Identification of the Zinc Ligands in Cobalamin-Independent Methionine Synthase (MetE) from *Escherichia coli*†

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ABSTRACT: Cobalamin-independent methionine synthase (MetE) from *Escherichia coli* catalyzes the transfer of a methyl group from methyltetrahydrofolate to homocysteine to form tetrahydrofolate and methionine. It contains 1 equiv of zinc that is essential for its catalytic activity. Extended X-ray absorption fine structure analysis of the zinc-binding site has suggested tetrahedral coordination with two sulfur (cysteine) and one nitrogen or oxygen ligands provided by the enzyme and an exchangeable oxygen or nitrogen ligand that is replaced by the homocysteine thiol group in the enzyme−substrate complex [González, J. C., Peariso, K., Penner-Hahn, J. E., and Matthews, R. G. (1996) *Biochemistry 35*, 12228−34]. Sequence alignment of MetE homologues shows that His641, Cys643, and Cys726 are the only conserved residues. We report here the construction, expression, and purification of the His641Gln, Cys643Ser, and Cys726Ser mutants of MetE. Each mutant displays significantly impaired activity and contains less than 1 equiv of zinc upon purification. Furthermore, each mutant binds zinc with lower binding affinity ($K_a \approx 10^{14}$ M^{-1}) compared to the wild-type enzyme ($K_a > 10^{16}$ M^{-1}). All the MetE mutants are able to bind homocysteine. X-ray absorption spectroscopy analysis of the zinc-binding sites in the mutants indicates that the four-coordinate zinc site is preserved but that the ligand sets are changed. Our results demonstrate that Cys643 and Cys726 are two of the zinc ligands in MetE from *E. coli* and suggest that His641 is a third endogenous ligand. The effects of the mutations on the specific activities of the mutant proteins suggest that zinc and homocysteine binding alone are not sufficient for activity; the chemical nature of the ligands is also a determining factor for catalytic activity in agreement with model studies of the alkylation of zinc-thiolate complexes.

Cobalamin-independent methionine synthase (MetE,[1] EC 2.1.1.14) catalyzes the transfer of a methyl group from 5-methyltetrahydropteroylpolyglutamate ($CH_3-H_4PteGlu_n$, $n \geq 2$) to the thiol group of L-homocysteine (Hcy) to form tetrahydropteroylpolyglutamate ($H_4PteGlu_n$, $n \geq 2$) and L-methionine (eq 1) in the terminal step of methionine

$$CH_3-H_4PteGlu_n + \text{L-homocysteine (RSH)} \rightarrow H_4PteGlu_n + \text{L-methionine (RSCH}_3\text{)} \quad (1)$$

biosynthesis in *Escherichia coli* (*1*, *2*). It has been established in our laboratory that the MetE enzyme contains 1 equiv of zinc, which is essential for the enzymatic activity, and that the homocysteine thiol group directly ligates to the active site zinc (*3*, *4*).

A growing number of proteins that catalyze thiol alkylation have also been found to contain an essential zinc (for a review, see ref *5*). These proteins include the *E. coli* Ada protein that responds to and regulates repair of DNA methylation (*6*), cobalamin-dependent methionine synthase from *E. coli* (*7*), protein farnesyltransferase (*8*−*10*; for a review, see ref *11*), methylcobamide:coenzyme M methyltransferase from *Methanobacterium barkeri* (*12*−*14*), human betaine-homocysteine methyltransferase (*15*), and most recently, epoxyalkane:coenzyme M transferase from *Xanthobacter* strain Py2 (*16*, *17*). The role of zinc in this family of proteins is apparently different from the well-established roles of zinc in providing structural integrity, as in zinc finger proteins; as a Lewis acid for electrophilic activation of a carbonyl group in alcohol dehydrogenase or in the activation of a water molecule for nucleophilic attack in metalloproteases, alkaline phosphatases, and carbonic anhydrases.

Progress has been made on the study of this new family of enzymes. Protein structures have been determined for the

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[1] Abbreviations: $CH^+{=}H_4$folate, methenyltetrahydrofolate; $CH_3-H_4PteGlu_n$, 5-methyltetrahydropteroylpolyglutamate with *n* glutamyl residues; $H_4PteGlu_n$, tetrahydropteroylpolyglutamate with *n* glutamyl residues; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; equiv, equivalent; EXAFS, extended X-ray absorption fine structure; Hcy, L-homocysteine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ICP, inductively coupled plasma-atomic emission spectrometry; IPTG, isopropyl β-D-thiogalactopyranoside; K_a, association constant; K_d, dissociation constant; MetE, cobalamin-independent methionine synthase (EC 2.1.1.14), the product of the *metE* gene; MetH, cobalamin-dependent methionine synthase (EC 2.1.1.13), the product of the *metH* gene; MMTS, methyl methanethiolsulfonate; PAR, 4-(2-pyridylazo)resorcinol; $PteGlu_3$, pteroyltriglutamate; PCR, polymerase chain reaction; PMPS, *p*-hydroxymercuriphenylsulfonic acid; SDS, sodium dodecyl sulfate; SSRL, Stanford Synchrotron Radiation Laboratory; Tris, tris(hydroxymethyl)aminomethane; XANES, X-ray absorption near edge structure; XAS, X-ray absorption spectroscopy.

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Entry	Gene Name	Source Organism	Sequence Motif 1 (641 643)	Sequence Motif 2 (726)
1	***metE***	*E. coli*	DTQIHT**H**M**C**YCEFNDI	ERLWVNPD**C**GLKTRGWP
2		*H. influenzae*	DTQIHT**H**M**C**YSEFNDI	ERLWVNPD**C**GLKTRGWT
3	*met6*	*S. cerevisiae*	KTQIHS**H**F**C**YSDLDP-	EKFWVNPD**C**GLKTRGWE
4		*S. pombe*	DTQIHS**H**F**C**YSDFNDI	DHLWLNPD**C**GLEDPRMA
5	*ATCIMS*	*A. thaliana*	STQIHT**H**M**C**YSHFNDI	NILWVNPD**C**GLKTRKYT
6	*metE*	*M. crystallinum*	TTQIHT**H**M**C**YSNFNDI	NVLWVNPD**C**GLKTRKYG
7	*metE*	*C. roseus*	TTQIHT**H**M**C**YSNFNDI	NILWVNPD**C**GLKTRKYA
8	*met*	*S. scutellarioides*	TTQIHT**H**M**C**YSNFNDI	NILWVNPD**C**GLKTRKYA
9	*metE*	*M. leprae*	STQIHT**H**L**C**YSEFGEV	QRLWVNPD**C**GLKTRSVD
10	*metE*	*M. tuberculosis*	ATQIHT**H**L**C**YSEFGEV	ERLWVNPD**C**GLKTRNVD
11	*metE*	*A. aeolicus*	ETQIHT**H**M**C**YSEFNEI	ELLWVNPD**C**GLKTRRWE
12	*TM1286*	*T. maritima*	ETQIHA**H**M**C**YSDFNEI	ELIWINPD**C**GLKTRNWD
13	*metC*	*B. subtilis*	ETQIHT**H**M**C**YSNFEDI	DRFWVNPD**C**GLKTRQQE
14	*metE*	*C. reinhardtii*	GTQVVT**H**L**C**YSDFQDI	DRIWVNPD**C**GLKTRGWP
15	*MS1*	*C. moewusii*	DVQIVT**H**L**C**YSNFEDI	DRLWVNPD**C**GLKTRAWS
16	*APE2048*	*A. pernix*	-AKRIV**H**I**C**YGELERI	EKVYVDPD**C**GLKRLPRD
17	*PH1090*	*P. horikoshii*	-IKVGL**H**V**C**YSNYYLL	ELLYINPD**C**GMKLLDRN
18	*metE*	*M. thermoautotrophicum*	DVDVSL**H**V**C**GDISGVI	DNLYIDPD**C**GMRKLPRE
19		*M. thermoautotrophicum*	DIEVSL**H**V**C**GDIRNVL	ENLYIDPD**C**GMRKLPRK
20		*M. jannaschii*	-IKFAM**H**V**C**GNVYNII	DNILIDPD**C**GMRLLPID
21		*Xanthobacter sp.*	-AKIIA**H**V**C**WGNWGGT	DRLGVTTD**C**GLILLQRY
22	***mtbA (cmtA)***	*M. barkeri*	-SVTVL**H**I**C**GKVNAIL	GVGVLAPG**C**GIAPMTPL
23	***mtaA (cmtM)***	*M. barkeri*	DVATVL**H**I**C**GNTTNGL	GIDLLTVG**C**GTVSMTPT
24	*mtsA*	*M. barkeri*	SCPLIL**H**I**C**GDTSKLL	QADIVGTA**C**DVSFGTSL
25	*cmuA*	*Methylobacterium sp.*	GGIGFT**H**T**C**TFTQPIW	GGFICMPG**C**DIDWTIPD

