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Cobalamin-Independent Methionine Synthase from *Escherichia coli*: A Zinc Metalloenzyme†

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ABSTRACT: Cobalamin-independent methionine synthase (MetE) from *Escherichia coli* catalyzes the transfer of a methyl group from methyltetrahydrofolate to homocysteine. Previous work had shown the existence of a reactive thiol group, cysteine726, whose alkylation led to loss of all detectable enzymatic activity [González, J. C., et al. (1992) *Biochemistry 31,* 6045−6056]. A site-directed mutation of MetE, Cys726Ser, was constructed to investigate the possible role of this cysteine. The Cys726Ser protein was purified to homogeneity, affording a protein with no detectable activity. To assess the possibility that cysteine726 functions as a metal ligand, inductively coupled plasma-atomic emission spectrometry was performed. The wild-type enzyme contains 1.02 equiv of zinc per subunit; the Cys726Ser mutant does not contain zinc, supporting the view that cysteine726 is required for metal binding. A loss of enzymatic activity is observed upon removal of zinc from the wild-type MetE by incubation in urea and EDTA; activity can subsequently be restored by zinc reconstitution, suggesting that zinc is required for catalysis. Circular dichroism measurements further suggest that there are no major differences in the secondary structures of the wild-type and the Cys726Ser mutant enzymes. Extended X-ray absorption fine structure analysis has established that the average zinc environment is different in the presence of homocysteine than in its absence and is consistent with the changes expected for displacement of an oxygen or nitrogen ligand by the sulfur of homocysteine. A possible model for zinc-dependent activation of homocysteine by MetE is presented.

Nature has evolved two routes for the *de novo* biosynthesis of methionine from homocysteine. One involves the cobalamin-dependent methionine synthase (EC 2.1.1.13) and the other the cobalamin-independent enzyme of the same name (EC 2.1.1.14). The former enzyme is found in organisms that synthesize vitamin B_{12} or obtain it from outside sources, while the latter is generally found in organisms that do not synthesize or transport vitamin B_{12}. In *Escherichia coli*, where both enzymes are found (Foster et al., 1964), the cobalamin-independent methionine synthase is specified by the *metE* gene and the cobalamin-dependent enzyme by *metH*. Both enzymes catalyze the same overall reaction, the transfer of a methyl group from methyltetrahydrofolate to homocysteine (eq 1). However, the cobalamin-independent enzyme differs from the cobalamin-dependent enzyme in showing an absolute requirement for a polyglutamate derivative of methyltetrahydrofolate (CH_3-H_4PteGlu$_n$,[1] where $n > 1$). MetE and MetH have other significant differences in their requirements for activity. MetE, unlike MetH, has a requirement for phosphate, and its activity is stimulated by divalent cations such as Mg^{2+} or Mn^{2+}. In contrast, MetH requires a reducing system and AdoMet for activity, while MetE has no such requirements. The turnover number of 22.6 min^{-1} determined for MetE in this study is ∼50-fold lower than that of 1128 min^{-1} determined for MetH (Banerjee et al., 1990).

$$CH_3\text{-}H_4\text{PteGlu}_n + \text{homocysteine (RSH)} \rightarrow H_4\text{PteGlu}_n + \text{methionine (RSCH}_3\text{)} \quad (1)$$

Since vitamin B_{12} is not found in the plant kingdom (Chanarin, 1990), the cobalamin-independent methionine synthase is of interest as it provides the only known route

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[1] Abbreviations: CH_3-H_4PteGlu$_n$, 5-methyltetrahydropteroylpolyglutamate with *n* glutamyl residues; H_4PteGlu$_n$, tetrahydropteroylpolyglutamate with *n* glutamyl residues; MetE, cobalamin-independent methionine synthase, the product of the *metE* gene (EC 2.1.1.14); MetH, cobalamin-dependent methionine synthase, the product of the *metH* gene (EC 2.1.1.13); Hcy, homocysteine; $PteGlu_3$, pteroyltriglutamate; TLCK, 1-tosylamido-l-lysyl chloromethylketone; TPCK, 1-tosylamido-l-phenylalanyl chloromethylketone; PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; ICP, inductively coupled plasma-atomic emission spectrometry; nt, nucleotide; EXAFS, extended X-ray absorption fine structure; XAS, X-ray absorption spectroscopy; XANES, X-ray absorption near-edge structure; SSRL, Stanford Synchrotron Radiation Laboratory; IPTG, isopropyl β-D-thiogalactopyranoside.

