-sheet that is
57
58 part of a single rigid body consisting of residues 405-410 , 504-510 , 514–544 , 571–593 and
59
60
61
62
63
64
65

1
2
3
4 618–635¹⁷. This rigid body rotates 11.4° with respect to the remaining C-terminal barrel along
5
6 an effective hinge axis marked in Figure 1a. This rotation axis is nearly parallel with the
7
8 interdomain rotation axis and the magnitude of motion is similar.
9

10
11
12
13
14
15
16
17 The Met6pA crystal used to capture the closed conformation was originally crystallized in the
18
19 open conformation. Soaking the substrates into the crystal induced the closed conformation so
20
21 it appears that the crystal lattice can accommodate both conformations. Although we soaked 5-
22
23 Me-THF-Glu₃ into the crystals, it appears that the methyl group was lost during soaking and the
24
25 product THF-Glu₃ was bound. The substrate induced movements described above have a
26
27 measurable effect on the orthorhombic unit cell; the *a* axis shrinks by 3% and *c* is elongated by
28
29 3%. Analysis by PISA shows that while the two structures have the same symmetry related
30
31 neighbors, the crystal contacts supporting each lattice differ. In the presence of both Hcy and
32
33 THF-Glu₃, the altered Met6pA overcomes weak crystal packing contacts holding the N and C
34
35 domains apart in the open conformation and makes new ones that favor the closed
36
37 conformation. It is probably important that the “tops” of the N and C-barrels, and the groove
38
39 separating them are unrestrained by crystal contacts, allowing considerable motion within the
40
41 cell. We recently reported the Hcy, Met and Glutamine induced restructuring of the C-domain
42
43
44
45
46¹⁶. However, previous attempts to soak both Hcy and 5-Me-THF-Glu₃ substrates caused the
47
48 crystals to crack, suggesting that the induced closed form strained the open form lattice to some
49
50 extent. The discovery of an intact ternary complex in the closed form has so far been a unique
51
52 event.
53
54
55
56
57
58
59
60
61
62
63
64
65

The closed interdomain interface

Our past analysis of the apo and various substrate bound open Met6pA structures identified the Hcy induced ordering of the solvent exposed $\beta\alpha$ loops 660-662, 680-688, 703-709, and 741-743 located in the C domain¹⁶. This ordering appears to be a pre-requisite for the open to closed transition; Hcy binding prepares the top of the C-terminal barrel to receive and pack against residues decorating the top of the N domain and loop 518-534. The surface rendering of the closed form in Figure 1c shows the extent of N and C domain contact. Analysis of the open and closed structures by PISA¹⁸ shows that the interdomain closure buries an additional 649 Å² of surface area for the N-domain and 620 Å² for the C-domain. Buried residues that line and completely desolvate the active site are displayed as sticks and include Met119, Lys121, His128, Tyr527, Arg530, Tyr660, His707 and Thr743. These residues are highly conserved and form a network of pi-stacking and hydrophobic interactions encasing the site of catalysis.

We further evaluated the residues surrounding the THF pyrazine by mutagenesis (to Ala) and found that while all mutants are less active, Lys121Ala and His707Ala retain less than 5% of the wild-type activity; these two mutants proteins are discussed below (Table 2). The N-domain residues Met119 and His128 appear to have the same structural role in both the open and closed conformations; that is, both help to embed the C-domain loop 518-534 into the top of the N-domain. The side chain of M119 makes hydrophobic interactions with the Tyr527 phenyl ring and the backbone amide and side-chain of His128 form hydrogen bonds with the backbone carbonyl and side-chain of Ser526, respectively. However, in the closed form, Met119 further strengthens the contact network that holds the N and C domains closed. It helps desolvate the active site near the folate by packing against C-domain residues Tyr660 and His707. Tyr660 appears to be consistently disordered in all open structures. We showed by site-direct mutagenesis, that Y660 is an important residue, and since it has no obvious function in the open

1
2
3
4 conformation we hypothesized that it may mediate the conformational switch and support
5
6 catalysis ¹⁶. In the closed form, a completely ordered Tyr660 serves as a point of contact
7
8 between the N and C domains and lines the catalytic pocket and confirms our previous
9
10 hypothesis.
11
12
13
14
15