FIGURE 1: Conserved zinc binding motifs of proteins with sequence similarity to *E. coli* MetE. These protein sequences were identified using the gapped BLAST program (*54*) and aligned using CLUSTAL W (*55*). Dashes are symbols for gaps allowing optimal alignment. Conserved and similar residues are boxed. The candidate ligands for zinc binding are in bold; the numbers shown are for the *E. coli* MetE sequence. Proteins that have been biochemically characterized are underlined, and those that have been shown to contain zinc are in bold. GenBank accession numbers and references for the protein sequences, and where performed, characterization, are (1) P25665 (*2*); (2) P45331 (*56*); (3) P05694 (*57*); (4) BAA13829 (*58*); (5) CAB38313 (*59*); (6) AAB41896 (*60*); (7) S57636 (*61*); (8) CAA89019 (*62*); (9) O05564 (*63*); (10) O06584 (*64*); (11) AAC07565 (*65*); (12) AAD36360 (*66*); (13) P80877 (*67*); (14) Q39586 (*68*); (15) AAD00267 (*69*); (16) BAA81058 (*70*); (17) BAA30189 (*71*); (18) P55299 (*72*); (19) O26869 (*73*); (20) Q58868 (*74*); (21) S47051 (*16*, *17*); (22) AAC38632 (*12*−*14*); (23) AAC44213 (*12*−*14*); (24) AAC46230 (*25*); (25) CAB39403 (*26*).

Ada protein (*18*) and protein farnesyltransferase (*10*, *19*). Zinc ligands have been identified for the Ada protein (*18*), cobalamin-dependent methionine synthase (*7*), and protein farnesyltransferase (*10*, *19*−*21*). Direct ligation of the substrate thiol group to the active site zinc has been established for the Ada protein (*18*), protein farnesyltransferase (*19*), and cobalamin-independent and cobalamin-dependent methionine synthases (*3*, *4*). There is evidence that the thiol substrate is ligated to the zinc as an ionized thiolate in protein farnesyltransferase (*22*) and cobalamin-dependent methionine synthase (*7*).

Even though the zinc-binding sites of these proteins are noticeably rich in thiol ligands, the protein scaffolds for zinc ligation are nevertheless distinct. The zinc ion in the apo form of protein farnesyltransferase ligates to His362, Cys299, both oxygen atoms of the carboxylate side chain of Asp297, and a water molecule in a distorted pentacoordinate geometry (*10*). In the ternary complex with a peptide substrate analogue, the cysteine thiol group from the peptide replaces the water molecule and ligates to the zinc, and the coordination geometry is changed to tetrahedral (*19*). In comparison, Cys247, Cys310, Cys311, and an exchangeable oxygen or nitrogen ligand are zinc ligands in the apo form of cobalamin-dependent methionine synthase (MetH) (*7*). The exchangeable ligand is replaced by the thiol group of the homocysteine substrate upon substrate binding. The tetrahedral geometry of the zinc site in the apo enzyme is preserved in the enzyme substrate complex (*4*). The arrangement of two adjacent cysteines as zinc ligands (Cys310 and Cys311) is rarely observed in zinc enzymes, but has precedence in metallothionines (*23*). As in the MetH−homocysteine complex, Ada protein has four thiol ligands to the zinc, but spacing in an arrangement of Cys−X_3−Cys−X_{26}−Cys−X_2−Cys (*18*). In this case, one of the protein ligands, Cys69, is the nucleophile that is methylated.

MetE from *E. coli* and its homologues belong to another distinct family of zinc enzymes involved in thiol alkylation. Analysis of the zinc environment of the native MetE enzyme and the complexes of the enzyme with the L-homocysteine or L-seleno-homocysteine substrate by X-ray absorption spectroscopy (XAS) suggests that the geometry of the active-site zinc is tetrahedral, and the native MetE enzyme provides two thiolate (cysteine) and one nitrogen or oxygen ligands, and another oxygen or nitrogen ligand, likely to be a water molecule, which is exchangeable with the thiol group from the homocysteine (*4*) or the selenol group from the L-seleno-homocysteine upon substrate binding (Pearlso, Zhou, Smith, Matthews, and Penner-Hahn, manuscript in preparation).

MetE from *E. coli* has high sequence homology to methionine synthases from plants, fungi, and other eubacteria (entries 1−15 in Figure 1). These MetE enzymes contain about 750 amino acid residues and share five conserved

Table 1: Sequences of Primers Used in the Construction of *metE* Mutants

mutant	primer sequence (5′-3′)[a]	nucleotide number[b]
His641Ala	GACACACAAATCCACACTGCCATGTGTTATTGCGAG	1903−1938
His641Cys	CACAAATCCACACTTGCATGTGTTATTGC	1907−1935
His641Thr	GACACACAAATCCACACTACCATGTGTTATTGCGAG	1903−1938
His641Lys	GACACACAAATCCACACTAAAATGTGTTATTGCGAG	1903−1938
His641Gln	GACACACAAATCCACACTCAGATGTGTTATTGCGAG	1903−1938
His641Asn	GACACACAAATCCACACTAATATGTGTTATTGCGAG	1903−1938
Cys643Ser	CCACACTCACATGAGCTATTGCGAGTTCAACGAC	1914−1947

[a] A pair of complementary primers was used for each mutant; only the sequence of one strand is shown. Changes in sequences are underlined. [b] Nucleotide number is assigned according to the GenBank sequence M87625 of *metE*. Translation of the *metE* gene begins at base pair 1 in the GenBank sequence.

blocks. Over 15% of the sequences are identical or highly similar. Interestingly, the N-terminal and C-terminal halves of MetE from eubacteria and eukaryotes share considerable sequence homology, which may have originated from a gene duplication event (*2*). The C-terminal half of MetE from *E. coli* also shares sequence homology to some other proteins (entries 16−25 in Figure 1), which are about half the size of *E. coli* MetE. Of these proteins, some (entries 16−20 in Figure 1) have been postulated to catalyze the methylation of homocysteine to form methionine. The *E. coli* MetE homologue from *M. thermoautotrophicum* (entry 18 in Figure 1) has been shown to be a methylcobalamin:homocysteine methyltransferase (*24*). The C-terminal half of MetE from *E. coli* also shares sequence homology to other enzymes (entries 21−25 in Figure 1) that catalyze alkylation of coenzyme M ($HO_3SCH_2CH_2SH$), a thiol cofactor mostly found in Archaea. These proteins include methylcobamide: coenzyme M methyltransferase from *Methanobacterium barkeri* (*12*−*14*, *25*); epoxyalkane:coenzyme M transferase from *Xanthobacter* strain Py2 (*16*, *17*); and a chloromethane utilization enzyme from *Methylobacterium* sp. CM4 (*26*) for which the physiological function is not clear. All the proteins listed in Figure 1 also share similar secondary structure patterns as predicted by the PHD program (*27*), suggesting that the overall tertiary structure could also be similar. Among these enzymes, several (indicated in bold type in Figure 1) have been found to contain catalytically essential zinc (*12*, *13*, *16*). Zinc ligands in these proteins have been proposed (*12*), but have not been identified. Residues aligning with His641, Cys643 and Cys726 in the *E. coli* MetE are the only conserved amino acids throughout the alignment, suggesting that these residues may be the zinc ligands in MetE from *E. coli* and its homologues.