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to methionine for these organisms (Eichel et al., 1995). Previous work had shown that these two enzymes share no sequence homology and are thus thought to have arisen by convergent evolution (González et al., 1992). While extensive studies on the mechanism of action [reviewed in Banerjee et al. (1990)] and structure (Drennan et al., 1994a,b) of MetH have previously been performed, little work has been carried out on MetE since the initial characterization of the protein by Whitfield et al. (1970), who first described the properties of a homogeneous preparation of MetE.

It had been previously established that cobalamin-independent methionine synthase is susceptible to alkylation by certain protease inhibitors, including those aimed at cysteine proteases (González et al., 1992). Two groups of alkylating agents had been studied: the iodoacetic acid family, with iodoacetamide resulting in a faster and more complete inactivation than its parent compound, and the chloromethyl ketones, with TPCK acting at a faster rate than TLCK. It was postulated from these observations that the alkylating agents might be binding to a region in, or in proximity to, the active site and that a cysteine may be playing an important or possibly direct role in catalysis.

The reaction catalyzed by cobalamin-independent methionine synthase is very challenging, in that nucleophilic displacement of a methyl group from a tertiary amine occurs in the apparent absence of a cofactor. MetE must possess an attacking group with strong enough nucleophilic character to displace the methyl group from methyltetrahydrofolate. This feat is accomplished in MetH by the cob(I)alamin cofactor (Banerjee, 1990), one of the most potent nucleophiles known (Brown, 1982). In addition, N^5-methyltetrahydrofolate must be activated for nucleophilic attack. It has been suggested that this activation may be effected by protonation of N^5 (Matthews & Drummond, 1990), and a similar activation may occur in the transfer of a methyl group from methyltetrahydrofolate to a corrinoid iron−sulfur protein catalyzed by a methyltranferase from *Clostridium thermoaceticum* (Zhao et al., 1995).

In the work presented here, inductively coupled plasma-atomic emission spectrometry (ICP) was used to demonstrate that the wild-type enzyme has one equivalent of zinc per protein subunit, and the metal has been shown to be essential for enzymatic activity. Circular dichroism studies do not suggest a structural role for zinc, implying a catalytic function for this metal. A Cys726Ser mutation was introduced into MetE, and the resultant mutant protein contained no bound zinc. Thus, it can be inferred that at least one function of cysteine726 is that of a ligand to zinc. The results of EXAFS analysis are consistent with a change in zinc coordination from two sulfur/two oxygen or nitrogen to three sulfur/one oxygen or nitrogen when homocysteine is added. The identification of a zinc cofactor in MetE is consistent with a model in which homocysteine is activated for nucleophilic attack by coordination to the zinc.

MATERIALS AND METHODS

Materials. The Geneclean Kit was purchased from Bio 101. Vent polymerase and *Sma*I restriction endonuclease were purchased from New England Biolabs. T4 DNA ligase was obtained from Boehringer Mannheim Biochemicals. Calf intestinal alkaline phosphatase and *Nsi*I, *Pst*I, and *Stu*I restriction enzymes were obtained from Promega. The Sequenase Version 2.0 DNA Sequencing Kit was purchased from United States Biochemical. All primers were made at the University of Michigan Protein and Carbohydrate Structure Facility. (6*S*)-CH_3-$H_4$$PteGlu_3$ was synthesized as described previously (Matthews, 1986) from $PteGlu_3$ purchased from Schircks Laboratories (Jona, Switzerland). *E. coli* strain XL1-Blue was purchased from Stratagene.

Site-Directed Mutagenesis. The cysteine726 codon (TGT) in the *metE* gene was changed to serine (TCT) using the method of site-directed mutagenesis by overlap extension PCR (Horton et al., 1993). For the construction of each mutation, four oligonucleotide primers (25-mers) were used: the sequence of primer A is identical to residues 1768−1792 of the noncoding strand of the published *metE* sequence (González et al., 1992) and begins 409 nt upstream of the first nucleotide of the cysteine726 codon; primer D is complementary to residues 2526−2550 of the noncoding strand of the *metE* gene, 289 nt downstream of the termination codon of *metE;* primer B is complementary to nt 2165−2189 on the noncoding strand, with the exception of the changes introduced at nt 2177 to convert the cysteine codon to one specifying serine; the sequence of primer C is identical to nt 2165−2189 of the noncoding strand, again with changes introduced at nt 2177. Two separate PCR reactions were carried out in a Perkin Elmer DNA Thermal Cycler 480, using Vent polymerase to minimize the possibility of errors in amplification. The first reaction contained primers A and B, the second one C and D. In both reactions, the pRSE562 plasmid (Maxon et al., 1989), containing the wild-type *metE* gene, was used as a template. The resulting products of amplification were isolated by electrophoresis on agarose gels and purified using the Geneclean system. The purified products were then used together in a single PCR reaction, so that fragments AB and CD served as templates for each other, in the presence of primers A and D. After purification, the product was digested with *Stu*I and *Nsi*I and isolated using the Geneclean system. The resulting fragment was reintroduced by ligation with T4 DNA ligase into the pRSE562 plasmid that had been previously cut with the same restriction enzymes and treated with alkaline phosphatase; the resulting plasmid is pJG11. Plasmids containing the wild-type and mutant *metE* genes were isolated after transformation of competent XL1-Blue cells as described by Chung and Miller (1988). The nucleotide sequences of the PCR products and their junctions with pRSE562 were confirmed by DNA sequencing (Sanger et al., 1977), using the Sequenase kit.

