Cobalamin-Dependent Methionine Synthase from *Escherichia coli:* Involvement of Zinc in Homocysteine Activation†

Celia W. Goulding‡ and Rowena G. Matthews*,‡

Biophysics Research Division and Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan, 48109-1055

Received August 11, 1997; Revised Manuscript Received October 13, 1997⊗

ABSTRACT: Methionine synthase (MetH) is a modular protein with at least four distinct regions; amino acids 2−353 comprise a region responsible for binding and activation of homocysteine, amino acids 345−649 are thought to be involved in the binding and activation of methyltetrahydrofolate, amino acids 650−896 are responsible for binding of the prosthetic group methylcobalamin, and amino acids 897−1227 are involved in binding adensylmethionine and are required for reductive activation of enzyme in the cob(II)-alamin form. Previous studies have shown that mutations of Cys310 or Cys311 to either alanine or serine result in loss of all detectable catalytic activity. These mutant proteins retain the ability to catalyze methyl transfer from methyltetrahydrofolate to exogenous cob(I)alamin, but have lost the ability to transfer methyl groups from exogenous methylcobalamin to homocysteine [Goulding, C. W., Postigo, D., and Matthews, R. G. (1997) *Biochemistry 36*, 8082−8091]. We now demonstrate that both MetH holoenzyme and a truncated MetH(2−649) protein, which lacks a cobalamin prosthetic group, contain 0.9 equiv of zinc, while the Cys310Ser and Cys311Ser mutant proteins contain less than 0.05 equiv of zinc. Addition of L-homocysteine to MetH(2−649) is accompanied by release of 1 equiv of protons/mol of protein, while addition of L-homocysteine to the Cys310Ser and Cys311Ser mutant truncated proteins does not result in proton release. The Cys310Ala and Cys311Ala mutant methylcobalamin holoenzymes have completely lost the ability to transfer the methyl group from methylcobalamin to homocysteine, suggesting that zinc is required for this reaction. Further evidence that zinc is required for catalytic activity comes from experiments in which the zinc is removed from MetH(2−1227). Removal of zinc from methylated wild-type holoenzyme by treatment with methyl methanethiolsulfonate and then with dithiothreitol and EDTA results in loss of the ability of the protein to catalyze methyl transfer from methyltetrahydrofolate to homocysteine. Reconstitution of the zinc-depleted holoenzyme results in incorporation of 0.4 equiv of zinc/mol of protein and partial restoration of the ability of the protein to catalyze homocysteine methylation.

Cobalamin-dependent methionine synthase (MetH) from *Escherichia coli* catalyzes three different methyl transfer reactions (Figure 1). In primary turnover, MetH catalyzes methyl transfer from enzyme-bound methylcobalamin to homocysteine to yield cob(I)alamin and methionine. Cob(I)-alamin is remethylated by methyltetrahydrofolate to regenerate methylcobalamin and produce tetrahydrofolate (*1*). Every 100−2000 turnovers cob(I)alamin undergoes oxidation to the inactive cob(II)alamin form of the cofactor. Cob(II)alamin must be reductively remethylated to place the cofactor back into primary turnover in the methylcobalamin form; in *E. coli*, reduced flavodoxin provides electrons, and adenosylmethionine (AdoMet)[1] is required as the methyl donor. The enzyme also catalyzes methyl transfer from exogenous methylcobalamin to homocysteine and from methyltetrahydrofolate to exogenous cob(I)alamin. These methyl transfer reactions are first-order in exogenous cobalamin and do not involve the bound cobalamin prosthetic group (*1*−*3*).

Methionine synthase is a monomeric protein containing 1227 amino acids residues and has a modular construction. The N-terminal module containing residues 2−353 can be expressed independently, and retains the ability to catalyze methyl transfer from exogenous methylcobalamin to homocysteine (*1*). This fragment shows significant sequence similarity to betaine-homocysteine methyltransferase (*4*). A fragment containing residues 2−649 catalyzes methyl transfer from exogenous methylcobalamin to homocysteine and from methyltetrahydrofolate to exogenous cob(I)alamin. Residues 354−649 exhibit significant sequence similarity to a corrinoid/iron-sulfur protein methyltransferase from *Clostridium thermoaceticum* (*5*), and this similarity has led us to postulate that this region contains the determinants for binding and activation of methyltetrahydrofolate. The third module, containing residues 650−896, binds the endogenous cobal-

† This research has been supported in part by NIH Research Grant R37 GM24908 (R.G.M.).

* Corresponding author, Department of Biological Chemistry. Correspondence should be directed to this author at: Biophysics Research Division, 4028 Chemistry Building, University of Michigan, 930 N. University Ave., Ann Arbor, MI 48019-1055. Fax: (313) 764-3323. Phone: (313)764-9459. E-mail: rmatthew@umich.edu.

‡ Biophysics Research Division.

⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1997.

[1] Abbreviations: AdoMet, *S*-adenosylmethionine; Hcy, L-homocysteine; H_4folate, 5,6,7,8-tetrahydrofolate; CH_3-H_4folate, 5-methyltetrahydrofolate; Tris, tris(hydroxymethyl)aminomethane; H_4MPT, tetrahydromethanopterin; CH_3-H_4MPT, methyltetrahydromethanopterin; HS-CoM, coenzyme M or 2-mercaptoethanesulfonate; CH_3-S-CoM, methyl-coenzyme M or 2(methylthio)ethanesulfonate; DTNB, dithiobis-(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; MMTS, methyl methanethiolsulfonate; PAR, 4-(2-pyridylazo)resorcinol; PMPS, *p*-hydroxymercuriphenylsulfonic acid; ICP, inductively coupled plasma-atomic emission spectrometry; EXAFS, extended X-ray absorption fine structure analysis.

S0006-2960(97)01988-0 CCC: $14.00

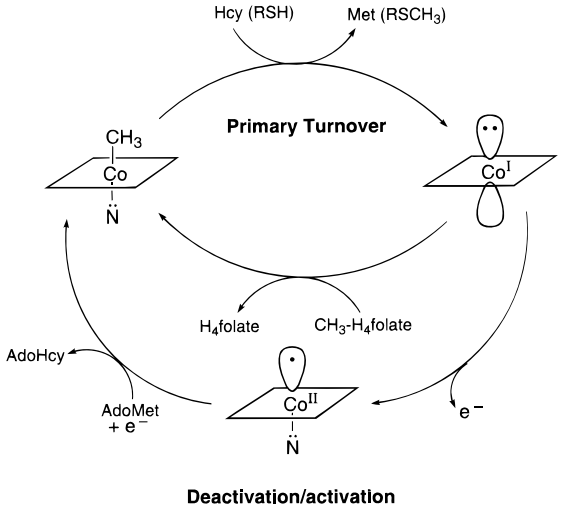

FIGURE 1: Schematic summary of the reactions carried out by methionine synthase. In the catalytic cycle, cobalamin-dependent methionine synthase transfers a methyl group from methylcobalamin to homocysteine, forming methionine and cob(I)alamin; the cob(I)alamin is remethylated by CH_3-H_4folate to produce H_4folate. Every 100−2000 turnovers, the cob(I)alamin species becomes oxidized to the inactive cob(II)alamin form (deactivation). In *E. coli*, return of the cob(II)alamin form to the catalytic cycle requires a reductive methylation to regenerate methylcobalamin (activation), in which an electron is supplied by reduced flavodoxin and a methyl group by AdoMet.

amin cofactor (*6*), while the fourth module, comprising residues 897−1227, binds adenosylmethionine and is required for reductive activation (*7*). The X-ray crystal structures of the cobalamin-binding region (*8*) and the AdoMet binding domain (*9*) have been solved recently, whereas the structure of the 71 kDa N-terminal substrate-binding region has yet to be elucidated.