16 **Substrate bound active site**

17
18
19 Figure 2 shows the major closed form substrate interactions, and Figure 3 shows OMIT electron
20
21 density around the bound substrates. The binding of Hcy in the closed form is essentially
22
23 indistinguishable from that seen in the open binary complex (PDB ID: 4L5Z).; details of
24
25 substrate binding in high resolution structures were published recently ¹⁶. In brief, Hcy is
26
27 anchored into the C terminal barrel by its backbone interactions, while the side chain thiol
28
29 coordinates with zinc through a 2.0 Å Zn-S_{Hcy} bond ¹⁶. In the open structure, residues Asn126,
30
31 Asp504, Tyr527, Arg530, Tyr531, Val532 and Trp576 were found to bind a folded form of folate
32
33 at the solvent exposed interdomain groove. These residues are part of a rigid body that moves
34
35 with folate upon closing (Figure 1b) ¹⁶. Interactions between these residues and folate are also
36
37 retained in the closed conformation; however, the closed form also creates novel interactions.
38
39
40
41
42
43
44

45 In the open structures the poly glutamate tail of the folate only has electron density for the first
46
47 glutamate, the second (pGlu₂) and third (pGlu₃) residues of the folate tri-glutamate tail are
48
49 disordered. However, in the closed conformation all three glutamates are clearly resolved
50
51 (Figure 2), and specific interactions with the protein can be defined. Conserved residues
52
53 Arg456 and Arg459 form salt bridges with the α-carboxylates in pGlu₁ and pGlu₂, respectively
54
55 (Figure 2). The conserved polar residue Gln451 is positioned to receive hydrogen bonds from
56
57 the α-amino group of pGlu₂ and from Ne and NH₂ of Arg456. These residues are located on a
58
59
60
61
62
63
64
65

1
2
3
4 C-terminal loop (residues 449-466) that folds into an α -helix and in the closed form translates ~ 3
5
6 $\text{\AA}$ towards the folate. This helix is enriched with polar and cationic residues that remain
7
8 disordered in the apo and substrate bound open Met6p structures, however, in the closed form,
9
10 the side chains are resolved and either make the above described interactions or exclude
11
12 solvent. Gln451, Arg456 and Arg459 were further evaluated by site directed mutagenesis to Ala
13
14 and found to be < 5 , 38 and < 5 % active, respectively (Table 2). The observed role of Gln451
15
16 and Arg459 in securing pGlu₂ is absolutely essential and corroborates the previously observed
17
18 requirement for at least a diglutamate tail for folate binding and catalysis^{1; 19}. The folate pGlu₃
19
20 does not appear to have an essential structural function; the γ -carboxylate forms salt bridges
21
22 with an unconserved Lys453 and a symmetry mate Lys330 (not shown). The ion pair between
23
24 Arg530 and the α -carboxylate of pGlu₁ observed in the open form is maintained in the closed
25
26 form by the 518-534 loop movement described above. In addition, relative movement of the
27
28 domains brings Arg16 from the N domain within ion pairing distance of the α -carboxylate in
29
30 pGlu₃; in a di-glutamate tail, Arg16 would be ideally positioned to ion pair with the γ -carboxylate
31
32 of pGlu₂.
33
34
35
36
37
38
39
40

41 The closed structure also employs a previously uncharacterized residue, Lys121, to reinforce
42
43 the acceptor/donor pairing between Asn126 and THF atoms N¹ and N⁸¹⁶. In the open form,
44
45 Lys121 is solvent exposed and makes no specific interactions with the enzyme or substrates.
46
47 However, in the closed form the side chain donates a 2.7 $\text{\AA}$ hydrogen bond to the Asn126 O ^{δ 1}
48
49 and forms a 2.6 $\text{\AA}$ salt bridge with a neighboring Glu620 O ^{ϵ 2}. As a result, Lys121 favors the
50
51 productive alignment of Asn126 and further maintains it by neutralizing a potentially disruptive
52
53 negative charge from Glu620. Lys121 serves an essential function that is abolished by
54
55 mutagenesis to Ala (Table 2).
56
57
58
59
60
61
62
63
64
65