Subcloning of Wild-Type and Mutant metE Genes and Expression of the Gene Products. The *metE* gene was isolated from plasmid pJG11 by cutting with *Sma*I and *Pst*I. Plasmid pKK223-3 (Pharmacia) was cut with same restriction enzymes and treated with alkaline phosphatase, and both products were isolated by electrophoresis on agarose gels, followed by purification using the Geneclean kit. The segment containing the *metE* gene was then ligated into pKK223-3, using T4 DNA ligase, and the products were used to transform competent cells of strain GW2531, which contains a transposon inserted into the *metE* gene and does not express wild-type MetE (Mulligan et al., 1982). Two new plasmids were obtained by this method: pJG301, containing mutation Cys726Ser; and pJG816, containing the wild-type gene. To isolate the *metE* gene products, *E. coli* strains were grown in 1 L of Luria-Bertani medium supplemented with 1 mM IPTG and 100 μg/mL ampicillin,

using 1 mL of an overnight growth of the same strain in Luria-Bertani medium as the inoculum. The cultures were incubated overnight (~18 h) in a shaker at 37 °C and grown to an approximate A_{420} of 6.0. The protein was then purified as described previously (González et al., 1992). For preparations in metal-supplemented media, zinc sulfate or cadmium chloride was added to a final concentration of 1 mM.

Determination of Protein Concentration. A stock solution of cobalamin-independent methionine synthase was prepared in 10 mM potassium phosphate buffer, pH 7.2, containing 500 μM dithiothreitol. The protein concentration was determined on three different occasions, following the standard Bio-Rad protein assay protocol, each time using fresh dilutions of bovine serum albumin and Bio-Rad dye for the standard curves. Two different MetE dilutions were used for each assay, with each dilution done in quadruplet for a total of eight data points per assay. The data for the three assays were combined, and an average concentration was determined for the stock solution. Three different dilutions of this MetE stock were then made and the samples submitted to the University of Michigan Protein and Carbohydrate Structure Facility for amino acid analysis by the phenylthiocarbamyl (PTC) method (Heinrikson & Meredith, 1984; Tarr, 1986). Each sample was analyzed twice. A fourth sample was then sent for analysis several days later.

Metal Content Determination. Divalent cations were removed from 25 mM triethanolamine buffer, pH 7.2, containing 500 μM dithiothreitol, by passing through a 1.5 × 23-cm column containing Chelex 100 resin (200−400 mesh) in the Na^+ form. A homogeneous sample of MetE was exchanged into the resulting buffer by washing 200 μL samples of enzyme three times with 2 mL volumes of the "scrubbed" buffer in Centricon 30 concentrators. The last filtrate was recovered and used as a blank. Samples were sent to the University of Michigan Department of Geological Sciences to determine metal content by ICP.

Zinc Removal from MetE. A sample of MetE was treated following a modification of a method described by Whitfield and Weissbach (1970). A buffer solution was prepared containing 0.5 M Tris chloride, 5 mM dithiothreitol, 75 mM EDTA, and 1 M urea, pH 7.8. This solution was chilled on ice, and MetE was added to a final concentration of 15 μM. The solution was left on ice for approximately 48 h, at which point the enzyme solution was concentrated in a Centricon 30 concentrator and washed twice with 2 mL volumes of 0.5 M Tris chloride buffer, pH 7.2, containing 5 mM dithiothreitol and 1 M urea, followed by two 2 mL volumes of "scrubbed" 25 mM triethanolamine buffer, pH 7.2, containing 500 μM dithiothreitol. The last filtrate was recovered and used as a blank for metal content measurements.

