Quantitation of Rate Enhancements Attained by the Binding of Cobalamin to Methionine Synthase†

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ABSTRACT: Cobalamin-dependent methionine synthase (MetH) catalyzes the methylation of homocysteine using methyltetrahydrofolate as the methyl donor. The cobalamin cofactor serves as an intermediate carrier of the methyl group from methyltetrahydrofolate to homocysteine. In the two half-reactions that comprise turnover for MetH, the cobalamin is alternatively methylated by methyltetrahydrofolate and demethylated by homocysteine to form methionine. Upon binding to the protein, the usual dimethylbenzimidazole ligand is replaced by the imidazole side chain of His759 [Drennan, C. L., Huang, S., Drummond, J. T., Matthews, R. G., and Ludwig, M. L. (1994) *Science 266*, 1669−1674]. Despite the ligand replacement that accompanies binding of cobalamin to the holo-MetH protein, a MetH(2−649) fragment of methionine synthase that contains the regions that bind homocysteine and methyltetrahydrofolate utilizes *exogenously* supplied cobalamin in methyl transfer reactions akin to those of the catalytic cycle. However, the interactions of MetH(2−649) with endogenous cobalamin are first order in cobalamin, while the half-reactions catalyzed by the holoenzyme are zero order in cobalamin, so rate constants for reactions of bound and exogenous cobalamins cannot be compared. In this paper, we investigate the catalytic rate enhancements generated by binding cobalamin to MetH after dividing the protein in half and reacting MetH(2−649) with a second fragment, MetH(649−1227), that harbors the cobalamin cofactor. The second-order rate constant for demethylation of methylcobalamin by Hcy is elevated 60-fold and that for methylation of cob(I)alamin is elevated 120-fold. Thus, binding of cobalamin to MetH is essential for efficient catalysis.

Cobalamin-dependent methionine synthase (MetH) catalyzes the terminal step of the de novo biosynthesis of methionine in *Escherichia coli*. In the first half of this reaction (*1*), homocysteine is methylated by the methylcobalamin cofactor to generate methionine and cob(I)alamin. In the second step, the enzyme-bound cob(I)alamin is remethylated by CH_3-H_4-folate[1] to regenerate the starting methylcobalamin form of the enzyme (Figure 1). Under aerobic conditions, the cob(I)alamin form of the cofactor is occasionally oxidized to a catalytically inactive cob(II)alamin form. The active methylcobalamin form of the enzyme is generated by reductive methylation with electrons that are derived from NADPH via a flavodoxin:NADPH flavodoxin oxidoreductase and flavodoxin (*7*) and with a methyl group that is derived from *S*-adenosylmethionine (*8*).

Biochemical studies of methionine synthase have shown this enzyme to be modular: distinct regions in the protein are responsible for binding and activating the substrates (*9*, *10*) (Figure 2). The cobalamin binding module of MetH is the focal point of methionine synthase: substrates required for turnover or reductive methylation are bound by separate regions of the polypeptide and presented to the cofactor in turn.[2] The modular nature of this protein has been reinforced by the competence of a truncated form of MetH(2−649) in reactions akin to those in the half-reactions of the catalytic cycle (*10*, *11*). This fragment binds Hcy and CH_3-H_4folate substrates and catalyzes the transfer of methyl groups between these and *exogenously* supplied cobalamin. The methyl transfers catalyzed by MetH(2−649) are first order in both protein and exogenous cobalamin components. The second-order rate constants measured with this fragment are similar to those obtained when substrates bound to the MetH holoenzyme are reacted with exogenous cobalamin (*10*). A smaller fragment, MetH(2−353), has also been expressed, and this fragment catalyzes methyl transfer between exogenously supplied cobalamin and Hcy with a rate which is comparable to those measured with MetH(2−649) and MetH(2−1227), suggesting that the determinants for binding and activation of the two substrates reside in distinct regions of the MetH(2−649) polypeptide (*11*).