In this paper, we report the effects of mutations of the candidate zinc ligands in MetE on catalytic activity, zinc and homocysteine binding. MetE mutants Cys726Ser, Cys643Ser, His641Gln, and His641Asn all display significantly impaired catalytic activities. All mutants not only contain less than 1 equiv of zinc as purified, but also bind zinc with lower affinity than the wild-type MetE. Furthermore, all mutants retain the ability to bind homocysteine and coordinate its thiol group to the active-site zinc. EXAFS analysis of the zinc environment in all mutant proteins shows that the four-coordinate zinc site is preserved and the ligand sets are changed. Our results demonstrate that Cys643 and Cys726 are two of the zinc ligands in MetE from *E. coli* and suggest that His641 is a third endogenous ligand.

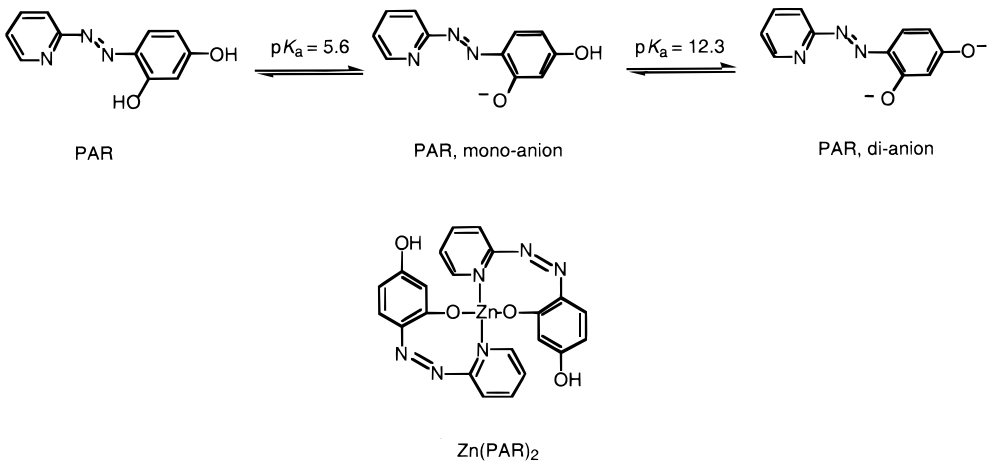
MATERIALS AND METHODS

Materials. Pfu DNA polymerase and restriction enzymes were obtained from Promega and New England Biolabs. Oligonucleotide primers were synthesized at the University of Michigan Protein and Carbohydrate Structure Facility. L-Homocysteine was prepared by hydrolysis of L-homocysteine thiolactone (Sigma) (*28*). (6S)-5-Methyltetrahydropteroyltriglutamate, (6S)-$CH_3-H_4PteGlu_3$, was prepared as previously described from pteroyltriglutamate, $PteGlu_3$ (*29*), which was purchased from Dr. B. Shircks Laboratories (Jona, Switzerland). The Butyl Sepharose 4 Fast Flow column was obtained from Amersham Pharmacia Biotech. Centricon concentrators (30 kDa molecular mass cutoff) were obtained from Amicon. *E. coli* strain GW2531 was obtained from Dr. G. C. Walker at Massachusetts Institute of Technology. Construction of *E. coli* strains GW2531/pJG816 and GW2531/pJG301 containing the wild-type and Cys726Ser mutant *metE* genes was previously reported (*3*). All metal-free solutions were prepared in 18.2 Ω water generated by a Millipore system, and passed through columns containing Chelex 100 resin (200−400 mesh) in the Na^+ form (Bio-Rad).

Construction of MetE His641 and Cys643 Mutants. The MetE mutants His641Ala, His641Cys, His641Lys, His641Thr, His641Gln, His641Asn, and Cys643Ser were constructed using the QuikChange protocol from Stratagene (San Diego, California) with plasmid pJG806 (*3*) as template. The sequences of primers used to introduce the mutations are listed in Table 1. The DNA products and plasmid pJG816 were digested with restriction enzymes *Stu*I and *Pst*I, which cut at nucleotide numbers 1870 and 2358 in GenBank sequence M87625 of *metE* (*2*), respectively. Fragments containing mutation sites were isolated by electrophoresis on agarose gels and ligated into digested plasmid pJG816 using T4 DNA ligase. Plasmids specifying the mutant genes were transformed into *E. coli* strain GW2531, which contains a Tn5 transposon insertion in the chromosomal *metE* gene and does not express wild-type MetE enzyme (*30*). Mutations were confirmed by DNA sequencing of both strands.

Expression and Purification of the MetE Proteins. The recombinant wild-type enzyme and all MetE mutants were overexpressed by growing the appropriate *E. coli* strains aerobically in Luria-Bertani medium supplemented with 100 μg/mL ampicillin, 0.5 mM zinc sulfate, and 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) as previously described (*3*). Most strains were grown at 37 °C to late log-phase or early stationary phase (A_{420} about 8.0); those strains containing His641Gln or His641Asn mutations were grown at 25 °C. The wild-type MetE, Cys643Ser, and Cys726Ser

Scheme 1



mutant proteins were purified to homogeneity by single step DEAE-Sepharose ion-exchange chromatography using a linear gradient of potassium phosphate buffer (180−500 mM at pH 7.2 containing 500 μM DTT) as previously described (*3*). For the His641Gln or His641Asn MetE mutants, an extra step was required to obtain homogeneous proteins. The partially purified proteins were loaded onto a Butyl Sepharose 4 Fast Flow column equilibrated with 0.75 M ammonium sulfate in 50 mM potassium phosphate buffer at pH 7.2. After washing the column with 0.5 and 0.25 M ammonium sulfate in 50 mM potassium phosphate buffer at pH 7.2, the desired proteins were eluted with 50 mM potassium phosphate buffer at pH 7.2. Protein purity was analyzed by electrophoresis in 12% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS−PAGE) and visualized by Coomassie blue staining. Mutant protein concentrations were determined by the standard Bio-Rad protein assay protocol based on the Bradford dye-binding procedure (*31*) using the wild-type enzyme as reference. The absorption coefficients for the mutants are found to be the same as the wild-type enzyme at 280 nm (1.62 mg^{-1} cm^2) (*1*, *3*). Purified proteins were concentrated to about 100 mg/mL and stored at −80 °C.

Metal Content Determination by ICP. Samples were extensively washed with metal-free buffers in Centricon concentrators until the filtrates contained no detectable divalent metal ions as judged by the spectroscopic assay described below. Metal contents of the protein samples and filtrate solutions were determined by inductively coupled plasma-atomic emission spectrometry (ICP) at the University of Michigan Department of Geological Sciences or the Garratt-Callahan Company (Millbrae, CA).