Zinc Reconstitution of MetE. Zinc sulfate was added to a sample of zinc-depleted MetE in 25 mM triethanolamine buffer, pH 7.2, containing 500 μM dithiothreitol, and left on ice for 12 h. To remove loosely bound metal, the enzyme solution was passed through a 400 μL volume Chelex 100 column (in a 1 mL pipettor tip) that had been previously equilibrated with the same buffer. The enzyme was then eluted with four 200 μL volumes of buffer. The protein concentration of the eluted solution was determined by the Bio-Rad protein assay before determining enzymatic activity and metal content.

Enzyme Assay. (a) Standard assay: The activity of methionine synthase was determined using a nonradioactive assay described by Drummond et al. (1995). The assay mixture contained 10 mM potassium phosphate, pH 7.2; 50 mM Tris chloride, pH 7.2; 100 μM magnesium sulfate; 10 mM dithiothreitol; 2 mM homocysteine; and 66 μM (6*S*)-CH_3-H_4PteGlu$_3$ in a total volume of 400 μL, including enzyme. MetE (27−72 pmol) was added to the standard assay. The assays were started by addition of homocysteine after a 5-min preequilibration at 37 °C. After another 5 min, the reaction was quenched by addition of 100 μL of 5 N HCl/60% formic acid, followed by heating to 88 °C for 12 min. After cooling, the reaction mixtures were centrifuged at 14000*g* for 5 min to remove precipitated protein, and the A_{350} was measured. The specific activity was determined from measurements made at two different enzyme concentrations using the correction factors for the cobalamin-independent methionine synthase assay established by Drummond et al. (1995). (b) Modified enzyme assay: The activity of MetE was measured using the nonradioactive assay described above with the following modifications: The assay solution was equilibrated at 37 °C without enzyme but with homocysteine present, and the reaction was initiated by addition of MetE. The amount of enzyme added to the assay was increased 10-fold over that in the standard assay, to 450−720 pmol, and the reaction time reduced to 30 s.

Circular Dichroism Changes Associated with Thermal Denaturation of MetE. Enzyme samples were suspended in 10 mM potassium phosphate buffer, pH 7.2, containing 500 μM dithiothreitol, at a protein concentration of 3.9 μM, equivalent to an A_{222} of 0.455. For the zinc-depleted MetE, "scrubbed" buffer was used. The enzyme solutions were placed in a 1-mm path length, strain-free quartz Suprasil cuvette, and measurements were made in an Aviv Circular Dichroism Spectrometer Model 62DS. The ellipticity at 222 nm was measured at 1 °C intervals as the temperature was raised, using a bandwidth of 5 nm and an average time constant of 1 s. A temperature-equilibration time of 12 s per °C was allowed. Measurements were taken from 10 to 85 °C. Blank spectra obtained over the same temperature range with buffer alone were subtracted.

XAS Sample Preparation, Data Collection, and Analysis. Wild-type MetE was equilibrated with 25 mM triethanolamine buffer, supplemented with 500 μM dithiothreitol. Sample I was treated for 1 h with 4 equiv of CH_3-H_4PteGlu$_3$ in 10 mM potasium phosphate and 50 mM Tris chloride buffer, pH 7.2, containing 100 μM $MgSO_4$ and 10 mM dithiothreitol, in order to remove any bound homocysteine; it was then reequilibrated with 25 mM triethanolamine buffer supplemented with 500 μM dithiothreitol. Four equivalents of homocysteine were added to sample II. The protein concentration of each enzyme solution was determined, and the samples were loaded in Lucite cuvettes with 40 μm kapton windows and frozen rapidly in liquid nitrogen.

XAS spectra were collected at the SSRL, on beamline VII-3, under dedicated conditions (3.0 GeV, 100), using a Si-(220) double crystal monochromator detuned by 50% to eliminate harmonics. X-ray energies were calibrated by simultaneous measurement of the absorption spectrum of a Zn foil, with the first inflection point assigned as 9659 eV. Samples were held at 10 K during data collection using a helium flow cryostat. Spectra were measured using 10 eV steps in the pre-edge region, 0.35 eV in the edge region,

and 0.05 $Å^{-1}$ in the EXAFS region with integration times of 1 s, 1 s, and k^3 weighted times from 1 to 25 s, respectively, for a total measurement time of ca. 35 min/scan. Data were measured as fluorescence excitation spectra, using a 13-element Ge solid-state detector array. The incident count rate for each channel was held below 90 kHz to avoid saturation; the windowed Zn Kα count rate was 15−20 kHz in the EXAFS region, giving a total of approximately 4×10^6 useful counts per scan at $k = 13$ $Å^{-1}$. Each channel of each scan was examined independently for glitches. Good channels (11 channels per scan) were then averaged for each sample to give the final spectrum. Seven and eight scans, respectively, were averaged for MetE and MetE + homocysteine samples.