A ternary intermediate resembling the likely transition state

In addition to rationalizing substrate binding, the closed form shows the Hcy and THF in juxtaposition with a geometry reflecting the likely transition state; if the observed THF was methylated at N⁵ it would most likely point toward the Hcy side chain. The active site is shown in stereo in Figure 2; Figure 3 shows a section of OMIT $2F_o - F_c$ electron density superimposed on the two substrates, the trapped catalytic water, the proton donor His707 and a few other surrounding residues in the closed conformation. The distance between S_{Hcy} and THF N⁵ is 3.6 Å and a separation of ≤ 3 Å can be expected between an N⁵-methyl and S_{Hcy}. The Hcy thiolate is oriented to attack the 5-Me group from the front, while an active site water protonates the leaving group from the rear. This water molecule is trapped 3.0 Å away from the His707 N^{ε2} and 3.2 Å from the THF N⁵. In terms of an organic reaction mechanism the methyl transfer involves a nucleophilic attack by the Hcy thiolate on the methyl group, and requires protonation at folate N⁵ to facilitate its leaving¹². Our structure strongly suggests that folate N⁵ is protonated by His707 via the water molecule trapped in the closed form. To support this contention, we mutated His707 to Ala which reduced the enzyme activity to < 5% (Table 2). Conversion of His707 to another potential acid of a similar size, Lys, does not rescue the enzyme activity; a stronger acid is likely required.

In a productive reaction, the Zn-S_{Hcy} bond must be weakened or disrupted to generate a free thiolate nucleophile. However in the observed closed form the thiolate remains liganded to the active site Zn. The zinc binding site elasticity has been well characterized^{16; 20}. Koutmos et al. proposed that a trigonal bipyramidal transition state with elongated Zn-S_{Hcy} bond could enhance the nucleophilicity of the Hcy thiolate, in which case the side chain of Glu679 would require access to the Hcy bound zinc²⁰. In the closed crystal conformation Glu679 is blocked from the

1
2
3
4 metal site by Tyr660 and it may be that crystal packing forces prevent this normal oscillation
5
6 from occurring.
7
8
9

10
11 The loss of 5-Me during the soak experiment allowed us to trap a nearly-productive ternary
12 structure that answers key substrate binding and reaction chemistry questions. The order in
13 which 5-Me is lost and the conformation changes (during the soak) is unknown and beyond the
14 scope of this paper. While this is the first reported Met6p structure bound with THF-Glu₃ and
15 Hcy, this ternary complex has been observed in solution¹². Like the methylated substrate, the
16 byproduct THF-Glu₃ is reportedly protonated at position N⁵ and its release from this
17 unproductive ternary complex is found to be a rate limiting step in the reaction mechanism¹².
18
19
20
21
22
23
24
25
26
27
28
29
30

31 Figure 4 is a cartoon drawing that summarizes a potential Met6p mechanism. It is important to
32 remember that this cartoon scenario artificially breaks the mechanism into discrete steps to aid
33 narration. It may well be that in solution several steps or aspects of steps are concerted. It may
34 be, for example, that protonation at N⁵ occurs simultaneously with an attack by the thiolate on
35 the methyl group.
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50

51 **Conclusion:**

52
53 In summary, substrate binding reorders the conformation of apo-Met6p to facilitate a direct S_N2
54 type methylation reaction. We have previously reported Hcy-induced ordering of β_α loops and
55 migration of Zn; events localized in the C-terminal barrel¹⁶. While binding of 5-Me-THF-Glu₃
56
57
58
59
60
61
62
63
64
65

1
2
3
4 had no effect on the open conformation, in combination with Hcy, the THF pGlu tail triggers a
5
6 change from the open to closed form; ion pairs to the second glutamate must contribute to the
7
8 differential energy that tips this movement since mono-glutamate substrates are inactive. The
9
10 closed active site desolvates the active site save one water that serves as a conduit from
11
12 His707 to protonate the THF N⁵. Oscillations around the Zn cluster allow the thiolate to attack
13
14 the methyl group and the protonated THF is already activated as a reasonable leaving group.
15
16 The transition state is likely to be a classic pyramidal structure with 3 methyl hydrogens in a
17
18 plane and the incoming thiolate and departing THF amine as axial ligands.
19
20
21
22
23
24