Spectroscopic Determination of Zinc Content and Association Constant for Zinc Binding. The zinc concentration was determined from the absorption change at 500 nm associated with a zinc complex formed with 4-(2-pyridylazo)resorcinol (PAR) in 50 mM Tris chloride buffer at pH 7 containing 400 mM NaCl. The concentration of the Zn−PAR complex was determined using an extinction coefficient of 66 000 M^{-1} cm^{-1} at 500 nm (*32*). To determine the total zinc content in MetE enzymes, the enzyme-bound zinc was released from the enzyme by the addition of *p*-hydroxymercuriphenylsulfonic acid (PMPS (*33*, *34*). To determine the equilibration of zinc in a competition assay between the MetE proteins and PAR, various concentrations of PAR (25−400 μM) and the MetE proteins (5−25 μM) were employed. All experiments were carried out at room temperature (23−25 °C).

As shown in Scheme 1, the pK_as of PAR are 5.6 and 12.3 (*35*). Zinc and PAR can form 1:1 and 1:2 complexes (*35*). The concentrations of the different zinc PAR complexes not only depend on the total concentrations of zinc and PAR, but also on the pH of the solution. The association constant of zinc to PAR monoanion to form the 1:2 complex, Zn(PAR)$_2$, had been reported to be 1.26 × 10^{17} M^{-2} (*35*). To simplify our calculations, an apparent association constant of zinc for PAR, K_a(PAR), is defined in eq 2 , where [Zn$_{free}$] is the concentration of zinc ion not chelated to PAR or the MetE proteins in solution and [PAR$_{free}$] is the total concentration of PAR not coordinated to zinc. At pH 7, when the concentration of PAR is 100 μM and the zinc concentration is under 12 μM, over 98% of the zinc is in the 1:2 Zn(PAR)$_2$ complex (*32*). Under these conditions, the K_a(PAR) had been calculated to be 2 × 10^{12} M^{-1} (*32*).

$$K_a(\text{PAR}) = [\text{Zn(PAR)}_2]/\{[\text{Zn}_{free}][\text{PAR}_{free}]\} \quad (2)$$

Similarly, under the same assay conditions, the apparent association constant of zinc for EDTA has been calculated to be 10^{14} M^{-1} (*32*). The association constant of zinc to the MetE proteins, K_a(MetE), is defined in eq 3 and calculated from eq 4

$$K_a(\text{MetE}) = [\text{Zn-MetE}]/\{[\text{Zn}_{free}][\text{MetE}_{apo}]\} \quad (3)$$

$$K_a(\text{MetE}) = K_a(\text{PAR})[\text{Zn-MetE}][\text{PAR}_{free}]/([\text{Zn(PAR)}_2][\text{MetE}_{apo}]) \quad (4)$$

Enzyme Activity Assays. Activity assays were performed as previously described (*28*), except that, for mutants, protein concentrations up to 45 μM were used. The product formed from methyltetrahydrofolate is tetrahydrofolate, which is measured in the assay by conversion to methenyltetrahydrofolate with formic acid and quantitation at 350 nm.

Homocysteine-Binding Assay. A centrifugation filtration method to measure ligand-protein binding was employed (Professors Martha Ludwig and Vincent Massey, University of Michigan, personal communication). The method was adapted from procedures described by Suter and Rosenbusch (*36*), and Centricon concentrators instead of nitrocellulose filters were used. When a protein binds a ligand, the fraction

of the ligand−protein complex depends on the dissociation constants (K_d's) of each ligand−protein complex and the concentration of free ligand but is independent of protein concentration. This is evident from general derivations of the expression for the ratio of total concentration of the ligand−protein complex to the total concentration of protein (for detailed discussion, see ref *37*). Since free ligand can pass through the Centricon concentrator membrane (just as it equilibrates across a dialysis membrane), the concentration of the free ligand can be conveniently measured in the filtrate obtained by brief centrifugation, and the concentration of the ligand bound to the protein can be obtained by the difference from the total. Although the protein concentration increases in the retentate during centrifugation, the concentration of free ligand and the ratio of the total concentration of the ligand−protein complex to the total concentration of protein will not change. This method thus provides a quick and convenient alternative to equilibrium dialysis. A similar method has been described to measure low-affinity ligand binding to proteins (*38*).

Centricon concentrators were first rinsed with 2 mL of metal-free 25 mM Hepes buffer at pH 7.7. Solutions of the MetE proteins (60−80 μM) and homocysteine (0−400 μM) in 485 μL of 25 mM Hepes buffer at pH 7.7 containing 0.5 mM DTT were centrifuged in the washed Centricon concentrators at about 1000*g* for 5−10 min at 4 °C, allowing about 110 μL of solution to pass through the membrane. Neither the MetE proteins nor homocysteine was found to bind to the concentrator. Homocysteine in the filtrate was reacted with PMPS, and then derivatized with fluorescamine and quantified by fluorescence measurements. Aliquots (35 μL) of the filtrate were mixed with 1.8 mL of 200 mM sodium borate buffer at pH 9.0 containing 100 μM PMPS, and then with vigorous vortexing, 0.6 mL of 1 mg/mL fluorescamine in acetone was added. To determine the concentration of total homocysteine in the protein solution, PMPS was added to the retentate solution to release all protein-bound homocysteine. The PMPS derivative of homocysteine was then separated from the protein by centrifugation filtration and was determined via the reaction with fluorescamine by the same procedure as for the concentration of free homocysteine. The fluorescence emission of the samples was measured at 485 nm with excitation at 390 nm. A range of homocysteine concentrations (0−400 μM) was used to generate standard curves. The dissociation constants, K_d, for the complexes of homocysteine and the MetE proteins were obtained by fitting the concentrations of bound homocysteine vs total homocysteine with eq 5 (*39*), using the

$$[ES] = \{([E] + [S] + K_d) - [([E] + [S] + K_d)^2 - [E][S] \times 4]^{1/2}\}/2 \quad (5)$$

KaleidaGraph 3.0 program (Synergy Software). [ES], [E], and [S] are the concentrations of protein−homocysteine complex, homocysteine-binding site at saturation, and total homocysteine, respectively.

XAS Sample Preparation, Data Collection, and Analysis. Samples of MetE His641Gln, Cys643Ser, and Cys726Ser mutants were equilibrated in 20 mM potassium phosphate buffer at pH 7.2. The protein concentrations were determined to be 2.8 mM for Cys643Ser, 3.3 mM, for Cys726Ser, and 1.2 mM for His641Gln, and the total zinc content for each mutant was 0.4, 0.8, and 0.4 equiv/mol of protein, respectively. Subsequently, the samples were loaded into Lucite cuvettes (~150 μL) with 40 μm Kapton windows and frozen rapidly in liquid nitrogen.

Zinc XAS data were obtained using three independently prepared samples of the Cys643Ser mutant and two samples of the Cys726Ser mutant at SSRL beamline VII-3, under dedicated conditions (3.0 GeV, 100 mA), using a Si(220) double-crystal monochromator detuned to 50% of the maximum intensity for harmonic rejection. These data were collected and calibrated by the procedure described previously (*3*), with the following experimental changes. The total count rate for each channel in the detector array was held below 80 kHz to avoid saturation, while the windowed ZnKα count rate was ~10 kHz/channel in the EXAFS region, giving a total of approximately 3×10^6 useful counts per scan at $k = 13$ Å^{-1}. All of the channels from each scan were examined for the presence of glitches, and the good channels (10 per scan) were then averaged for each sample to give the final spectrum. Six scans were averaged for each mutant sample.