Edge data were normalized by fitting the data both below and above the edge to tabulated X-ray absorption cross sections (McMaster et al., 1969), using a single, low-order polynomial and a single scale factor (Waldo, 1991). EXAFS data reduction followed standard procedures for pre-edge subtraction and spline background removal (Teo, 1986). Data were converted to k space, $k = [2m_e(E - E_o)/\hbar^2]^{1/2}$, using $E_o = 9675$ eV. Fourier transforms were calculated using k^3-weighted data over a k range of 2.0−12.5 $Å^{-1}$. Fits to Fourier filtered ($R = 0.8$−2.8 Å) and unfiltered data gave the same structural parameters.

EXAFS data are described by eq 2, where $\chi(k)$ is the

$$\chi(k) = \sum_s \frac{N_s A_s(k) S}{kR_{as}^2} \exp(-2k^2 {\sigma_{as}}^2) \sin(2kR_{as} + \phi_{as}(k)) \quad (2)$$

fractional modulation in the absorption coefficient above the edge, N_s is the number of scatterers at a distance R_{as}, $A_s(k)$ is the effective backscattering amplitude, S is a scale factor, σ^2_{as} is the mean-square deviation in R_{as}, $\phi_{as}(k)$ is the phase-shift that the photoelectron wave undergoes in passing through the potentials of the absorbing and scattering atoms, and the sum is taken over all scattering interactions.

Experimental data were fit ($k = 2.5$−12 $Å^{-1}$) with theoretical EXAFS amplitude and phase functions, $A_s(k)$ and $\phi_{as}(k)$, calculated using FEFF 6.01 (Rehr et al., 1991, 1992). Single scattering parameters were calculated for Zn−O, Zn−N, and Zn−S using bond lengths of 2.00, 2.04, and 2.31 Å, respectively. The scale factors, $S = 1.02$ (S) and 0.85 (N/O), and threshold energy, $E_o = 9$ eV, were calibrated by fitting EXAFS data for crystallographically characterized zinc models (K. Clark, D. L. Tierney, K. Govindaswamy, E. Gruff, C. Kim, J. Berg, S. Koch, and J. E. Penner-Hahn, unpublished data). R and σ were varied, and N was held fixed at reasonable integer values (Clark, 1993; Garcia et al., 1996).

RESULTS

Mutation of Cys726. The Cys726Ser MetE protein was purified to homogeneity. In order to insure the identity of this inactive protein, five cycles of Edman degradation were carried out on a homogeneous sample of the mutant protein at the University of Michigan Protein and Carbohydrate Structure Facility. The sequence Met-Thr-Ile-Leu-Asn was obtained, in agreement with that found for the N-terminal end of the wild-type enzyme. A western blot was performed using polyclonal rabbit antibodies raised against the wild-type methionine synthase, and it showed a band of equal mobility to that of a wild-type sample. Preimmunized rabbit serum was used in the control, and no band was visualized.

The Cys726Ser MetE had no detectable activity using either the nonradioactive assay for methionine synthase or the radioactive assay, which relies upon the detection of the transferred radioactive methyl group from (6*S*)-$^{14}CH_3$-H_4-$PteGlu_3$ to homocysteine (González et al., 1992; Whitfield et al., 1970). This result is in agreement with previous experiments that showed that MetE loses all detectable activity upon alkylation of cysteine726. The lack of detectable activity for this mutant is especially interesting in light of the conservation of cysteine726 in deduced amino acid sequences of the cobalamin-independent methionine synthase from *Catharanthus roseus* (Eichel et al., 1995), *Haemophilus influenzae* (White, 1995), *Saccharomyces cerevisiae* (Korch et al., 1995), *Oryza sativa* (Sasaki et al., 1994), and *Methanobacterium thermoautotrophicum* (Vaupel, 1995). These sequences also contain conserved residues in the region surrounding cysteine726 at the C-terminal end of MetE, reaffirming the importance of this region in catalysis.