25 **Materials and Methods:**

26 *Protein preparation*

27
28 The Met6pA variant from *Candida albicans* was previously created and transformed into the *E.*
29
30 *coli* expression vector pNIC28-Bsa4⁷. The Met119Ala, Lys121Ala, His128Ala, Gln451Ala,
31
32 Arg456Ala, Arg459Ala, His707Ala and His707Lys mutants were made in the Met6pA
33
34 background using PCR-based site-directed mutagenesis by overlap primer extension. DNA
35
36 primers were designed with QuickChange Primer Design (Agilent Technologies). Each mutant
37
38 gene sequence was verified by DNA sequencing. Each construct was transformed into One
39
40 Shot® BL21 DE3 *E. coli* (Invitrogen) and four liter cultures were grown for protein expression
41
42 and purification using methods described previously⁷. Protein purity was evaluated by SDS-
43
44 PAGE gel and estimated to be >95%. Protein concentration was measured from the UV
45
46 absorbance at 280 nm and an extinction coefficient was calculated from the protein sequence (ϵ
47
48 = 90,760 M⁻¹ cm⁻¹). Protein was concentrated to 15-20 mg/ml in 20 mM Tris-HCl, 150 mM
49
50 NaCl, 10 mM β -mercaptoethanol (BME), pH 7.4 and aliquots were flash frozen and stored at -
51
52 80° C.
53
54
55
56
57
58
59
60
61
62
63
64
65

Substrate preparation

(6R,S)-5-methyl-tetrahydrofolate-Glu₃ (5-Me-THF-Glu₃) was purchased from the Schircks Laboratories (Jona, Switzerland) and re-suspended in 10 mM Tris pH 7.4. L-homocysteine (Hcy) was made by alkaline hydrolysis of L-homocysteine thiolactone ²¹. Both substrates were flushed with N₂ gas, protected from light and stored at -80 °C.

Crystallization

Met6pA crystals were grown at 20 °C using the hanging drop vapor diffusion method by combining 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, 20 mM Tris-HCl, pH 7.4. Each 3 µL drop was equilibrated against 500 µL of the well solution. Crystals appeared within a week; large 200 X 600 µm crystals were incubated for 3 hours in a fresh drop of reservoir solution supplemented with 250 µM BME and 150 µM zinc sulfate followed by an 8-12 hour soak in reservoir solution containing 250 µM BME. Zinc-replete crystals were soaked for 4 hours in 5 mM 5-Me-THF-Glu₃ and 2 mM Hcy and cryo-protected in the soak condition containing 30% (w/v) PEG 3350 prior to flash-cooling.

Data Collection

Diffraction data were collected at 100 K at the Advanced Light Source beam line 5.0.3 at the Lawrence Berkeley National Laboratory. The data set was integrated and scaled using HKL2000 ²². The molecular replacement solution was determined with Phaser ²³ using the structure of Met6pA in complex with Hcy (PDB ID: 4L5Z) ¹⁶ as the search model. The N (residues 1-400) and C (residues 401-767) terminal domains were treated as independent entities. The initial cycle of rigid body refinement was performed using Phenix ²⁴. The model

was subsequently improved through iterative rounds of model building in Coot²⁵, improvement with Molprobit²⁶ and refinement using Refmac^{27; 28}.

Fluorescence Activity Assay

The protein preparations of a fully active Met6pA and mutants Met119Ala, Lys121Ala, His128Ala, Gln451Ala, Arg456Ala, Arg459Ala, His707Ala and His707Lys were tested for enzymatic activity using a standard fluorescence-based assay¹⁶ but with 2X the steady state protein concentration. All reagents and buffers were first flushed with N₂ gas and if necessary protected from light and kept on ice. Prior to use, each protein sample was buffer exchanged into 20 mM Tris-HCl, 150 mM NaCl, pH 7.4. The replicate control and test reaction vials contain 50 mM potassium phosphate (pH 7.0), 50 mM Tris-HCl (pH 7.4), 500 μM 5-Me-THF-Glu₃ and 150 μM Hcy. The 10 μl reaction was initiated by adding 0 or 0.2 μg of enzyme (226 nM) into the control and test vials, respectively, and incubated for 10 min at 37 °C. The reaction was stopped by incubation on ice and transferred to a 96 well polypropylene plate containing 100 μL of the 1:100 Measure-iTTM thiol quantization reagent per well. The plate was sealed, centrifuged at 1000 RPM for 1 minute and the fluorescence was measured using excitation/emission filters of 464/510 nm. The fluorescence reading was converted to μM Hcy using a standard curve. The change in Hcy concentration during the reaction is calculated as the difference between a control sample (to which no enzyme was added) and the test sample. The mutant activity is reported in Table 2 as a simple fraction of the Met6pA (wild-type) activity.