Since fragments of MetH that lack a binding site for the cobalamin prosthetic group retain the ability to transfer methyl groups to and from exogenous cobalamin, one may infer that homocysteine and methyltetrahydrofolate must be activated for methyl transfer by residues contained in the 71 kDa N-terminal substrate-binding region. *E. coli* cells produce a second enzyme that catalyzes methyl transfer from methyltetrahydrofolate polyglutamates to homocysteine, cobalamin-independent methionine synthase, or MetE (*10*). This enzyme exhibits no sequence similarities with MetH (*11*), but has been shown to require 1 equiv of zinc for catalytic activity (*12*). Fourier-transformed EXAFS spectra are best fitted on the assumption that the zinc is coordinated to two sulfur ligands and two oxygen or nitrogen ligands in the MetE enzyme as isolated. Addition of homocysteine to the enzyme results in a change in the coordination of the zinc (*12*), and the spectrum is best fitted on the assumption that the zinc is now coordinated to three sulfur ligands. Mutation of the conserved cysteine residue Cys 726 to serine leads to loss of catalytic activity and of enzyme-bound zinc. Given the observation of conserved cysteines in the N-terminal module of MetH, Cys310, and Cys311, which when mutated result in loss in catalytic activity (*1*), it seemed possible that the two enzymes might employ similar catalytic strategies to activate their homocysteine substrate for methyl transfer, even in the absence of detectable sequence similarity.

In this paper, we investigated whether zinc is required for catalytic activity of MetH, and if so, whether the zinc is required specifically for the binding and activation of homocysteine. We have used inductively coupled plasma emission spectroscopy (ICP) to demonstrate that both wild-type MetH holoenzyme and the truncated MetH(2−649) fragment contain 1 equiv of enzyme-bound zinc/ mol of protein, whereas the Cys310Ala and Cys311Ala mutant proteins contain no significant enzyme-bound zinc. Our studies suggest that zinc is required for binding of homocysteine as a thiolate anion and for catalysis of methyl transfer from methylcobalamin to homocysteine and support the idea that the catalytic strategies employed by cobalamin-independent and cobalamin-dependent methionine synthase for activation of homocysteine are remarkably similar.

MATERIALS AND METHODS

Expression and Purification of Wild-Type and Mutant Methionine Synthase Holoenzymes and MetH(2−649) Proteins. The recombinant wild-type methionine synthase MetH(2−1227) from *E. coli* K-12 strain XL1-Blue/pMMA-07 was overproduced and purified as described previously (*13*). Construction, expression, and purification of mutant methionine synthase proteins, MetH(2−649) and mutant MetH(2−649), were previously described (*1*). For preparations in metal-supplemented media, zinc chloride or cadmium chloride was added to a final concentration of 0.5 mM.

Metal Content Determination by ICP. Samples were prepared as described earlier (*12*) and were sent to the University of Michigan Department of Geological Sciences or the Garratt-Callahan Company (Millbrae, CA) to determine the metal content by ICP.

Spectroscopic Metal Content Determination. The zinc content was determined from the absorption change at 500 nm associated with a zinc complex formed with 4-(2-pyridylazo)-resorcinol (PAR) as enzyme-bound zinc was released from the enzyme by the addition of *p*-hydroxymercuriphenylsulfonic acid (PMPS) according to the protocol of Giedroc et al. (*14*). A 15 μM sample of enzyme in 50 mM Tris chloride buffer, pH 7.2/0.4 M NaCl/0.1 mM PAR, was titrated with 1 mM PMPS in the same buffer/NaCl/PAR to release all cysteine-bound metal. The titrant was added using plastic pipette tips and not a metal syringe needle, as divalent metals in the syringe needle complex with PAR and perturb the result. The titration was judged to be complete when no further change at 500 nm was observed. The concentration of the $Zn(PAR)_2$ complex was determined assuming an extinction coefficient for $Zn(PAR)_2$ at 500 nm of 66 000 M^{-1} cm^{-1} (*15*).

Determination of Proton Release in Equilibrium Titrations. Proton release accompanying the binding or reaction of homocysteine with MetH(2−649) protein was monitored by titration in the presence of phenol red. The methods used for the proton-release experiments and for the calculation of results have been reported in the preceding paper in this issue (*16*). When truncated MetH proteins, lacking a cobalamin binding region, are used, the analysis of proton release is simplified because the absorbance changes due to proton release do not have to be corrected for changes in the cobalamin spectra.

Homocysteine-Methylcobalamin Methyltransferase Assay in the Presence of EDTA. The reaction rates were monitored spectrophotometrically in the dark at 37 °C using a procedure modified from an earlier paper (*1*). The MetH(2−353)

protein (500 μM) was preincubated with 0−1.2 mM EDTA (pH 7.2) for 10 min at 37 °C before initiating the methyl transfer reaction with the enzyme/EDTA solution instead of with homocysteine.

Methyltetrahydrofolate-Homocysteine Methyl Transferase Assay. Methionine synthase activity was determined using a nonradioactive assay that measures the conversion of CH_3-H_4 folate to H_4folate by derivatization of the latter to form CH^+═H_4folate. This assay has previously been described *(17)*.

Zinc Removal from MetH(2−1227). The preparation of zinc-depleted methylated MetH(2−1227) protein was modified from a method described by Qui et al. (*18*), which consists of *S*-methylation of the cysteine residues of the protein, removal of zinc with EDTA, and then removal of the *S*-methyl groups with dithiothreitol. The cobalamin of MetH(2−1227) protein was methylated as previously described (*19, 20*). Methylated MetH(2−1227) protein was exchanged into 50 mM Tris chloride buffer, pH 7.2, and was incubated with 20 equiv of MMTS at 4 °C. After 1 h, the *S*-methylated protein was dialyzed against 50 mM Tris chloride, pH 7.2, and 1 mM EDTA at 4 °C for 24 h, and the protein was then dialyzed against 2 mM DTT and 50 mM Tris chloride buffer at pH 7.2 to remove the methyl groups from the cysteines and to ensure that they were fully reduced (*21, 22*). The zinc-depleted methylated MetH(2−1227) was then washed three times with metal-free Tris chloride buffer, pH 7.2, using Centricon 30 concentrators.