Determination of MetE Protein Concentration, Zinc Content, and Specific Activity. In order to determine accurately the concentration of methionine synthase in solutions, a correction factor was established for the Bio-Rad assay, which is based on the Bradford method (1976). A ratio of 1.11 ± 0.07 relates the MetE protein concentration determined by Bio-Rad assay to that obtained by amino acid analysis. ICP spectrometry demonstrated the presence of one zinc equivalent per subunit of cobalamin-independent methionine synthase. Enzyme preparations obtained from cells grown in unsupplemented Luria-Bertani medium contain an average of 0.738 ± 0.029 zinc equivalents per protein subunit and have a specific acitivity of 0.207 ± 0.006 μmol min^{-1} mg^{-1}. Zinc supplementation yields protein with an average of 1.02 ± 0.17 zinc equivalents per protein subunit and a specific activity of 0.271 ± 0.030 μmol min^{-1} mg^{-1}. ICP spectrometry on the Cys726Ser mutant protein showed 0.03 zinc equivalents per protein subunit. This sample containes concentrations of magnesium, calcium, manganese, iron, cobalt, copper, molybdenum, and cadmium that are below the limits of detection. The specific activity value for zinc-supplemented MetE preparations is higher than the values published by Whitfield et al. (1970) of 0.17 μmol min^{-1} mg^{-1} and those found previously in this laboratory, 0.15 μmol min^{-1} mg^{-1} (González et al., 1992). The observed differences in specific activity are very likely the result of zinc supplementation of the growth medium for the MetE-overproducing strain. The overproduction of MetE enzyme in the absence of zinc supplementation appears to result in a fraction of enzyme that lacks zinc, which in turn gives rise to protein of lower specific activity. Also, the results shown here take into account the correction factor for protein concentration measurements obtained by means of the Bio-Rad assay, whereas previous studies in this laboratory did not. This correction would raise the specific activity of earlier preparations in this laboratory to 0.17 μmol min^{-1} mg^{-1}.

Removal of Zinc from MetE. The method of zinc removal described here is a variation of a method developed by Whitfield and Weissbach (1970) to remove tightly bound magnesium from MetE. As shown in Table 1, ICP analysis indicates removal of zinc from MetE by treatment with urea and EDTA. When the standard assay was used to measure

Table 1: MetE Activity in Modified Assays

enzyme	standard assay (μmol min^{-1} mg^{-1})	modified assay 0.5-min incubation (μmol min^{-1} mg^{-1})	Zn per protein
MetE holoenzyme	0.297	0.282	0.83
MetE apoenzyme	0.145	<0.005	0.017
MetE[a] + 1.1 equiv zinc	0.231	0.241	1.13

[a] 40 nmol of MetE apoenzyme was incubated with 44 nmol of zinc sulfate.

the specific activity of the zinc-depleted enzyme, ~50% residual activity was observed. However, when the assay was modified to eliminate pre-equilibration of the enzyme and to reduce the assay time from 5 min to 30 s, the specific activity for the zinc-depleted enzyme dropped to undetectable levels, suggesting that zinc is required for MetE activity and that the enzyme affinity for zinc is very high. ICP results have shown that a standard reaction mixture contains 0.52−0.64 μM zinc, enough to reconstitute zinc-depleted enzyme that is present at 0.07−0.18 μM concentration in standard assays.

Reconstitution of MetE Holoenzyme by Addition of Zinc. A sample of zinc-depleted, wild-type MetE was supplemented with zinc, as described in Materials and Methods. ICP results indicated that the reconstituted enzyme contained 1.13 zinc equivalents per protein subunit, as shown in Table 1. The specific activity of the reconstituted enzyme in the modified assay was 74% that of the original holoenzyme. The observed decrease in enzymatic activity is probably due to irreversible protein damage caused by urea treatment of MetE.

Isolation of MetE from Cells Grown in Medium Supplemented with Cadmium Chloride. The overexpressing strain GW2531/pJG816 was grown in LB supplemented with 1 mM $CdCl_2$. The enzyme purified from cells grown under these conditions contained 0.44 equiv of zinc and 0.48 equiv of cadmium, as measured by ICP, and had a specific activity of 0.098 μmol min^{-1} mg^{-1}, or 36% of the specific activity of enzyme containing 1 equiv of zinc. These results, while preliminary, suggest that cadmium cannot substitute for zinc in generating active enzyme. Figure 1 shows a plot of the specific activity of different preparations of MetE vs the equivalents of zinc per mol enzyme determined by ICP; the cadmium-supplemented enzyme lies on this line.

Circular Dichroism Studies. Studies of the ellipticity at 222 nm for the wild-type and Cys726Ser MetE proteins indicated that the approximate "melting" temperatures of the proteins are very similar, with the transition half-maximal at ~55 °C (data not shown). These data suggest that the absence of zinc in the Cys726Ser mutant protein does not grossly perturb the stability of the protein and are consistent with the high level of expression of the mutant protein and its purification to homogeneity in good yield.