Atomic coordinates

The atomic coordinates have been deposited to the Protein Data Bank with entry code 4QQU.

1
2
3
4 **Acknowledgments:**
5

6
7 This work was supported by Robert A. Welch Foundation grant F1225, and by the College of
8
9 Natural Sciences support to the Center for Structural Biology.
10

11
12 Instrumentation and technical assistance for this work were provided by the Macromolecular
13
14 Crystallography Facility, with financial support from the College of Natural Sciences, the Office
15
16 of the Executive Vice President and Provost, and the Institute for Cellular and Molecular Biology
17
18 at the University of Texas at Austin.
19

20
21 Portions of this research were conducted at the Advanced Light Source with the support of the
22
23 Berkeley Center for Structural Biology. The Berkeley Center for Structural Biology is supported
24
25 in part by the National Institutes of Health, National Institute of General Medical Sciences, and
26
27 the Howard Hughes Medical Institute. The Advanced Light Source is supported by the Director,
28
29 Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under
30
31 Contract No. DE-AC02-05CH11231.
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

References:

1. Suliman, H. S., Sawyer, G. M., Appling, D. R. & Robertus, J. D. (2005). Purification and properties of cobalamin-independent methionine synthase from *Candida albicans* and *Saccharomyces cerevisiae*. *Arch Biochem Biophys* **441**, 56-63.
2. Fu, T. F., Scarsdale, J. N., Kazanina, G., Schirch, V. & Wright, H. T. (2003). Location of the pteroylpolyglutamate-binding site on rabbit cytosolic serine hydroxymethyltransferase. *J Biol Chem* **278**, 2645-53.
3. Schirch, V. & Strong, W. B. (1989). Interaction of folypolyglutamates with enzymes in one-carbon metabolism. *Arch Biochem Biophys* **269**, 371-80.
4. Ferrer, J. L., Ravanel, S., Robert, M. & Dumas, R. (2004). Crystal structures of cobalamin-independent methionine synthase complexed with zinc, homocysteine, and methyltetrahydrofolate. *J Biol Chem* **279**, 44235-8.
5. Pejchal, R. & Ludwig, M. L. (2005). Cobalamin-independent methionine synthase (MetE): a face-to-face double barrel that evolved by gene duplication. *PLoS Biol* **3**, e31.
6. Fu, T. M., Almqvist, J., Liang, Y. H., Li, L., Huang, Y. & Su, X. D. (2011). Crystal structures of cobalamin-independent methionine synthase (MetE) from *Streptococcus mutans*: a dynamic zinc-inversion model. *J Mol Biol* **412**, 688-97.
7. Ubhi, D., Kavanagh, K. L., Monzingo, A. F. & Robertus, J. D. (2011). Structure of *Candida albicans* methionine synthase determined by employing surface residue mutagenesis. *Arch Biochem Biophys* **513**, 19-26.
8. Evans, J. C., Huddler, D. P., Hilgers, M. T., Romanchuk, G., Matthews, R. G. & Ludwig, M. L. (2004). Structures of the N-terminal modules imply large domain motions during catalysis by methionine synthase. *Proc Natl Acad Sci U S A* **101**, 3729-36.
9. Dixon, M. M., Huang, S., Matthews, R. G. & Ludwig, M. (1996). The structure of the C-terminal domain of methionine synthase: presenting S-adenosylmethionine for reductive methylation of B12. *Structure* **4**, 1263-75.
10. Matthews, R. G., Smith, A. E., Zhou, Z. S., Taurog, R. E., Bandarian, V., Evans, J. C. & Ludwig, M. (2003). Cobalamin-Dependent and Cobalamin-Independent Methionine Synthases: Are There Two Solutions to the Same Chemical Problem? *Helvetica Chimica Acta* **86**, 3939-3954.
11. Taurog, R. E., Jakubowski, H. & Matthews, R. G. (2006). Synergistic, random sequential binding of substrates in cobalamin-independent methionine synthase. *Biochemistry* **45**, 5083-91.
12. Taurog, R. E. & Matthews, R. G. (2006). Activation of methyltetrahydrofolate by cobalamin-independent methionine synthase. *Biochemistry* **45**, 5092-102.
13. Ragsdale, S. W. (2008). Catalysis of methyl group transfers involving tetrahydrofolate and B(12). *Vitam Horm* **79**, 293-324.
14. Suliman, H. S., Appling, D. R. & Robertus, J. D. (2007). The gene for cobalamin-independent methionine synthase is essential in *Candida albicans*: a potential antifungal target. *Arch Biochem Biophys* **467**, 218-26.
15. Prasannan, P., Suliman, H. S. & Robertus, J. D. (2009). Kinetic analysis of site-directed mutants of methionine synthase from *Candida albicans*. *Biochem Biophys Res Commun* **382**, 730-4.