Data were also collected on individual samples of His641Gln in the absence of added homocysteine and His641Gln, Cys643Ser, and Cys726Ser in the presence of 4 equiv of L-homocysteine/mol of protein. These data were collected at SSRL beamline 9-3, using a Si(220) double-crystal monochromator and an Rh coated upstream mirror for harmonic rejection. These data were also collected and calibrated in the manner previously described (*3*, *4*), utilizing a 30-element Ge solid-state detector array. The total integrated count rates were held below 90 kHz/channel to avoid saturation, and the windowed ZnKα count rates were ~18 kHz/channel in the EXAFS region, which gives ~10.8×10^6 usable counts/scan at $k = 13$ Å^{-1}. All of these channels were also examined for glitches, and the good channels (24 for His641Gln both with and without L-homocysteine; 25 for Cys643Ser + L-homocysteine and Cys726Ser + L-homocysteine) were averaged to produce the final EXAFS spectrum. Three scans were averaged for the Cys643Ser + L-homocysteine sample, while four scans were averaged for the three remaining samples.

X-ray absorption near edge structure (XANES) data normalization and the EXAFS data reduction followed procedures described previously (*3*). Fourier transforms were calculated using k^3-weighted data over a range 2−12.8 Å^{-1} for Cys643Ser and Cys726Ser and a range 2−12.4 Å^{-1} for the remaining four samples. The first shell ($R + \alpha = 0.9$−3.9 Å for Cys643Ser and Cys726Ser; $R + \alpha = 0.8$−3.0 Å for His641Gln and His641Gln, Cys643Ser, and Cys726Ser + L-homocysteine) was then backtransformed over the same range. Fits to both the filtered and unfiltered data were performed as described (*40*), using the program Feff v. 6.0.1 to calculate the theoretical phase and amplitude functions for a zinc−nitrogen bond at 2.05 Å and a zinc−sulfur bond at 2.35 Å (*41*, *42*). Filtered and unfiltered data gave equivalent structural parameters.

RESULTS

Expression and Purification of MetE Mutants. The Cys643Ser and Cys726Ser mutants were expressed as soluble proteins at 37 °C. The His641Ala, His641Ser, His641Thr,

Table 2: Zinc Content, Zinc Association Constants (K_a), and Specific Activities for the MetE Proteins

	Zn per mol of protein[a]	K_a of Zn with MetE (M^{-1})	specific activity (μmol min^{-1} mg^{-1})
wild-type	1.0	$>10^{16}$	0.27
His641Gln	0.3−0.4	1.3×10^{14}	0.0004
Cys634Ser	0.8−0.9	5.7×10^{13}	not detectable[b]
Cys726Ser	0.4−0.7	1.4×10^{14}	0.0007

[a] Isolated from cells grown in Luria-Bertani medium supplemented with 0.5 mM zinc sulfate. See discussion for the description of the variation of zinc content in the mutant proteins. [b] Detection limit for specific activity was about 0.0002 μmol min^{-1} mg^{-1}.

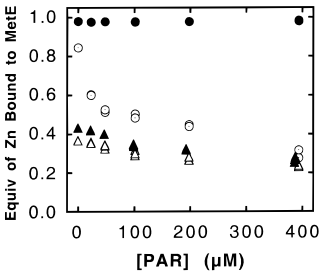


FIGURE 2: Dependence of the equilibration of zinc between the MetE proteins and PAR on the PAR concentration. Assays were carried out in 50 mM Tris chloride containing 400 mM NaCl at pH 7 and room temperature. Total protein concentrations of the wild-type MetE (●), His641Gln (△), Cys643Ser (○), and Cys726Ser (▲) mutants were 11.9, 23.8, 10.2, and 21.9 μM, respectively.

His641Lys, and Cys726Ala mutants all overexpressed as insoluble protein at 37 °C. His641Gln and His641Asn mutants were partially expressed as soluble proteins at 37 °C. Growing strains specifying the His641Gln and His641Asn mutant proteins at 25 °C yielded soluble mutant proteins. The His641Cys mutant was predominantly expressed in soluble, but truncated forms (molecular weights of 47 000 and 43 000 as judged by SDS−PAGE) at 37 °C. All soluble mutants were expressed as about 30% of the total protein in crude cell extracts, and the purified proteins were obtained with yields of ∼100 mg/L growth medium. All soluble untruncated proteins were purified to homogeneity as judged by SDS−PAGE.

Zinc Content of the Wild-Type and Mutant MetE Proteins. The metal contents determined from ICP and spectroscopic assays with PAR were almost identical, and the results are summarized in Table 2. The wild-type enzyme contains 1 equiv of zinc when purified from cells grown in medium containing 0.5 mM zinc sulfate. Under the same conditions, the purified mutant enzymes contain less than 1 equiv of zinc, and the exact amount varies with each preparation, even though the same purification procedure was carefully followed. The variation in zinc content may be due to the reduced zinc-binding affinity for the mutants and is discussed in detail later. For cells grown in medium without zinc supplement (zinc concentration in the growth medium is about 10 μM), purified wild-type enzyme contains ∼0.7 equiv of zinc; the purified Cys726Ser protein contains less than 0.03 equiv of zinc.

Binding Affinity of Zinc to the MetE Protein. PAR by itself cannot extract zinc from the wild-type MetE enzyme, as shown in Figure 2 and Figure 3. On the other hand, significant amounts of zinc are extracted from the mutants with PAR under the same assay conditions, and the equilibrium is dependent both on the concentration of PAR (Figure 2) and the concentration of MetE proteins (Figure 3). Equilibria were established upon mixing the protein and PAR solutions (∼5 s), and the Zn−PAR complex concentrations did not change upon further incubation (data not shown). On the basis of eq 4, the association constants for zinc binding to the His641Gln, Cys643Ser, and Cys726Ser mutant proteins are calculated to be in the neighborhood of 10^{14} M^{-1}, as summarized in Table 2. It has been previously shown that millimolar EDTA could not extract zinc from the wild-type MetE (3) in the absence of added denaturants. Therefore, the association constant for zinc binding to the wild-type enzyme is over 10^{16} M^{-1}. On the basis of these association constants, each mutant MetE protein binds zinc more weakly than the wild-type enzyme by a factor of at least 100.

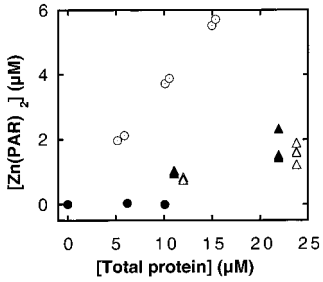


FIGURE 3: Variation of the Zn−PAR complex concentration with the total concentration of MetE. Assays were carried out with 100 μM PAR in 50 mM Tris chloride containing 400 mM NaCl at pH 7 and room temperature. Zinc contents of the wild-type MetE (●), His641Gln (△), Cys643Ser (○), and Cys726Ser (▲) mutants were 0.98, 0.37, 0.89, and 0.39, respectively.

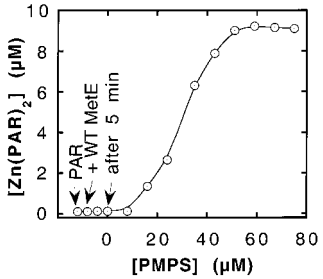


FIGURE 4: PMPS-promoted zinc release from the wild-type MetE to a solution of PAR. Assays were carried out with 100 μM PAR in 50 mM Tris chloride containing 400 mM NaCl at pH 7 and room temperature. The total protein concentration was 13 μM, and the total zinc concentration was 9.5 μM by ICP analysis. The wild-type MetE enzyme was first added to the PAR solution and incubated for 5 min before PMPS was added.