Zinc Reconstitution of the Zinc-Depleted MetH(2−1227) Protein. Zinc chloride (1.5 M equiv) was added to a zinc-depleted methylated MetH(2−1227) protein solution (100 μM) containing 1 M urea and 2 mM dithiothreitol. The enzyme solution was dialyzed against 50 mM Tris chloride for 48 h. To ensure that all nonspecifically bound zinc was removed, the enzyme solution was passed through a 400 μl Chelex 100 column (Bio-Rad) that had been equilibrated with 50 mM Tris chloride buffer, pH 7.2. The enzyme solution was eluted with the same buffer, and the pink fractions were collected and concentrated in a Centricon 30 concentrator. The concentration of methylated MetH(2−1227) was determined spectroscopically by monitoring the absorbance of the enzyme-bound methylcobalamin at 525 nm using the extinction coefficient of 8910 M^{-1} cm^{-1} (*23*). The amount of enzyme-bound zinc was determined using the colorimetric PMPS/PAR assay described earlier in this section.

Quantitation of Thiols. The number of sulfhydryl groups in MetH(2−1227), zinc-depleted MetH(2−1227), and reconstituted MetH(2−1227) were determined by a modification of the method described by Guo and co-workers, using a colorimetric assay (*22*). In a cuvette, 400 μL of protein samples (10−15 μM) in 50 mM Tris chloride buffer, pH 7.2, containing 5 M urea/2.5 mM EDTA/100 mM KCl, were mixed with 10 μL of 8 mM DTNB. The reaction of DTNB with the denatured protein was monitored at 412 nm for 10 min. The concentration of 2-nitro-5-thiobenzoate anions released on reaction with reduced thiol groups on the protein was determined using the extinction coefficient at 412 nm of 14 150 M^{-1} cm^{-1} (*24*), after subtraction of the absorbance obtained for the buffer/urea/EDTA/KCl mixture alone.

Stopped-Flow Studies of Turnover and Demethylation of Methylated MetH(2−1227) by Homocysteine. The rate of approach to steady state and the turnover number were determined by stopped-flow spectroscopy at 25 °C under anaerobic conditions, as described earlier (*23*). The methylcobalamin holoenzyme was reacted with homocysteine (100 μM) and (6-*R*,*S*)-CH_3-H_4folate (1 mM) at 25 °C, with both the enzyme and the substrates in 50 mM Tris chloride, pH 7.2, and 100 mM KCl. The reaction was followed at 525 nm to observe the demethylation of methylcobalamin. The initial rate of cob(I)alamin formation and the turnover number were calculated as previously described (*23, 25*).

The rate of demethylation of methylated holoenzyme by homocysteine was determined at 25 °C under aerobic conditions. Methylcobalamin enzyme (10 μM) in 50 mM Tris chloride buffer, pH 7.2, 100 mM KCl was reacted with homocysteine (100 μM) in 50 mM Tris chloride buffer, pH 7.2, 100 mM KCl. The formation of cob(I)alamin and subsequent oxidation to cob(III)alamin were observed at 386 nm. The data points were fitted to an equation with two exponential terms (eq 1), where k_1 is the rate constant for

$$\Delta A_{386\ nm} = A + B_{exp}^{(-k_1 t)} + C\ exp^{(-k_2 t)}$$

the formation of cob(I)alamin, and k_2 is the rate constant for the oxidation of cob(I)alamin to cob(III)alamin. The assigned rate constants, k_1 and k_2, and constants *A*, *B*, and *C* are adjusted in stimulations to obtain the values that best fit the data.

RESULTS

Determination of the Zinc Content of Wild-Type and Mutant MetH Proteins. Recent studies on cobalamin-independent methionine synthase (MetE) have shown that zinc is required for catalytic activity and have suggested that homocysteine is activated for nucleophilic attack on methyltetrahydrofolate by coordination to zinc in its thiolate form (*12*). At physiological pH, homocysteine, which has a microscopic pK_a of 10.0 for the thiol group (*26*), is largely present in the thiol form, so the deprotonation of the thiol to form a thiolate coordinated to zinc should enhance the nucleophilic character of homocysteine. In cobalamin-dependent methionine synthase (MetH), homocysteine could possibly be activated in the same manner to accept a methyl group from endogenous methylcobalamin. Therefore, the zinc content of MetH(2−1227) and MetH(2−649) protein were determined by ICP (Table 1). For cells grown in unsupplemented medium, holoenzyme MetH(2−1227) and MetH(2−649) contain 0.7−0.8 M equiv of zinc/mol of protein present. When cells are grown in $ZnCl_2$ supplemented media, as described in Materials and Methods, holoenzyme MetH(2−1227) and MetH(2−649) contain 0.9−1.0 M equiv of zinc/mol of protein. In contrast, the Cys310Ser and Cys311Ser MetH(2−1227) and MetH(2−649) mutant proteins contain ≤0.06 equiv of zinc/mol of protein, as does the wild-type MetH(2−353) truncated protein.

Measurement of Proton Release Associated with Binding of Homocysteine to Wild-type and Mutant MetH (2−649) Proteins. When homocysteine is added to MetH(2−649) protein in the presence of phenol red, there is a decrease in absorbance at 560 nm due to proton release from the enzyme: homocysteine complex and the subsequent protonation of phenol red. Averaging data from four experiments conducted at pH ∼7.7 with enzyme from cells grown in zinc-supplemented medium, this change in absorbance corre-

Table 1: Results of ICP and Colorimetric Assays of MetH Zinc Content

holoenzymes	Zn per mol of protein	truncated enzymes	Zn per mol of protein
MetH(2−1227) holoenzyme	0.69	MetH(2−649) protein	0.82
MetH(2−1227) holoenzyme[a]	0.89	MetH(2−649) protein[b]	0.97
MetH(2−1227) Cys310Ser	0.06	MetH(2−649) Cys310Ala	0.01
MetH(2−1227) Cys311Ser	0.05	MetH(2−649) Cys311Ala	0.01
zinc-depleted MetH(2−1227)	0.04	zinc-depleted MetH(2−649)	0.02
		MetH(2−353) protein	0.04

[a] Isolated from cells grown in glucose minimal MOPS medium supplemented with 500 μM $ZnCl_2$ [b] Isolated from cells grown in Luria-Bertani medium supplemented with 500 μM $ZnCl_2$.

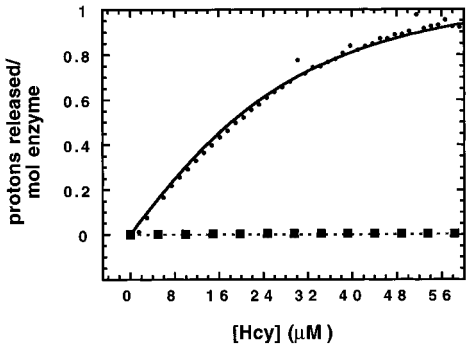

FIGURE 2: Proton release associated with binding of homocysteine to wild-type and mutant MetH(2−649) proteins at pH 7.7. Titration of wild-type MetH(2−649) with L-homocysteine is depicted by the filled circles and resulted in release of 1.0 equiv of protons/mol of protein. The data points were fitted to a quadratic binding curve with the enzyme concentration fixed at 25 μM and gave an apparent K_d of 9 μM. When homocysteine was titrated into solutions of the Cys310Ala (large solid square) and Cys311Ala (small solid square) mutant proteins, no proton release was observed. Enzyme (25 μM) in 50 μM potassium phosphate buffer, initially at pH 7.2/100 mM NaCl/40 μM phenol red, was transferred to an anaerobic cuvette, degassed, and then titrated with a homocysteine solution in the same buffer. The release of protons was determined from the increase in absorbance of phenol red at 560 nm, as described in the preceding paper in this issue (*16*). The actual pH range during the titration can be determined from the absorbance of the phenol red; in the experiment shown the pH decreased from 7.68 to 7.64.