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[1] Abbreviations: AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; CH_3-H_4folate, 5-methyltetrahydrofolate; H_4-folate, tetrahydrofolate; NADPH, nicotinamide adenine diphosphate, reduced form; DTT, dithiothreitol; Hcy, L-homocysteine; IPTG, isopropyl *β*-D-thiogalactopyranoside; KPB, potassium phosphate buffer, pH 7.2.

[2] Steady-state and rapid-reaction kinetic studies led to the tentative conclusion that the enzyme catalyzes methyl transfers within a ternary complex (*1*), although it was noted that the stopped-flow data were also consistent with a ping-pong mechanism. However, structural studies strongly suggest that substrate-presenting modules occupy mutually exclusive sites vis-à-vis the cobalamin cofactor, leading to the proposal of a ping-pong mechanism (*2*).

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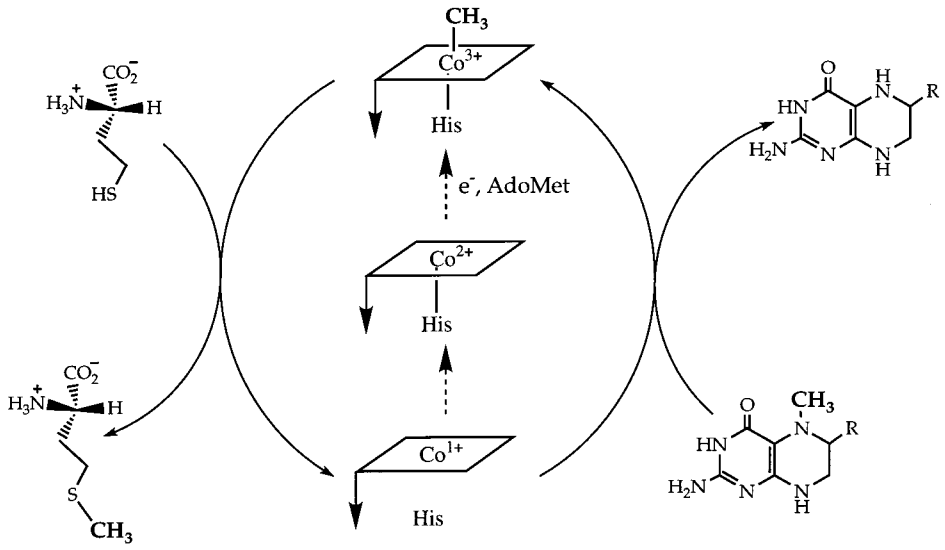

FIGURE 1: Schematic view of the reactions catalyzed by methionine synthase. The substrates, homocysteine and methyltetrahydrofolate, are bound to separate sites within the methionine synthase polypeptide. During turnover, the enzyme-bound cobalamin cycles between the methylcob(III)alamin and cob(I)alamin forms. In the first half-reaction, methylcobalamin is presented to the binding site containing homocysteine to form methylcobalamin and cob(I)alamin. In the second half-reaction, cob(I)alamin is methylated by CH_3-H_4folate to regenerate the methylcobalamin form of the enzyme.

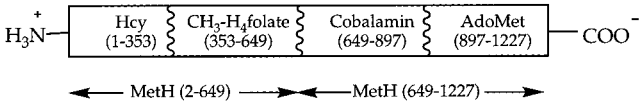

FIGURE 2: Schematic representation of the modules in methionine synthase. The substrates for primary turnover, homocysteine and CH_3-H_4folate, and the methyl donor for reductive methylation, AdoMet, are bound to separate regions of the MetH polypeptide. The substrates are presented to the cobalamin cofactor, in turn, during the turnover or reductive methylation cycles. The regions of the protein encompassed by the fragments used in the studies described in this paper are shown below the figure.

This apparent modularity in MetH is reinforced by the ability to selectively diminish the rate of one methyl transfer or another by site-directed mutagenesis (*10*). Extended X-ray absorption fine structure studies have shown that MetH supplies three cysteine ligands to a zinc ion that binds and activates the Hcy substrate (*12*). Site-directed mutants that lack one of these zinc ligands have been prepared (*11*, *12*); while these proteins are devoid of zinc and are incapable of methylating homocysteine with exogenously supplied methylcobalamin, methyl group transfer from CH_3-H_4folate to cob(I)alamin is not impeded. Likewise, variants of MetH(2−649) have been prepared which are impaired in the methyl transfer between CH_3-H_4folate and exogenous cob(I)alamin but can catalyze the transfer between methylcobalamin and Hcy at rates comparable to those observed with the wild-type fragment (*13*).