Upon modification of the cysteine thiol groups in the wild-type MetE by PMPS, zinc is released into solution and chelated by PAR (Figure 4). The formation of a PMPS−mercaptide bond was evident from the increase of absorption at 250 nm upon PMPS addition (data not shown). The formation of a PMPS−mercaptide bond and the formation of a Zn−PAR complex were completed upon mixing the solutions (∼5 s, data not shown). While the wild-type MetE enzyme contains six cysteine residues, only 4 equiv of PMPS (50 μM) was required to completely release all bound zinc from the enzyme (13 μM). With excess PMPS, the amount of zinc released to the PAR solution was the same as the total amount of zinc bound to the protein as determined by ICP analysis. Treatment of the wild-type MetE with methyl methanethiolsulfonate (MMTS), another thiol modification

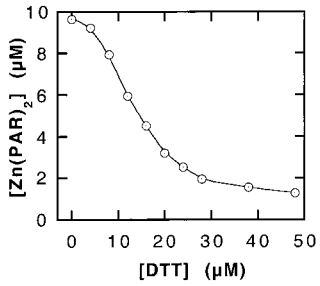


FIGURE 5: Extraction of zinc from a Zn−PAR complex by apo-MetE regenerated by the addition of DTT. The wild-type MetE (13 μM containing 9.5 μM zinc) was treated with 60 μM PMPS (see Figure 4), in the presence of 100 μM PAR. Aliquots of DTT were then added and the decrease of absorption at 500 nm due to the zinc-PAR complex was measured. Assays were carried out in 50 mM Tris chloride containing 400 mM NaCl at pH 7 and room temperature.

reagent (*43*), also effectively released the protein-bound zinc (data not shown). Similar to the wild-type enzyme, modification of the cysteine residues of the mutant proteins with PMPS and MMTS effectively releases all of the protein-bound zinc into the solution containing PAR (data not shown).

Upon regeneration of the thiol groups of the cysteine residues by treatment with DTT, zinc that was released to the PAR solution was back extracted into the apo-enzyme of the wild-type MetE enzyme (Figure 5). Again, equilibration of zinc between the MetE protein and PAR was completed within 5 s (data not shown). With higher concentrations of DTT (500 μM), all the zinc ions are transferred from PAR into MetE (data not shown). DTT at concentrations lower than 500 μM does not affect zinc binding to 100 μM PAR in the absence of MetE protein (data not shown).

Activities of the MetE Proteins. The specific activities of the wild-type and mutant MetE proteins are also listed in Table 2. The Cys643Ser mutant shows no detectable activity, while the His641Gln and Cys726Ser mutants display measurable, albeit low, activities (0.1 and 0.3% relative to the wild-type enzyme, respectively). Because the *E. coli* strain GW231 used to express the MetE mutants does not produce wild-type enzyme, the purified mutant proteins did not contain any wild-type enzyme. Therefore, the observed activities of the mutant proteins were not due to contamination by the wild-type enzyme. It has been previously reported that the Cys726Ser mutant protein is catalytically inactive (*3*). The difference between the observed activities for the same mutant protein in this paper and the prior report is attributed to the different zinc content in the purified proteins. The Cys726Ser mutant was previously isolated from cells grown in unsupplemented medium and contained 0.03 equiv of zinc, while the mutant protein examined in this paper was isolated from cells grown in zinc-supplemented medium and contained 0.4 equiv of zinc.

Homocysteine Binding to the MetE Proteins. The dissociation constants (K_d) of the homocysteine−MetE complexes are listed in Table 3. The amount of homocysteine bound to each MetE protein at saturation was approximately the same as the amount of zinc bound to that protein. Interestingly, we found that even after all exogenous zinc had been removed by repeated washing with metal-free buffer, there were still ~0.4 and 0.07 equiv of homocysteine/total protein that were bound to the purified Cys643Ser and His641Gln mutant proteins, respectively. This bound homocysteine was detected in the fluorescamine assay without addition of exogenous homocysteine. Further extensive washing with buffer can remove all the bound homocysteine.

Table 3: Binding of L-Homocysteine to the MetE Proteins[a]

	[protein] (μM)[b]	[zinc] (μM)[c]	[Hcy] (μM)[d]	K_d (μM)[e]
wild-type	82	59	65	57
His641Gln	103	31	35	196
Cys634Ser	100	89	81	9
Cys726Ser	96	38	48	118

[a] Binding assay was carried out in 25 mM Hepes buffer at pH 7.7 containing 0.5 mM DTT at 4 °C. [b] Concentrations of total protein. [c] Concentrations of protein-bound zinc. [d] Concentrations of homocysteine bound to the MetE proteins at saturation as determined from eq 2. [e] Dissociation constants of the homocysteine−protein complexes.

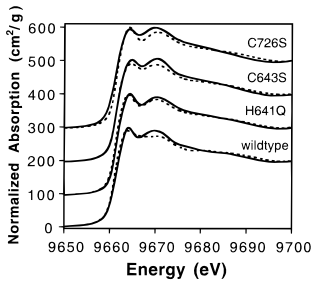


FIGURE 6: Normalized XANES spectra of wild-type and mutant MetE proteins (solid lines) and the proteins with addition of 4 equiv of L-homocysteine/mol of protein (dashed lines). All spectra were plotted on the same scale and have been offset vertically for clarity.

X-ray Absorption Spectroscopy Analysis of Zinc-Binding Sites in Mutants. XANES spectra for the wild-type, His641Gln, Cys643Ser, and Cys726Ser MetE proteins are given in Figure 6. Comparing the XANES spectra for the wild-type MetE (*3*) with that of the mutants, the Cys to Ser mutations at the zinc site produce an edge structure that is similar to but distinct from that observed in the wild-type enzyme. The differences are seen most easily in the ratio of the intensities of the peaks at 9664 and 9670 eV. This ratio is slightly greater than one for the wild-type enzyme and slightly less than one for the Cys → Ser mutants. The decrease in this ratio is similar to the changes observed in zinc model complexes when one of the sulfur ligands is replaced by a low-Z (N/O) ligand (*40*). In contrast, the XANES spectrum for the His641 mutant has a structure very similar to that of the wild-type enzyme. Binding of homocysteine to wild-type MetE causes changes in the XANES spectrum due to the perturbation in zinc coordination. Changes of a similar magnitude are seen in the XANES spectra of each of the mutants when homocysteine is bound.

The Fourier transformed EXAFS data (Figure 7) also demonstrate differences both between the mutant and wild-type MetE enzymes and for each protein when homocysteine is bound. The differences between wild-type and Cys to Ser mutant MetE proteins can be seen qualitatively as an increase in the height of the unresolved peak corresponding to Zn−(N/O) ($R + \alpha \approx 1.3$ Å) relative to that for Zn−S ($R + \alpha \approx 1.8$ Å) and as a slight decrease in the overall amplitude of the Fourier transform. These differences are compatible with a decrease in the numbers of sulfur ligands to the zinc. For each protein, addition of L-homocysteine causes an increase

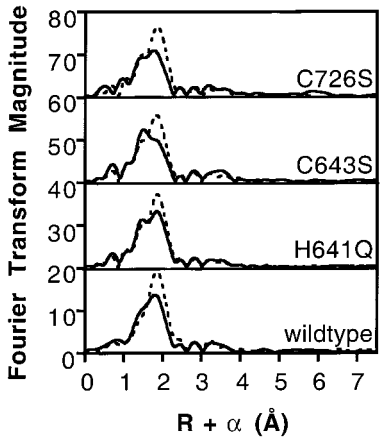


FIGURE 7: Fourier transformed EXAFS spectra of the wild-type and mutant MetE proteins without (solid line) and with (dashed line) addition of 4 equiv of L-homocysteine.