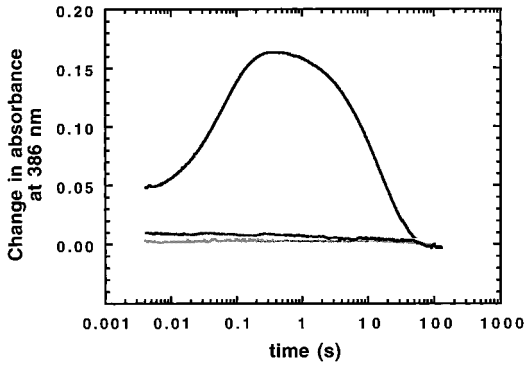

FIGURE 3: Stopped-flow measurements of methyl transfer from methylated MetH(2−1227) to L-homocysteine under aerobic conditions at 25 °C. Enzyme in the methylcobalamin form (~10 μM) was mixed with 100 μM homocysteine. The cob(I)alamin concentration was monitored at 386 nm; the absorbance increases as the demethylation of methylated enzyme to cob(I)alamin enzyme occurs, and then the oxidation of cob(I)alamin enzyme to cob(III)alamin enzyme results in a decrease in absorbance at 386 nm. The black curve shows the demethylation of MetH(2−1227) grown in zinc-supplemented glucose minimal MOPS medium; the data have been fitted to a biexponential rate equation as described in the text. The two curves that show no demethylation present data recorded for the Cys310Ala (lower trace) and Cys311Ala mutant MetH(2−1227) (upper trace) proteins.

sponds to the release of 0.9 ± 0.2 protons, and the quadratic binding curve fits yield a K_d(observed) of ~7 ± 3 μM, in good agreement with measurements of homocysteine binding to MetH(2−1227) (*16*). The observed release of a proton suggests that the enzyme-bound zinc binds the homocysteine thiolate anion at pH 7.7, despite the relatively high microscopic pK_a of 10.0 measured for the homocysteine thiol (*26*). The apparent K_d for homocysteine is also very similar to that observed for MetH(2−1227) holoenzyme at pH 7.8 (*16*). The Cys310Ala MetH(2−649) and Cys311Ala MetH(2−649) mutant proteins do not catalyze methyl transfer from exogenous methylcobalamin to homocysteine (*1*), and by metal analysis they contain only 0.05 equiv of zinc/mol of protein. Due to the lack of enzyme-bound zinc in the MetH(2−649) Cys310Ala and Cys311Ala mutant proteins, one might expect that there would be no proton release upon homocysteine binding to these mutant proteins. Indeed, upon addition of homocysteine to the Cys310Ala and Cys311Ala MetH(2−649) mutants, no proton release is observed (Figure 2).

Effect of Cys310Ser and Cys311Ser MetH(2−1227) Mutants on the Demethylation of Methylated Enzyme. In previous work, we have shown that the methyl transfer from exogenous methylcobalamin to homocysteine is not catalyzed by Cys310Ala and Cys311Ala MetH(2−649) mutants and mutants of MetH(2−1227) in which Cys310 or Cys311 are replaced by either alanine or serine are not catalytically active (*1*). We now show that the rate of demethylation of the methylcobalamin prosthetic group of MetH(2−1227) protein by homocysteine is affected by the Cys310Ser and Cys311Ser mutations.

By stopped-flow analysis, we followed the demethylation of the wild-type methylcobalamin form of MetH(2−1227) by homocysteine under aerobic conditions (Figure 3). We monitored the reaction at 386 nm in a stopped-flow spectrophotometer after mixing 9 μM methylated enzyme with 100 μM homocysteine. At 386 nm, we observed the formation of cob(I)alamin and then oxidation of cob(I)alamin to cob(III)alamin. The spectrum of the final product was that of MetH(2−1227) in the cob(III)alamin form. The rate constant for the demethylation of methylcobalamin by homocysteine in this experiment is 11.3 s^{-1}, and approximately 85% of the methylated holoenzyme was demethylated before cob(I)alamin began to oxidize to cob(III)alamin. The rate constant varies somewhat from one experiment to another, with a range of values 8.5−15 s^{-1} seen with five different enzyme preparations. We observed no demethylation of the methylated Cys310Ser and Cys311Ser MetH(2−1227) mutant proteins with homocysteine over 30 min, confirming that the cysteine mutations abolish the demethylation of the endogenous methylcobalamin cofactor, as well as the demethylation of exogenous methylcobalamin.

The rate constant for demethylation of the wild-type enzyme measured in these experiments is ~10-fold slower than the rate constant of 140 s^{-1} reported by Banerjee et al. (*27*). In that study, the stopped-flow experiments were carried out in 10 mM potassium phosphate buffer, pH 7.2, and 25 mM dithiothreitol, under anaerobic condtions. Due to high zinc contamination in potassium phosphate buffer, all our samples were exchanged into metal-free Tris chloride buffer, pH 7.2, in the absence of dithiothreitol. The buffer exchange does not result in loss of zinc, since all the protein samples analyzed for zinc content in Table 1 had undergone a similar exchange procedure. To ensure that the slow rate constants observed in Tris buffer were not simply a consequence of the use of inactive preparations of enzyme, samples of the same batch of MetH(2−1227) were exchanged either into 10 mM phosphate buffer/25 mM dithiothreitol or into Tris chloride buffer/100 mM KCl and then were demethylated. The rate constant for demethylation of methylated MetH(2−1227) by homocysteine in phosphate buffer was 127 s^{-1}, in good agreement with the previous study (*27*), while the rate constant for demethylation in Tris buffer was 8.5 s^{-1}. It should be noted that phosphate buffer also stimulates the rate of methyl transfer to homocysteine in cobalamin-independent methionine synthase (*10*, *11*).

Effect of EDTA on the Methyl Transfer from Exogenous Methylcobalamin to Homocysteine Catalyzed by MetH(2−353) Protein. The proton release experiments with MetH(2−649) protein and Cys310Ser and Cys311Ser MetH(2−649) mutant proteins imply that zinc is required for deprotonation of homocysteine on binding to the enzyme. Furthermore, the Cys310Ala and Cys311Ala MetH(2−1227) mutant proteins do not undergo demethylation in the presence of homocysteine, and they both lack enzyme-bound zinc. MetH(2−353) protein has been shown previously to catalyze methyl transfer from exogenous methylcobalamin to homocysteine, yielding methionine and cob(III)alamin under aerobic conditions (*1*). The ICP results show that purified MetH(2−353) contains 0.04 equiv of zinc/mol protein (Table 1). Since MetH(2−353) is the module required for homocysteine binding, we suspected that its activity measured under assay conditions was achieved by scavenging zinc from the assay solution. The zinc content of our methylcobalamin-homocysteine methyl transfer assay solution is 3−5 μM, as measured using PAR, while the concentration of enzyme in these assays is 5 μM. If zinc is weakly bound to MetH(2−353), then one would expect the metal chelator EDTA to inhibit the ability of MetH(2−353) protein to catalyze methyl transfer from exogenous methylcobalamin to homocysteine.