The X-ray structure of the cobalamin binding region of MetH has been determined (*14*). The structure revealed that, upon binding of methylcobalamin to MetH, the dimethylbenzimidazole substituent which coordinates the lower axial position of cobalt in free methylcobalamin is replaced by the imidazole side chain of His759. The dimethylbenzimidazole is inserted into a cavern where it no longer has direct access to the cobalt. The new axial ligand to the cobalt, His759, is part of a hydrogen-bonded triad that involves the carboxylate side chain of Asp757 and the hydroxyl group of Ser810. Such "ligand replacement" has emerged as a general theme in a number of cobalamin-dependent methyltransferases (*15*−*17*).

Site-directed mutations of the triad residues in MetH indicate that they play a role in catalysis (*18*). Mutation of His759, the lower axial ligand to the cobalt, to a glycine yields an inactive protein. The effect of alterations of the other residues of the triad, while not as extreme, are still substantial. The role of these residues in enhancing the reactivity of the cobalamin is hard to gauge. While the reactions of *exogenous* cobalamin and MetH(2−649) can serve as excellent model reactions, these reactions are first order in both cobalamin and the enzyme, and their rates cannot be compared to those with full-length MetH. The substrates are bound to a single polypeptide in the holo-MetH as is the tightly bound cobalamin cofactor with which they interact. Therefore, the rate constants that are measured for the individual methyl transfer reactions are zero order in cobalamin. We reasoned that the ability to reconstitute MetH activity by combining the first and second halves of the protein would afford the opportunity to measure rate constants for the individual methyl transfer reactions and that these measurements would be directly comparable to those with MetH(649−1227) and *exogenous* cobalamin.

This paper presents the expression, purification, and characterization of the protein fragment MetH(649−1227) corresponding to the second half of methionine synthase. Results of reconstitution of enzymatic activity *in trans* using a combination of the two halves of MetH are discussed.

EXPERIMENTAL PROCEDURES

Materials. (6*R*,*S*)-5-Methyltetrahydrofolate (calcium salt) was purchased from Schircks Laboratories (Jona, Switzerland); [*methyl*-^{14}C]CH_3-H_4folate (barium salt) was from Amersham; [*methyl*-^{3}H]AdoMet was purchased from Moravek. AdoMet, dithiothreitol, aquocobalamin, DEAE-Sepharose, *p*-(hydroxymercuri)phenylsulfonic acid (monosidum salt),

and L-homocysteine thiolactone were purchased from Sigma Chemical Co. L-Homocysteine thiolactone was converted to L-homocysteine and quantitated as described (*19*). XL1-Blue cells were obtained from Novagen. Ethanolamine (free base) and 4-(2-pyridylazo)resorcinol (monosodium salt hydrate) were from Aldrich; ampicillin was from Research Products International. Q-Sepharose (fast flow), phenyl-Sepharose CL-4B, and MonoQ (HR16/10) were from Pharmacia. Bio-Rex 70, AG 50 X8 (100−200 mesh, H^+ form), and AG 1 X8 (100−200 mesh, Cl^- form) resins were from Bio-Rad. EcoLite scintillation cocktail was from ICN.

Construction of the Expression Vector for MetH(649−1227). The plasmid used in these studies to overexpress MetH(649−1227) in *E. coli* was constructed by Dr. Mohan Amaratunga, using the synthetic gene encoding the cobalamin binding domain (*20*), which contains restriction sites every ∼100 base pairs. The plasmid pMMA1-38 (*20*) was digested with *Sac*I and *Bgl*II and ligated to a cassette which was prepared by mixing equal quantities of oligonucleotides 1 and 2, where the mutated residues are shown in lower case.