Table 4: EXAFS Fitting Results for MetE Mutant Proteins[a]

	high Z			low Z			
sample	N	R (Å)	σ^2 (Å^2 × 10^3)	N	R (Å)	σ^2 (Å^2 × 10^3)	F^b
wild-type	1S	2.32	0	3N	2.06	4.4	0.548
	2S	**2.31**	**3.4**	**2N**	**2.04**	**3.8**	**0.370**
	3S	2.31	5.9	1N	2.03	2.0	0.457
wild-type	1S	2.34	0	3N	2.11	4.5	1.001
+ Hcy	2S	2.34	1.3	2N	2.08	2.9	0.458
	3S	**2.33**	**3.6**	**1N**	**2.07**	**2.3**	**0.439**
Cys726Ser	**1S**	**2.30**	**1.1**	**3N**	**2.04**	**6.8**	**0.624**
	2S	2.29	1.8	2N	2.04	7.7	0.728
	3S	2.28	8.8	1N	2.07	9.3	0.870
Cys726Ser	1S	2.32	0	3N	2.07	6.2	0.766
+ Hcy	**2S**	**2.32**	**1.9**	**2N**	**2.04**	**3.8**	**0.356**
	3S	2.31	4.3	1N	2.01	1.6	0.441
Cys643Ser	**1S**	**2.31**	**0.5**	**3N**	**2.03**	**3.3**	**0.301**
	2S	2.30	3.7	2N	2.02	4.5	0.371
	3S	2.29	7.4	3N	2.00	0	0.543
Cys643Ser	1S	2.33	2.6	3N	2.08	3.3	0.641
+ Hcy	**2S**	**2.31**	**2.4**	**2N**	**2.04**	**4.0**	**0.356**
	3S	2.31	4.9	1N	2.02	2.5	0.465
His641Gln	1S	2.32	0	3N	2.05	4.8	0.492
	2S	**2.31**	**2.8**	**2N**	**2.02**	**2.9**	**0.378**
	3S	2.30	5.4	1N	2.00	0.3	0.529
His641Gln	1S	2.33	0	3N	2.08	6.1	0.808
+ Hcy	**2S**	**2.32**	**1.7**	**2N**	**2.05**	**3.7**	**0.330**
	3S	2.32	4.0	1N	2.02	1.6	0.420

[a] Results given are for fits to filtered data. The best fits are in boldtype. Similar bond distances and Debye−Waller factors (σ^2) were obtained from fits to unfiltered data but with higher F values due to the neglect of outer shell scattering. [b] $F = [k^6(\text{data-fit})^2]/N$, where N is the number of data points.

in the amplitude of the Fourier transform and a decrease in the relative height of the low-R Zn−(N/O) peak. These qualitative conclusions are supported by quantitative curve-fitting results (Table 4).

As was seen for the XANES, the Fourier transform for the His641Gln mutant is similar to that for the wild-type enzyme. Surprisingly, the features at ($R + \alpha \geq 2.6$ Å), which are similar to those observed in zinc model peptides with histidine ligands (*40*) and are generally attributed to outer-shell scattering of the imidazole ring, are still present in the His641Gln Fourier transform and, indeed, are very similar for all four proteins.

The EXAFS fits to the Cys726Ser and Cys643Ser mutants in the presence and absence of L-homocysteine confirm that the amplitude increase observed in the Fourier transform on adding homocysteine is due to replacing one of the low-Z (N/O) ligands at the zinc site with a sulfur ligand. By replacing homocysteine with selenohomocysteine, these changes have been shown to be due to the thiolate directly binding to the zinc atom in the wild-type MetE (Peariso, Zhou, Smith, Matthews, and Penner-Hahn, manuscript in preparation). The similarity of the changes observed in the mutant samples suggests that homocysteine binding perturbs the zinc site in a similar manner for the mutants and for the wild-type enzyme.

In contrast, the fitting results for the His641Gln mutant are unexpected. Despite clear changes in the Fourier transform, the best integer fits for both His641Gln and His641Gln with homocysteine suggest 2S + 2(N/O) ligand sets (Table 4). This is a consequence of round-off error; fits with quarter-interger coordination give apparent ligations of 1.5S + 2.5 N for His641Gln and 2.25S + 1.75N for His641Gln with L-homocysteine. These numbers show the expected increase in S coordination number when homocysteine binds, but still give total sulfur coordination that is significantly lower than expected (expected values are 2S + 2N and 3S + N, respectively). In addition to the low sulfur content, the His641Gln mutant is also surprising in that the outer shell scattering, attributed to histidine ligation, is identical to that in the wild-type enzyme, suggesting that a single histidine ligand is coordinated to the Zn in both proteins. The reasons for these anomalies are not clear. It is possible that His641 is not a ligand to the Zn in the wild-type enzyme. This would explain the retention of outer shell scattering even when His641 is replaced, but would not explain the decrease in sulfur coordination in His641Gln. Moreover, as noted above, His641 is the only conserved histidine in the MetE-like sequences. The simplest explanation is that His641 is a ligand in the wild-type enzyme but that there is a change in Zn ligation for His641Gln. One possibility is a nonconserved histidine at position 639. This is predicted to be part of the same β-sheet that contains His641. If His639 becomes a Zn ligand in His641Gln, this could strain the Zn site such that one of the cysteines is no longer coordinated. This would explain the lower than expected sulfur coordination, the retention of histidine outer shell scattering, and the loss of activity, but not of homocysteine binding in His641Gln. The observation of an apparent S coordination number greater than 1 for His641Gln may indicate that one of the cysteines is in equilibrium with a low Z ligand or may simply reflect binding of a small fraction of residual homocysteine. For His641Gln, the extensive washing needed to remove homocysteine (see above) also results in loss of Zn. We estimate that up to 20% of the Zn sites may retain bound homocysteine in the His641Gln mutant.

DISCUSSION

The results presented in this paper demonstrate that Cys643 and Cys726 are zinc ligands in cobalamin-independent methionine synthase (MetE) from *E. coli* and suggest that His641 is a third Zn ligand. Due to the apparent change in Zn ligation from His641 to His639 in the His641Gln mutant, it is not possible to be conclusive regarding His641. Definitive demonstration that His641 is a ligand will likely require determination of the three-dimensional structure of MetE.

The zinc ligand set in MetE shares some common features with other zinc-containing enzymes that catalyze thiol alkylation. Most noticeably, the zinc sites of all these proteins contain cysteine sulfur ligands. In addition, many of the noncysteine ligands are potentially negatively charged ligands, thus making it likely that the substrate−zinc complexes have net negative charges. If the cysteines are assumed to be ionized, then all of the zinc alkyl-transfer sites for which the ligation is defined are anionic. These include Ada protein (−2 charge, four cysteine ligands) (*6*), the complex of *E. coli* MetH and the homocysteine substrate (−2 charge, three cysteine and one homocysteine ligands) (*4*), and the complex of farnesyl transferase and its substrate (−1 charge, two cysteine, one aspartate and one histidine ligands) (*19*−*21*).