A series of experiments were carried out using 5 μM MetH(2−353) protein to catalyze methyl transfer from exogenous methylcobalamin (0.25 mM) to homocysteine (0.5 mM) in the presence of 0−10 μM EDTA, as described in Materials and Methods. As the amount of EDTA increased in the assay mixture, the second-order rate constant for the demethylation of methylcobalamin decreased (Figure 4). At 10 μM EDTA, the methyl transfer reaction rate of MetH(2−353) is reduced 10-fold. We infer that EDTA is chelating any zinc in the reaction solution, and therefore preventing the binding of zinc to MetH(2−353) and rendering MetH(2−353) inactive. MetH(2−353) appears to bind zinc more weakly than MetH(2−649) so that enzyme-bound zinc is lost during purification.

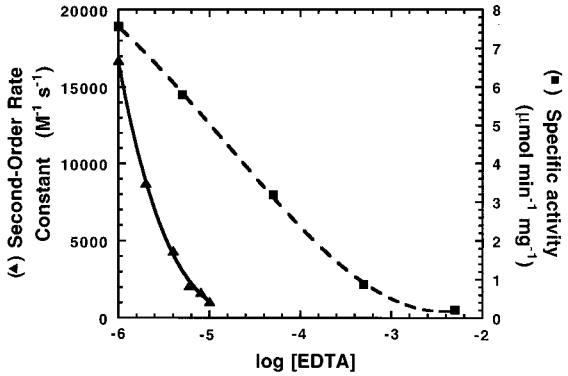

FIGURE 4: The effect of EDTA on the rate of methyl transfer from exogenous methylcobalamin to homocysteine catalyzed by MetH(2−353) and on the specific activity of wild-type MetH(2−1227). (▲) The demethylation of methylcobalamin (0.25 mM) by homocysteine (0.5 mM) was catalyzed by 5 μM MetH(2−353) with 0−10 μM EDTA present in the assay mixture. The inital rates of reaction were calculated as described previously (*1*), and the observed second-order rate constants are plotted versus the log of the concentration of EDTA in the assay. (■) Assays were carried out using 4.6 nM enzyme as described in Materials and Methods and were conducted in the presence of 0−5 mM EDTA. The specific activity of wild-type MetH(2−1227) is plotted versus the log of the concentration of EDTA in the assay.

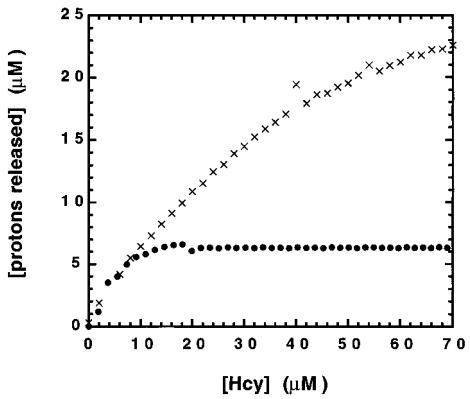

FIGURE 5: Proton release associated with binding of L-homocysteine to MetH(2−353) protein (●). MetH(2−353) protein, 22 μM, in 50 μM potassium phosphate, pH 7.2/100 mM NaCl/40 μM phenol red, was degassed in an anaerobic cuvette and then titrated with homocysteine made up in the same buffer. The release of protons was determined from the increase in absorbance of phenol red at 560 nm as described in the preceding paper in this issue (*16*). The final concentration of protons released was 6 μM. Proton release due to the titration of 25 μM MetH(2−649) with homocysteine is shown for comparison (×).

Proton Release Associated with the Addition of Homocysteine to MetH(2−353). Although no zinc is bound to MetH(2−353), if MetH(2−353) is scavenging zinc from the surrounding solution, one should observe proton release upon addition of homocysteine to MetH(2−353). However, if the concentration of MetH(2−353) is greater than the concentration of zinc in the assay mixture, only substoichiometric proton release should be observed. The proton release experiment shown in Figure 5 was performed with 22 μM MetH(2−353). Initial aliquots of homocysteine added resulted in proton release, but after the addition of 10 μM homocysteine, proton release was no longer observed. The decrease in absorbance at 560 nm, due to the change in absorbance of phenol red, was equivalent to 6 μM protons being released into solution, indicating that MetH(2−353) had scavenged 6 μM zinc. Analysis of several buffer samples used for proton release experiments, using PAR,

indicated 6 ± 2 μM zinc was present.

Effect of EDTA on the Specific Activity of Holoenzyme. Since EDTA inhibited the catalytic activity of MetH(2−353), we wanted to determine whether EDTA would also inhibit the holoenzyme in primary turnover. The standard methyltetrahydrofolate-homocysteine methyltransferase assay contains 4.6 nM MetH(2−1227) holoenzyme and this assay was performed in the presence of 0−5 mM EDTA. The specific activity of MetH(2−1227) is decreased as the concentration of EDTA in the assay increases, and the specific activity of MetH(2−1227) in the presence of 1 mM EDTA is reduced 55-fold (Figure 4).

Isolation of MetH(2−1227) from Cells Grown in Medium Supplemented with Cadmium Chloride. The overexpressing strain XL1-Blue/pMMA-07 was grown in B_{12}-supplemented minimal medium (*13*) containing 500 μM $CdCl_2$. Metal determination by ICP showed that MetH(2−1227) isolated from cells grown in cadmium-supplemented medium contained 1.1 equiv of cadmium/mol of protein, and it also contained 0.1 equiv of zinc/mol of protein. The specific activity of MetH(2−1227) purified under these conditions was 4.6 μmol min^{-1} mg^{-1}, which is a 60% reduction in specific activity compared to MetH(2−1227) grown in zinc-supplemented medium.

Effect of EDTA on the Demethylation of Methylated MetH(2−1227) by Homocysteine and the Effect of Cd-Substituted Enzyme. We observed the rate of demethylation of methylated MetH(2−1227) by monitoring the reaction at 386 nm in a stopped-flow spectrophotometer after mixing 10 μM methylated enzyme with 200 μM homocysteine under aerobic conditions (Figure 6A). When 300 μM EDTA is added to the methylated holoenzyme mixture, the maximum concentration of cob(I)alamin formed is reduced (Figure 6A), but the rate constant for its formation is approximately the same (Figure 6B). These results suggest that EDTA removes zinc from the holoenzyme, but does not significantly affect the rate constant for demethylation of residual zinc-replete methylated holoenzyme by homocysteine.