1: CGCCGAGAAATATCGCGGCACGAAAACCGA-
CGACACCGCC**Atg**GCCCAGCAGGCGGAGTGGA

2: GATCTCCACTCCGCCTGCTGGGC**caT**GGCGG-
TGTCGTCGGTTTTCGTGCCGCGATATTT-
CTCGGCGAGCT

Oligonucleotide 1 contains nt 1902−1963 of the sense strand of *metH* from the synthetic gene and primer 2 is complementary to nt 1898−1967 of the synthetic *metH* gene. The cassette incorporates a mutation to introduce an **ATG** to replace Asn648 by a Met, as well as introducing a new *Nco*I site (underlined). The resulting vector, pMMA1-45, was digested with *Spl*I and *Xho*I and ligated to a cassette which was prepared by mixing equal quantities of oligonucleotides 3 and 4.

3: GTACGTATaCAGCACGGGCGC**tAG**tAACC-
GCGCACACCACCGGTCACGC

4: TCGAGCGTGACCGGTGGTGTGCGCGGTTa-
CTaGCGCCCGTGCTGtATAC

Oligonucleotide 3 corresponds to nt 2668−2716 of the sense strand of *metH* from the synthetic gene and incorporates a **TAG** stop codon immediately proceeding Arg896. Oligonucleotide 4 is complementary to nt 2668−2717 of the *metH* synthetic gene. The resulting vector, pMMA1-48, was digested with *Spl*I and *Nco*I to liberate nt 1941−2667. Next, the vector pKF5A, which contains the coding region for MetH (*20*), was digested with *Spl*I and *Hin*dIII to release nt 2668−3687, which specify amino acids Val890−Asp1227 of the MetH coding sequence. The *pTrc99a* (Pharmacia) vector was digested with *Nco*I and *Hin*dIII and combined with the fragment obtained from the *NcoI*/*Spl*I digest of the plasmid pMMA1-48 and the *NcoI*/*Hin*dIII fragment derived from digesting pKF5A. The resulting expression vector, pMMA11, was transformed into XL1-Blue cells to obtain the MetH(649−1227) overproducing *E. coli* strain XL1-blue/pMMA11 that was used in the subsequent experiments. Expression of MetH(649−1227) in this strain is IPTG-inducible.

Media for MetH(649−1227) Fermentations. XL1-Blue cells harboring the pMMA11 expression vector were grown aerobically at 37 °C in a modified M9 medium (*21*) where ethanolamine (25 mM) replaced glucose and NH_4Cl as a source of carbon and nitrogen, respectively. Previous experiments had shown that when *E. coli* is grown in the presence of ethanolamine *and* cobalamin, the adenosylcobalamin-dependent enzyme ethanolamine ammonia-lyase is induced (*22*). Ethanolamine ammonia-lyase converts ethanolamine to acetaldehyde and ammonia, allowing the cells to derive the carbon and nitrogen required for growth from ethanolamine (*22*). We reasoned that under these conditions the cells would be forced to take up more cobalamin than normal, which would enhance the pool of cobalamin available in the cell for incorporation into methionine synthase. The medium was also supplemented with 0.1 mg/mL ampicillin, 5 μM hydroxocobalamin, micronutrients (*23*), 10 μM thiamin, and 20 amino acids at levels previously employed by Wanner et al. (*24*). A single colony was used to inoculate 5 mL of medium and allowed to grow into stationary phase (typically ∼24 h). A 0.1 L starter culture was inoculated with the 5 mL culture and grown until turbid ($A_{420\,nm} < 1$, ∼24 h) and used to inoculate six 3.5 L flasks each containing 1 L of medium. The cultures were grown to an $OD_{420\,nm}$ of ∼1, and expression of MetH(649−1227) was induced by the addition of 0.5 mM IPTG. Cultures were allowed to grow ∼18 h after induction to a final $OD_{420\,nm}$ ∼2. Cells were collected by 15 min centrifugation at 4440*g* (4 °C) and frozen at −80 °C.