Negatively charged zinc clusters are thought to be important to increase the nucleophilicity of thiol substrates at physiological pH and thus to promote thiol alkylation. Zinc has been postulated to function as a Lewis acid, lowering the pK_a of thiol group of the substrate and increasing the fraction of thiolate present at physiological pH. A thiolate anion is much more reactive that the corresponding thiol, with differences in nucleophilicity reported to be over 10^9-fold (*44*, *45*). Homocysteine has a microscopic pK_a of 10 for its thiol group (*46*), so its thiol group is present predominantly in the protonated form at physiological pH. Coordination to the zinc in MetE could effectively lower the pK_a value of the homocysteine thiol group to near or below physiological pH. In fact, proton release has been observed following homocysteine binding both to cobalamin-dependent methionine synthase (MetH) at pH 7.7 (*7*) and to cobalamin-independent methionine synthase (MetE) (Zhou and Matthews, unpublished results). Dependence of substrate binding on pH in farnesyltransferase also suggests that the substrate binds as an ionized thiolate at physiological pH (*22*).

The notion that the net charge and number of thiolate ligands influences the reactivity of the thiolate in a zinc complex is supported by model studies of methyl transfer from methyl phosphotriester to complexes of zinc and benzenethiolate (PhS^-) (*47*, *48*). Kinetic analysis revealed that a dianionic complex such as $[Zn(SPh)_4]^{2-}$ is 10 times more reactive than a monoanionic complex such as $[Zn(SPh)_3(MeIm)]^{1-}$, where MeIm is 1-methylimidazole; and 1000 times more reactive than a neutral complex such as $[Zn(SPh)_4(MeIm)_2]$. The observed reactivity of the thiol ligands in this model system has been attributed to an equilibrium between ligated and free thiolates, where the net negative charge on the complex increases thiolate dissociation and hence reactivity. The thiolate was postulated to dissociate from zinc prior to alkylation, although zinc-bound thiolates may also be reactive under some conditions (*49*, *50*). Theoretical calculations indicate that an increase either in the negative charge of a zinc complex or in the number of thiolate ligands will increase the negative charge on the thiolate ligands and raise the energy of the highest occupied molecular orbital (HOMO) of the nucleophiles, consequently enhancing their reactivity (*51*, *52*).

The wild-type enzyme binds zinc with high affinity, $K_a > 10^{16}$ M^{-1}, as expected for a zinc complex rich in thiolate ligands. Partial denaturation with 1 M urea is required for extraction of zinc from the wild-type MetE by EDTA (*3*). The tight binding of zinc has also been observed for the Ada protein (*53*), the MetH enzyme from *E. coli* (*7*), and other proteins with zinc-binding scaffolds similar to MetE (*12*, *16*). The association constants for binding of zinc to the two methylcobamide:coenzyme M methyltransferases isozymes from *M. barkeri* were determined to be about 10^{10} M^{-1} under partially denaturating conditions (0.2% SDS at pH 7.0) (*13*).

With a limited supply of zinc in the growth medium, the wild-type enzyme is able to incorporate over 0.7 equiv of zinc as compared to 0.03 equiv of zinc for the Cys726Ser mutant, as previously reported (*7*). These results suggest that the wild-type MetE is capable of competing with other molecules inside the cell for zinc binding, while the mutant proteins are unable to do so, consistent with the difference in zinc-binding affinities we report here. All purified MetE mutant proteins contain less than 1 equiv of zinc and exhibit diminished zinc-binding affinities ($K_a \approx 10^{14}$ M^{-1}). The variance of zinc contents in the isolated mutant proteins could be due to their reduced binding affinities for zinc which lead to loss of zinc during biosynthesis and purification. Mutant proteins that do not have proper side-chain functional groups for zinc binding, e.g., His641Ala, His641Lys, and Cys726Ala, are expressed as insoluble proteins. The His641Cys mutant protein was expressed mostly in truncated form, indicating a change of protein tertiary structure. These observations suggest that zinc binding is critical for proper protein folding during the biosynthesis of these proteins, although it may not be required to maintain the folded conformation. A similar phenomenon has been observed for the expression of Ada protein; zinc must be added to the growth medium to isolate properly folded protein in vivo (*53*).

The effective release of zinc from both the wild-type MetE and the mutants by the thiol modification reagents PMPS and MMTS supports the involvement of cysteine ligands in zinc binding. Zinc release promoted by thiol modification reagents, such as PMPS and MMTS, has also been observed in other proteins with sequence homology to *E. coli* MetE (*12*) and other zinc enzymes with cysteine ligands, such as *E. coli* aspartate transcarbamoylase (*33*) and Gene 32 protein (*34*), *E. coli* Ada protein (*53*), and MetH from *E. coli* (*7*). The apo-enzyme generated by addition of DTT can extract back all the released zinc from PAR, again indicating that the wild-type MetE binds zinc more tightly than PAR.

EXAFS analysis suggests that homocysteine coordinates to the zinc ion of the mutant proteins through direct thiol ligation, as previously observed with the wild-type enzyme (*4*). The amount of homocysteine bound to the MetE proteins at saturation is approximately the same as the amount of zinc bound to these proteins (see Table 3), suggesting that zinc coordination is essential for homocysteine binding to both the wild-type and the mutant MetE proteins. The His641Gln and Cys726Ser mutant proteins, which display low activities, bind homocysteine 2−4-fold more weakly than the wild-type enzyme. In the case of the His641Gln mutant protein, weaker homocysteine binding and low activity may result from a rearrangement of the ligand set. The Cys643Ser mutant, which displays no detectable activity, binds homocysteine almost 7 times tighter than the wild-type MetE enzyme. A slower off rate of homocysteine release from the zinc ion in the Cys643Ser mutant might contribute to the loss of activity and might also be the reason for retaining exogenous homocysteine in the Cys643Ser mutant upon purification. Ensign and co-workers reported that coenzyme

M substrate is co-purified with an epoxyalkane:coenzyme M transferase, which is homologous to MetE and which contains 0.83 equiv of zinc (*16*). Further investigation of the rates of homocysteine binding and release for the wild-type MetE and the mutants is in progress, which may give deeper insight into the mechanism of thiol activation in this enzyme.

Even with conservative mutation of the zinc ligands, the catalytic activity is markedly impaired. The extreme sensitivity of the activity to mutation of zinc ligands has also been observed with protein farnesyltransferase, where 15−500-fold decreases in activities on ligand mutation have been reported (*20*, *21*). As noted previously, structural changes caused by mutation of zinc ligands have been observed for other zinc enzymes, such as carbonic anhydrase. Therefore, without detailed information on the three-dimensional structures of the wild-type MetE and mutant proteins, we cannot rule out some conformational changes in the zinc sites or other portions of the proteins that could contribute to the loss of activities of the mutants. However, if we assume the overall structures of the zinc site and the protein in the cysteine mutants are generally similar to those of the wild-type enzyme, the effects of the mutations on the catalytic activity could be due to the changes in chemical nature of the ligands. The carboxamide and oxygen ligands in the mutants are considerably harder than the sulfur ligands in the wild-type enzyme. They less efficiently donate electrons to the zinc complex and, therefore, reduce the nucleophilicity of the thiol group of bound homocysteine for methyl transfer and/or affect the equilibrium between ligated and dissociated thiolates. Zinc and homocysteine binding are not sufficient for thiol activation and catalytic activity. Rather, the chemical nature of the ligands is a determining factor for catalysis.

On the basis of the high sequence homology and similar secondary structure patterns between the *E. coli* MetE enzyme and its homologues listed in Figure 1, our results would suggest that all these proteins contain zinc with the conserved histidine and cysteines as the zinc ligands in those proteins and that they all catalyze thiol alkylation. Detailed studies of other systems, along with further investigation of the MetE enzyme, will be invaluable to a better understanding of the mechanism of this new family of zinc proteins that catalyze thiol alkylation.

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SUPPORTING INFORMATION AVAILABLE

Figures showing the measured EXAFS spectra and best fits of the MetE mutant proteins (Figures S1, S2, and S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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