The holoenzyme isolated from cells grown in cadmium-supplemented medium shows different characteristics than the holoenzyme from cells grown in zinc-supplemented medium. The cadmium-substituted holoenzyme contains 1.1 equiv of cadmium and 0.1 equiv of zinc/mol of protein. The rate of formation of cob(I)alamin in MetH(Cd) (rate constant of 0.84 s^{-1}) is considerably slower than that of methylated MetH(Zn) in the presence of homocysteine (Figure 6B). The rate constant for the demethylation of methylated MetH(Cd) is sufficiently slow that oxidation of cob(I)alamin to cob(III)alamin (rate constant of 0.15 s^{-1}) competes with formation of cob(I)alamin, which explains why one observes only 50% cob(I)alamin during the demethylation of methylated MetH(Cd) protein (Figure 6A). The final spectrum of the methylated MetH(Cd) enzyme reacted with homocysteine is ∼85% cob(III)alamin, indicating that at least 85% of methylated MetH(Cd) undergoes demethylation by homocysteine. Sulfur ligands bind cadmium more tightly than zinc; one may assume that the dissociation of the homocysteine thiolate ion from cadmium is slower than that from zinc and gives rise to the observed 12-fold reduction in the rate constant for demethylation of methylated MetH(Cd) by homocysteine. There is no fast phase in the demethylation of MetH(Cd), indicating that the 0.1 equiv of zinc detected by ICP are not bound at the active site of the enzyme.

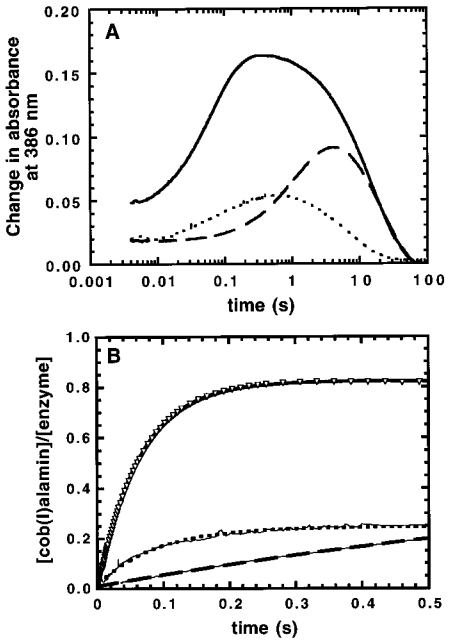

FIGURE 6: Stopped-flow studies of methyl transfer from methylated MetH(2−1227) to L-homocysteine under aerobic conditions at 25 °C. Enzyme in the methylcobalamin form (∼10 μM) was mixed with 100 μM homocysteine. (A) The cob(I)alamin concentration was monitored at 386 nm; absorbance at 386 nm is plotted versus time. One observes an increase in absorbance as the demethylation of methylated enzyme to cob(I)alamin enzyme occurs, and then the oxidation of cob(I)alamin enzyme to cob(III)alamin enzyme results in a decrease in absorbance at 386 nm. The curves show the demethylation of MetH(2−1227) isolated from cells grown in zinc-supplemented medium (—), MetH(2−1227) from cells grown in cadmium-supplemented medium (− − −) and MetH(2−1227) incubated with EDTA (300 μM) (···). The data points have been fitted to a bi-exponential rate equation as described in Materials and Methods. (B) Comparison of the initial rates of demethylation of methylated enzyme by homocysteine. The observed changes in absorbance at 386 nm under aerobic conditions were normalized using the absorbance increase at 386 nm determined by complete demethylation of the methylated enzyme to cob(I)alamin enzyme under anaerobic conditions; absorbance at 386 nm is plotted as a linear function of time. The curves show the demethylation of MetH(2−1227) isolated from cells grown in zinc-supplemented medium (—), MetH(2−1227) from cells grown in cadmium-supplemented medium (− − −), and MetH(2−1227) after incubation with 300 μM EDTA (···). The normalized data were fitted to single exponential equations.

Effects of Zinc Removal and Reconstitution on the Specific Activity of MetH(2−1227). The addition of MMTS to MetH(2−1227) was followed by dialysis with EDTA and dithiothreitol, resulting in a zinc-depleted MetH(2−1227) enzyme. Metal determination by ICP showed only 0.06 equiv of zinc/protein. The specific activity of zinc-depleted MetH(2−1227) in the standard enzyme assay is reduced by 80% from 10.1 μmol min^{-1} mg^{-1} to 2.1 μmol min^{-1} mg^{-1} for the zinc-depleted MetH(2−1227). Residual activity is assumed to reflect scavenging of zinc from the assay mixture. Reconstitution of zinc-depleted MetH(2-1227) by dialysis against zinc chloride, urea, and dithiothreitol resulted in incorporation of 0.37 equiv of enzyme-bound zinc/mol of protein, as determined by the PMPS/PAR colorimetric assay. The specific activity of reconstituted MetH(2−1227) was 4.3 μmol min^{-1} mg^{-1}, which is approximately 40% of the original specific activity of MetH(2−1227).

Effect of Zinc Removal and Reconstitution on Enzyme-Monitored Steady-State Turnover of MetH(2−1227) and on

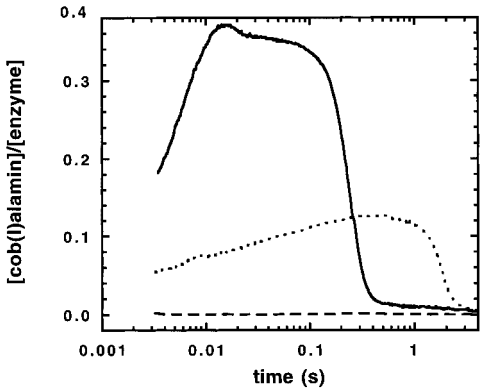

FIGURE 7: Enzyme-monitored turnover of untreated, zinc-depleted, and zinc-reconstituted methylated MetH (2−1227). Enzyme-monitored turnover was observed under anaerobic conditions in a stopped-flow spectrophotometer at 25 °C. Enzyme in the methylcobalamin form (∼10 μM) was mixed with 10 equiv of homocysteine and 50 equiv of methyltetrahydrofolate (where both solutions are in 50 mM Tris chloride, pH 7.2, and 100 mM KCl) as described in Materials and Methods. The cob(I)alamin concentration was monitored at 525 nm and normalized using the absorbance change at 525 nm determined by complete demethylation of the methylated enzyme to cob(I)alamin enzyme using excess homocysteine. Data are shown for MetH(2−1227) (—), zinc-depleted MetH(2−1227) (− − −), and zinc-reconstituted MetH(2−1227) (•••) .