EXAFS Spectroscopy of MetE in the Presence and Absence of Homocysteine. There are obvious changes in both the XANES (Figure 2, inset) and the EXAFS (Figure 2) when homocysteine is added to MetE. The changes in the XANES are typical of those seen when a low-Z ligand (N or O) is replaced by a high-Z ligand such as sulfur (Clark, 1993). This is shown even more clearly by the EXAFS. There is a small increase in the average bond length and a significant increase in the EXAFS amplitude in the sample containing

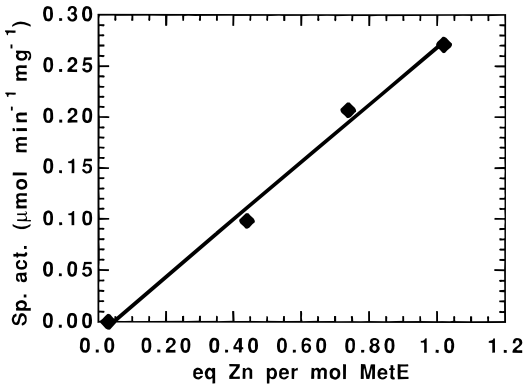

FIGURE 1: Correlation between the zinc content of preparations of MetE and the specific activity of the enzyme. In order of increasing zinc content, the preparations are as follows: Cys726Ser mutant protein, 0.03 equiv zinc; MetE from cells grown in medium supplemented with cadmium chloride, 0.44 equiv zinc + 0.48 equiv cadmium; MetE from cells grown in unsupplemented medium, 0.74 equiv zinc; and MetE from cells grown in medium supplemented with zinc, 1.03 equiv zinc.

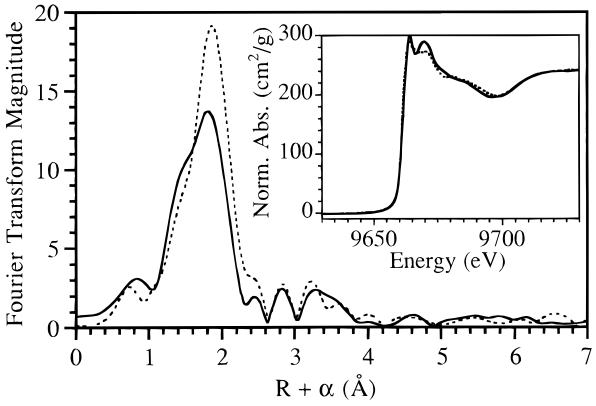

FIGURE 2: XAS data for MetE. Fourier transforms of EXAFS data are shown that have been calculated as described in Materials and Methods. The inset shows normalized XANES data. Solid line, MetE; dashed line, MetE + homocysteine.

homocysteine. In both cases, good fits to the EXAFS data could only be obtained by using two shells of scatterers, one with high Z (S or Cl) and one with low Z (N or O). The former almost certainly represents thiolate sulfur ligands. The latter could represent either imidazole nitrogen from histidines or oxygen from a variety of possible ligands (water, carboxylate, phosphate, etc.). These possibilities cannot be distinguished by EXAFS.

The fit parameters are summarized in Table S1 of the Supporting Information. For the native enzyme, slightly better fits were obtained for 2 (N/O) + 2 S ligation than for 1 (N/O) + 3 S ligation. Moreover, the latter gave Debye−Waller factors that were unreasonably small for N/O and unreasonably large for S, thus demonstrating that the 2 (N/O) + 2 S fit is the correct description of the ligation. The presence of two low-Z ligands is further supported by the observation of a low-R shoulder in the Fourier transform. This feature is generally much less pronounced in structures having only a single low-Z ligand (Garcia et al., 1996). As expected from the large changes in the Fourier transform, the addition of homocysteine gives rise to significant changes in the fitting results. The MetE + homocysteine data give significantly better fits for 1 (N/O) + 3 S than for other possible ligations, and only the 1 (N/O) + 3 S fits give

reasonable Debye−Waller factors for both the S and the N/O shells.

In addition to the first-shell peak, the EXAFS data for both MetE and MetE + Hcy show two outer-shell features that are significantly above the noise level. It is possible that these features are due to scattering from the ring carbons in one or more coordinated histidines. Alternatively, it is possible that they arise from other features within the active site, such as a coordinated phosphate or the magnesium ion required for enzyme activity. Experiments aimed at identifying the origin of these peaks are in progress. Whatever their origin, these features do not appear to change with homocysteine binding.