- 1
2
3
4 16. Ubhi, D., Kago, G., Monzingo, A. F. & Robertus, J. D. (2014). Structural analysis of a fungal
5 methionine synthase with substrates and inhibitors. *J Mol Biol* **426**, 1839-47.
6
7 17. Hayward, S. & Lee, R. A. (2002). Improvements in the analysis of domain motions in proteins
8 from conformational change: DynDom version 1.50. *J Mol Graph Model* **21**, 181-3.
9
10 18. Krissinel, E. & Henrick, K. (2007). Inference of macromolecular assemblies from crystalline state.
11 *J Mol Biol* **372**, 774-97.
12
13 19. Whitfield, C. D. & Weissbach, H. (1970). Binding of the folate substrate to 5-
14 methyltetrahydropteroyltriglutamate-homocysteine transmethylase. *J Biol Chem* **245**, 402-9.
15
16 20. Koutmos, M., Pejchal, R., Bomer, T. M., Matthews, R. G., Smith, J. L. & Ludwig, M. L. (2008).
17 Metal active site elasticity linked to activation of homocysteine in methionine synthases. *Proc Natl*
18 *Acad Sci U S A* **105**, 3286-91.
19
20 21. Drummond, J. T., Jarrett, J., Gonzalez, J. C., Huang, S. & Matthews, R. G. (1995).
21 Characterization of nonradioactive assays for cobalamin-dependent and cobalamin-independent
22 methionine synthase enzymes. *Anal Biochem* **228**, 323-9.
23
24 22. Otwinowski, Z. & Minor, W. (1997). Processing of X-Ray Diffraction Data Collected in Oscillation
25 Mode. *Methods in Enzymology* **276**, 307-326.
26
27 23. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J.
28 (2007). Phaser crystallographic software. *J Appl Crystallogr* **40**, 658-674.
29
30 24. Adams, P. D., Afonine, P. V., Bunkoczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J.,
31 Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R.,
32 Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C. & Zwart, P. H. (2010).
33 PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta*
34 *Crystallogr D Biol Crystallogr* **66**, 213-21.
35
36 25. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). Features and development of Coot.
37 *Acta Crystallogr D Biol Crystallogr* **66**, 486-501.
38
39 26. Davis, I. W., Leaver-Fay, A., Chen, V. B., Block, J. N., Kapral, G. J., Wang, X., Murray, L. W.,
40 Arendall, W. B., 3rd, Snoeyink, J., Richardson, J. S. & Richardson, D. C. (2007). MolProbity: all-
41 atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Res* **35**,
42 W375-83.
43
44 27. Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). Refinement of macromolecular structures
45 by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* **53**, 240-55.
46
47 28. Winn, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., Keegan, R.
48 M., Krissinel, E. B., Leslie, A. G., McCoy, A., McNicholas, S. J., Murshudov, G. N., Pannu, N. S.,
49 Potterton, E. A., Powell, H. R., Read, R. J., Vagin, A. & Wilson, K. S. (2011). Overview of the
50 CCP4 suite and current developments. *Acta Crystallogr D Biol Crystallogr* **67**, 235-42.
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure and Table Legends:

Figure 1: The overall structure of Met6p in complex with Hcy and THF-Glu₃. (a) The closed and open Met6pA structures are superimposed. The closed N and C terminal domains are colored blue and red, respectively, and the open N and C domains are colored grey and black, respectively. (b) Additional features of the superimposed closed (red) and open (grey) active site are shown in stereo. The substrates and Zn are shown in yellow for the closed form. (c) A surface rendering of the closed form with the N domain shown in blue and the C domain colored red. The substrates (yellow) and relevant residues are shown as sticks. A water molecule trapped in the active site is labeled W.