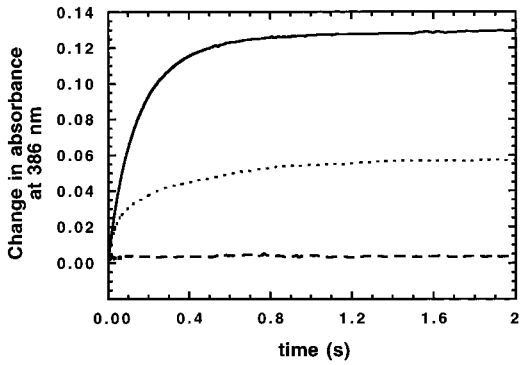

FIGURE 8: Stopped-flow measurements of the rate of methyl transfer from methylated MetH(2−1227) to homocysteine under aerobic conditions at 25 °C. Demethylation by homocysteine is shown for MetH(2−1227) (—), zinc-depleted MetH(2−1227) (− − −), and zinc-reconstituted MetH(2−1227) (•••). Enzyme in the methylcobalamin form (∼10 μM) was mixed with 100 μM homocysteine; both enzyme and substrates were dissolved in 50 mM Tris chloride, pH 7.2/100 mM KCl. These experiments differ from those shown in Figure 7 in that CH_3-H_4folate is not present. As noted in the text, the observed rate constant is ∼10-fold slower for untreated enzyme equilibrated in Tris/KCl buffer than for enzyme in phosphate/dithiothreitol buffer.

the Rate of Demethylation of Methylated MetH(2−1227) by Homocysteine. The primary turnover reactions involve methyl transfers that occur in a ternary complex of the enzyme with both of its substrates (*27*). In Figure 7, one can observe steady state turnover of methylated MetH(2−1227) with limiting but saturating amounts of homocysteine and an excess of methyltetrahydrofolate; the formation of cob(I)alamin and its remethylation are monitored at 525 nm under anaerobic conditions. Initially, the untreated methylated enzyme is rapidly demethylated by homocysteine, a slight overshoot of steady state cob(I)alamin levels is seen, and then steady state [∼35% cob(I)alamin] is achieved. After limiting homocysteine has been exhausted, the cob(I)alamin is remethylated by the excess methyltetrahydrofolate to regenerate methylated MetH(2−1227). Analysis of the approach to steady state gives a rate constant for demethylation of methylated MetH(2−1227) by homocysteine of 125 s^{-1}, which is similar to previously observed rate constants (*23, 27*). Zinc-depleted methylated MetH(2−1227) which has been treated with MMTS and then with dithiothreitol and EDTA to remove enzyme-bound zinc, does not turn over under these conditions (Figure 7). Titration of the zinc-depleted enzyme with DTNB under denaturing conditions established that all thiols had been unblocked.

Zinc reconstitution of zinc-depleted MetH(2−1227), which incorporates 0.37 equiv of zinc/mol of protein, restores approximately 35% of primary turnover as determined by the steady state concentration of cob(I)alamin produced. The turnover number is reduced from 26.2 s^{-1} with MetH(2−1227) to 2.1 s^{-1} with reconstituted MetH(2−1227). This represents a 5-fold reduction in activity if we take into account that only 37% of reconstituted MetH(2−1227) is zinc-replete (Figure 7). The rate constant for the approach to steady state associated with demethylation of zinc-reconstituted methylated MetH(2−1227) is also reduced by 5-fold, from 125 s^{-1} to 27 s^{-1}, again taking into account that only 37% of the enzyme is zinc replete. The loss in activity in turnover may be due to irreversible damage caused to MetH(2−1227) by treatment with 1 M urea.

The effect of zinc-reconstitution on the initial rate of demethylation of methylated enzyme may be better studied by observing the demethylation of reconstituted methylcobalamin MetH(2−1227) by homocysteine in the absence of methyltetrahydrofolate. Stopped-flow analyses of the demethylation of the methylated enzymes by homocysteine in the absence of methyltetrahydrofolate were performed under similar conditions as the experiment shown in Figure 3 and are shown in Figure 8. Zinc-depleted methylated MetH(2−1227) does not demethylate in the presence of homocysteine under aerobic conditions. With zinc-reconstituted methylated MetH(2−1227) containing 0.37 equiv of zinc/mol of protein, the rate constant for demethylation of reconstituted methylated MetH(2−1227) is approximately the same as that for the wild-type enzyme. These results suggest that zinc is required for catalytic activity, and in particular, that zinc is required for the methyl transfer reaction from enzyme-bound methylcobalamin to homocysteine.

DISCUSSION

These studies strongly support the concept that two enzymes with no detectable homology, cobalamin-independent and cobalamin-dependent methionine synthase, employ basically similar strategies for the binding of homocysteine, i.e., they both coordinate the homocysteine thiolate to enzyme-bound zinc. Recent work in other laboratories suggests that this strategy for binding and activation of thiolates is employed by a variety of other proteins, and the role of zinc in thiol activation has recently been the subject of a review (*28*). The precedent for thiol activation by protein-bound divalent zinc comes from studies on the mechanism of methylthioether formation at a specific cysteine residue during the repair of DNA methylphosphotriesters by the Ada protein from *E. coli* (*29, 30*). In the Ada system, spectroscopic evidence shows that zinc is ligated to the thiolate of cysteine 69, activating this residue for nucleophilic attack on the methyl group of a DNA meth-

ylphosphotriester. The divalent zinc ion in the Ada protein is coordinated to four thiolate ligands, and this coordination is postulated to enhance the nucleophilicity of cysteine 69 at neutral pH (*31*).

Thus far, two other methyltransferase enzymes have been shown to contain zinc *(32)*. These enzymes from *Methanosarcina barkerii* catalyze methyl transfer from methylcobamide to coenzyme M (methanethiolsulfonate). They contain 1.0 equivalent of zinc/mol of protein, and enzyme activity is inhibited in the presence of EDTA (*32*). Although these proteins exhibit no significant identities with cobalamin-independent and cobalamin-dependent methionine synthases, the role of zinc in these proteins may be similar, in that both archaebacterial proteins also catalyze methyl transfer to a thiol substrate.

The prenyl:protein transferases have also been found to contain zinc, which is required for binding the peptide that becomes prenylated (*33, 34*). Farnesyltransferase catalyzes the transfer of a farnesyl group from farnesyl pyrophosphate to a cysteine residue near the C-terminus of acceptor proteins such as $p21^{ras}$. The X-ray structure of farnesyl:protein transferase has recently been determined (*35*) and reveals that the zinc is pentacoordinate, with bidentate ligation to an aspartyl residue, and monodentate ligation to additional cysteine, histidine, and water ligands. It is postulated that the substrate cysteinyl residue that will be farnesylated displaces the water ligated to the zinc. Elegant studies by Carol Fierke and her colleagues with cobalt-substituted enzyme (*36*) have established that the coordination of the cobalt is altered by binding of peptide substrates, with the visible absorbance spectrum of the protein changing from one characteristic of a single cobalt-thiolate charge transfer band to a spectrum characteristic of a complex with two thiolate-cobalt charge transfer bands. These studies also demonstrated that the change in coordination occurs in a kinetically competent fashion.