DISCUSSION

In enzymes, zinc ions seem to play one of two possible roles: stabilization of holoprotein structure, due to a large free energy decrease associated with the binding of zinc to the protein, or direct involvement in catalysis. Several properties make zinc an important cofactor in enzymes. First, zinc has the unique capacity to adopt four-, five-, or six-coordinate geometries, with small energetic barriers between these states. Secondly, zinc-ligand bonds are kinetically labile (Bertini et al., 1985) and can rapidly be exchanged (Bertini & Luchinat, 1994). These two properties allow zinc to bind a substrate by displacement of a solvent ligand and/or by increasing its coordination number. Zinc is also a powerful Lewis acid and can activate a substrate for nucleophilic attack, e.g., water in hydrolytic enzymes.

There is also precedence for the activation of a sulfhydryl group by a divalent zinc. In *E. coli*, the Ada protein plays an important role in the repair of alkylated DNA strands (Sedwick et al., 1988). Ada carries out demethylation reactions on O^6-methylguanines, O^4-methylthymines and S_p-methylphosphotriesters in DNA (Demple et al., 1982, 1985; McCarthy & Lindahl, 1985). When the latter reaction takes place, the Ada protein undergoes a conformational change, which gives rise to a sequence-specific, DNA-binding activity that recognizes a promoter element, "the Ada box", in this manner activating its own expression and that of other repair enzymes at the transcriptional level (Saget & Walker, 1994). The demethylation of methylphosphotriesters is catalyzed by nucleophilic attack on, and subsequent displacement of, the methyl group by the sulfhydryl group on cysteine69 of the Ada protein (Sedwick et al., 1988) and is irreversible. A divalent zinc metal in Ada is required for activity (Myers et al., 1992) and is believed to act by enhancing the nucleophilicity of the cysteine group under physiological conditions (Myers et al., 1993). The zinc is coordinated by four cysteinyl sulfurs (Myers et al., 1993) and model studies suggest that this arrangement, which imparts a net charge of −2 to the zinc center, is optimal for nucleophilic activation of the cysteine ligand (Wilker & Lippard, 1995). If zinc is coordinated to two cysteines and two histidines, the coordinated thiol is less activated for nucleophilic attack.

A second protein in which zinc may serve to activate a thiol for nucleophilic attack is rat farnesyltransferase (Andres et al., 1993). This protein catalyzes the transfer of a farnesyl group from farnesyl pyrophosphate to cysteine residues near the C terminus of acceptor proteins such as $p21^{ras}$. Zinc is essential for the binding of the peptide substrates, and it has been proposed that it coordinates the cysteine sulfhydryl group on the acceptor protein.

In an analogous manner, it can be hypothesized that the cobalamin-independent methionine synthase carries out the demethylation of 5-methyltetrahydrofolate by a direct attack of homocysteine bound transiently to a zinc ligand. The observation that the zinc environment is altered when homocysteine is added to the enzyme is consistent with such a proposal. The amino and thiol p*K* values for homocysteine are nearly equivalent and are associated with macroscopic dissociation constants of 8.87 and 10.86 (Ryklan & Schmidt, 1944). Spectrophotometric titrations have shown the concentration of thiolate and thiol to be equal at a pH of 10.0 (Benesch & Benesch, 1955). A thiolate coordinated to zinc is not expected to be as strong a nucleophile as a naked thiolate due to the decreased electronic charge on the sulfur. However, at physiological pH, where homocysteine is largely present as the thiol, the effect of ligation to zinc should more than compensate for the decrease in the nucleophilic character of the coordinated thiolate. Recent calculations have suggested that water is activated by zinc in a similar manner (Bertini, 1990).

The Ada protein is catalyzing a thermodynamically less-challenging reaction than MetE by virtue of having a better leaving group, namely, the phosphate diester. The cobalamin-dependent methionine synthase, on the other hand, possesses a very powerful nucleophile, cob(I)alamin, that enables it to displace a methyl group attached to a much poorer leaving group, a secondary amine. While it remains to be elucidated how the cobalamin-independent methionine synthase is able to carry out the same overall reaction, the finding of a zinc cofactor opens the possibility for this enzyme to catalyze a difficult reaction by simultaneously activating the nucleophilic agent (homocysteine) by deprotonation, and the methyl donor (methyltetrahydrofolate) by protonation.

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SUPPORTING INFORMATION AVAILABLE

Plots of EXAFS and best fits and table of curve fitting results (3 pages). Ordering information is given on any current masthead page.

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