Figure 2: Stereogram of the closed Met6p active site. The Hcy and folate are shown in the center surrounded by active site residues and an ordered water molecule (W).

Figure 3: Stereogram of the OMIT $2F_o - F_c$ electron density contoured at 1σ . Density is shown for both substrates, the catalytic water molecule (W), His707 and a few other residues. Both substrates and the active site water were omitted from the phasing of this map.

Figure 4: The likely mechanism of methyl transfer by Met6p. (a) The apo protein in the open form. The open form is symbolized by the two curved substrate pockets which are separated in space. The active site zinc is pseudo tetrahedrally liganded to its protein ligands Cys659, Cys739, and His657 (indicated by bonds) and Glu679 (**PDB: 3PPG**). (b) Hcy binds the C-terminal barrel and replaces Glu679 as the fourth ligand, causing the Zn to translate and its hand to invert (**PDB: 4L5Z**). (c) The binding of 5-me-THF triggers a conformational change to

1
2
3
4 the closed form, as symbolized by the fusing of binding site curves. His707 moves into the
5
6 active site cavity and traps a water molecule near 5-me-THF; in this panel we show 5-me-THF
7
8 as roughly flat at position N⁵. His707 is in the acidic imidazolium form and its proton is
9
10 transferred to the N⁵ of the folate via the water as indicated by the curving arrows. **(d)** The
11
12 protonation event converts the folate into a good leaving group and the tetrahedral geometry at
13
14 position N⁵ displaces the methyl group towards the Hcy thiol. An attack on the Zn by Glu679,
15
16 alters the metal geometry to trigonal bipyramidal with two elongated bonds to axial ligands
17
18 Glu679 and Hcy; upon release, the thiolate attacks the methyl group on N⁵. **(e)** The methyl
19
20 transfer reaction is complete, creating the methionine and the byproduct THF; the folate
21
22 geometry is more flattened at N⁵.
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Table 1: Data collection and refinement statistics of the Met6pA ternary complex.

Space group	Met6pA + Hcy + THF-Glu ₃ P2 ₁ 2 ₁ 2 ₁
<i>Cell dimensions</i>	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	75.0, 99.1, 104.0
$\alpha\beta\gamma$ (°)	90, 90, 90
Resolution (Å)	72-3.0 (3.03-2.98) ^a
R _{merge} (%)	0.172 (0.601)
$\langle I/\sigma_I \rangle$	14.2 (3.4)
Completeness (%)	100 (100)
Unique reflections	16,415
Redundancy	7.8
R _{working}	0.175
R _{free}	0.276
<i>B-factor</i>	
For Zinc (Å ²)	33.4
For the molecule W ^b (Å ²)	17.6
Average for protein atoms (Å ²)	32.7
Average for solvent atoms (Å ²)	24.8
Average for Hcy atoms (Å ²)	30.4
Average for THF-Glu ₃ atoms (Å ²)	25.6
<i>Rms deviation from ideality</i>	
Bond length (Å)	0.012
Bond angle (°)	1.635
<i>Ramachandran plot</i>	
% of residues in favored regions	87.6
% of residues in additional allowed regions	12.1
% of residues in generously allowed regions	0.3
% of residues in disallowed regions	0.0
<i>MolProbity validation</i>	
Clashscore	6.71 (100 th percentile)
MolProbity score	1.88 (100 th percentile)
% of poor rotamers ^c	1.18
% of Cβ deviations >0.25 Å	0
% of bad backbone bonds	0
% of bad backbone angles	0

^aHighest resolution shell is shown in parenthesis.

^bThe water molecule labeled as W in Figures 1, 2 and 3.

^c766 residues are present in the refined model.

Table 2: Mutant activity relative to Met6pA (wild-type). Activity measurements with a low signal to noise ratio are marked as < 5% (the observed standard deviation).

Protein	% Activity
Met6pA_Met119Ala	22
Met6pA_Lys121Ala	< 5
Met6pA_His128Ala	26
Met6pA_Gln451Ala	< 5
Met6pA_Arg456Ala	38
Met6pA_Arg459Ala	< 5
Met6pA_His707Ala	< 5
Met6pA_His707Lys	< 5

Figure1

Figure2

Figure3

Figure4