EXAFS of cobalamin-dependent methionine synthase has indicated that the enzyme-bound zinc is coordinated by three thiolate residues from the protein, and binding of L-homocysteine results in the formation of a tetrathiolate complex (K. Peariso, C. W. Goulding, S. Huang, R. G. Matthews, and J. Penner-Hahn, manuscript in preparation). The challenge now is to determine the degree to which ligation of homocysteine to enzyme-bound zinc alters the reactivity of the thiolate as compared to the reactivity of the uncoordinated thiolate form of homocysteine. Studies on the rates of reaction of thiolates have generally suggested that the thiol form is unreactive (*37, 38*). The model studies of Wilker and Lippard (*39*) measured the reactivity of benzyl thiolate coordinated in a $[Zn(SC_6H_5)_4]^{2-}$ complex on reaction with $(CH_3O)_3PO$ in dimethylsulfoxide and compared the reactivity with that of uncomplexed benzyl thiolate. The first-order rate constant for reaction of the uncomplexed thiolate was 1.1×10^{-4} s^{-1}, while that for the complexed thiolate was 8.2×10^{-5} s^{-1}. These studies concluded that all reactivity of the zinc tetrathiolate complex could be attributed to dissociated thiolate, indicating that ~75% of the complex was dissociated to form $[Zn(SC_6H_5)_3]$ and (SC_6H_5) in dimethyl sulfoxide. If one or two of the ligands in $[Zn(SC_6H_5)_4]^{2-}$ were replaced by methylimidazole ligands, the reactivity of the remaining thiolate ligands decreased to 6×10^{-6} s^{-1} or $<5 \times 10^{-8}$ s^{-1}, respectively. Thus, these studies suggest that the net charge of −2 for the zinc complex is critical for maximal thiolate reactivity. These predictions can now be tested by mutagenesis of cobalamin-dependent methionine synthase, with substitution of histidine residues for the cysteine ligands to zinc.

ACKNOWLEDGMENT

We thank Professor David Ballou (University of Michigan) for the use of his stopped-flow apparatus. We are indebted to Dr. Theodore J. Huston at the University of Michigan Department of Geological Sciences for performing ICP metal concentration determinations.

REFERENCES

1. Goulding, C. W., Postigo, D., and Matthews, R. G. (1997) *Biochemistry 36*, 8082−8091.
2. Guest, J. R., Friedman, S., Woods, D. D., and Smith, E. L. (1962) *Nature 195*, 340−342.
3. Taylor, R. T. (1971) *Arch. Biochem. Biophys. 144*, 352−362.
4. Garrow, T. A. (1996) *J. Biol. Chem. 271*, 22831−22838.
5. Roberts, D. L., Zhao, S., Doukov, T., and Ragsdale, S. W. (1994) *J. Bacteriol. 176*, 6127−6130.
6. Banerjee, R. V., Johnston, N. L., Sobeski, J. K., Datta, P., and Matthews, R. G. (1989) *J. Biol. Chem. 264*, 13888−13895.
7. Drummond, J. T., Huang, S., Blumenthal, R. M., and Matthews, R. G. (1993) *Biochemistry 32*, 9290−9295.
8. Drennan, C. L., Huang, S., Drummond, J. T., Matthews, R. G., and Ludwig, M. L. (1994) *Science 266*, 1669−1674.
9. Dixon, M. M., Huang, S., Matthews, R. G., and Ludwig, M. (1996) *Structure 4*, 1263−1275.
10. González, J. C., Banerjee, R. V., Huang, S., Sumner, J. S., and Matthews, R. G. (1992) *Biochemistry 31*, 6045−6056.
11. Whitfield, C. D., Steers, E. J., and Weissbach, H. (1970) *J. Biol. Chem. 245*, 390−401.
12. González, J. C., Peariso, K., Penner-Hahn, J. E., and Matthews, R. G. (1996) *Biochemistry 35*, 12228−12234.
13. Amaratunga, M., Fluhr, K., Jarrett, J. T., Drennan, C. L., Ludwig, M. L., Matthews, R. G., and Scholten, J. D. (1996) *Biochemistry 35*, 2453−2463.
14. Giedroc, D. P., Keating, K. M., Williams, K. R., Konigsberg, W. H., and Coleman, J. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8452−8456.
15. Hunt, J. B., Neece, S. H., Schachman, H. K., and Ginsberg, A. (1984) *J. Biol. Chem. 259*, 14793−14803.
16. Jarrett, J. T., Choi, C. Y., and Matthews, R. G. (1997) *Biochemistry 36*, 15739−15748.
17. Drummond, J. T., Jarrett, J., Gonzalez, J. C., Huang, S., and Matthews, R. G. (1995) *Anal. Biochem. 228*, 323−329.
18. Qui, H., Kodadek, T., and Giedroc, D. P. (1994) *J. Biol. Chem. 269*, 2773−2781.
19. Jarrett, J. T., Goulding, C. W., Fluhr, K., Huang, S., and Matthews, R. G. (1997) *Methods Enzymol. 281*, 196−213.
20. Luschinsky, C. L., Drummond, J. T., Matthews, R. G., and Ludwig, M. L. (1992) *J. Mol. Biol. 225*, 557−560.
21. Smith, D. J., Maggio, E. T., and Kenyon, G. L. (1975) *Biochemistry 14*, 766−771.
22. Guo, J., and Giedroc, D. P. (1997) *Biochemistry 36*, 730−742.
23. Jarrett, J. T., Amaratunga, M., Drennan, C. L., Scholten, J. D., Sands, R. H., Ludwig, M. L., and Matthews, R. G. (1996) *Biochemistry 35*, 2464−2475.
24. Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979) *Anal. Biochem. 94*, 75−81.
25. Massey, V., and Hemmerich, P. (1978) *Biochemistry 17*, 9−17.
26. Benesch, R. E., and Benesch, R. (1955) *J. Am. Chem. Soc. 77*, 5877−5881.
27. Banerjee, R. V., Frasca, V., Ballou, D. P., and Matthews, R. G. (1990) *Biochemistry 29*, 11101−11109.
28. Matthews, R. G., and Goulding, C. W. (1997) *Curr. Opin. Chem. Biol. 1*, 332−339.

29. Myers, L. C., Terranova, M. P., Nash, H. M., Markus, M. A., and Verdine, G. L. (1992) *Biochemistry 31*, 4541−4547.
30. Sedwick, B., Robins, P., Totty, N., and Lindahl, T. (1988) *J. Biol. Chem. 263*, 4430−4433.
31. Myers, L. C., Terranova, M. P., Nash, H. M., Markus, M. A., and Verdine, G. L. (1993) *Science 261*, 1164−1167.
32. LeClerc, G. M., and Grahame, D. A. (1996) *J. Biol. Chem. 271*, 18725−18731.
33. Reiss, Y., Brown, M. S., and Goldstein, J. L. (1992) *J. Biol. Chem. 267*, 6403−6408.
34. Moomaw, J. F., and Casey, P. J. (1992) *J. Biol. Chem. 267*, 17438−17443.
35. Park, H. W., Boduluri, S. R., Moomaw, J. F., Casey, P. J., and Beese, L. S. (1997) *Science 275*, 1800−1804.
36. Huang, C. C., Casey, P. J., and Fierke, C. A. (1997) *J. Biol. Chem. 272*, 20−23.
37. Fava, A., Illiceto, I., and Camera, E. (1957) *J. Am. Chem. Soc. 79*, 833−836
38. Kharasch, N. (1961) *Organic Sulfur Compounds*, Vol. 1, Pergamon Press, Elmsford, NY.
39. Wilker, J. J., and Lippard, S. J. (1997) *Inorg. Chem. 36*, 969−978.

BI971988L