Purification of MetH(649−1227). Cells from 2 × 6 L fermentations were combined and thawed in ∼50−100 mL of 10 mM potassium phosphate buffer (KPB), pH 7.2, containing the protease inhibitors phenylmethanesulfonyl flouride (0.1 mL of 20 mg/mL 2-propanol solution) and tosyl-L-lysine chloromethyl ketone (0.2 mL of a 1 mg/mL solution in water). The cells were disrupted by sonication, and cell debris was removed by centrifugation at 64000*g* for 1 h. The cleared lysate was applied to a DEAE-Sepharose column that had been equilibrated with 10 mM KPB. The column was washed until free cobalamin was eluted from the column (0.1−0.15 L) and eluted with a linear gradient (0.4 L) of 10−250 mM KPB. The red fractions containing MetH(649−1227) were pooled and dialyzed against 4 L of 10 mM KPB. The dialysate was applied to a Q-Sepharose column equilibrated with the same buffer and washed with 0.15 L of 10 mM KPB. MetH(649−1227) was eluted as for the DEAE-Sepharose column. Red fractions containing MetH(649−1227) were pooled and dialyzed against 4 L of 25 mM KPB. The dialysate was taken to ∼1.2 M $(NH_4)_2SO_4$, and the resulting solution was applied to a column of phenyl-Sepharose CL-4B which had been equilibrated with 25 mM KPB containing 1.2 M $(NH_4)_2SO_4$. The column was washed with 100 mL of the same buffer and eluted with a linear gradient from 0.6 to 0 M $(NH_4)_2SO_4$ in 25 mM KPB in a total volume of 400 mL. Fractions containing MetH(649−1227) were combined and dialyzed against 4 L of 10 mM KPB. The dialysate was chromatographed on a MonoQ column (Pharmacia) which had been equilibrated with the same buffer. The sample was loaded onto the column, and the column was washed with 80 mL of 10 mM KPB. MetH-(649−1227) was eluted with a linear gradient from 10 to 160 mM KPB in 150 mL total volume. Protein was eluted at ∼130 mM KPB. Fractions containing MetH(649−1227)

were pooled and dialyzed against 4 L of 10 mM KPB. The dialysate was concentrated with Centricon concentrators (YM30 membrane) and frozen at −80 °C. The extinction coefficient of the methylcobalamin cofactor bound to MetH(649−1227) was determined as described for the full-length enzyme (*18*). Protein used in all subsequent experiments was quantitated according to its cobalamin content. The N-terminal sequence of the protein was obtained at the Biomedical Research Core Facility of the University of Michigan.

Expression and Purification of Wild Type and D522N and C310A Variants of MetH(2−649). Wild-type, C310A, and D522N MetH(2−649) were overexpressed in an *E. coli* XL1-Blue strain and purified as previously described (*10*, *13*). Protein concentration was determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) [$\epsilon_{412\,nm}$ 13 600 M^{-1} cm^{-1} (*25*)] in 6 M guanidinium hydrochloride buffered with 0.05 M Tris-chloride (pH 8.0). Zinc content was determined by titrating with 4-(2-pyridylazo)resorcinol in the presence of *p-(*hydroxymercuri)phenylsulfonic acid as described previously (*11*).

Turnover Assays with MetH(2−649) + MetH(649−1227). Methionine synthesis activity was monitored by measuring the transfer of ^{14}C label from [*methyl*-^{14}C]CH_3-H_4folate to methionine. Assay solutions (0.2 mL) contained 0.1 M KPB, dithiothreitol (25 μM), AdoMet (0.38 mM), [*methyl*-^{14}C]CH_3-H_4folate (0.42 mM, specific activity 1270 dpm/nmol), hydroxocobalamin (50 μM), L-homocysteine (∼0.9 mM), and variable quantities of Met(2−649) and MetH(649−1227). The assay mixtures were prepared by mixing all of the components except Hcy and incubating for 5 min at 37 °C. Samples were withdrawn prior to addition of Hcy and, at set times after addition of the substrate, were quenched by mixing with ∼1 mL aliquots of water, in 1.5 mL Eppendorf tubes, which were kept in a 70−80 °C water bath. The mixtures were incubated in the water bath for 3 min and cooled on ice, and [^{14}C]methionine was isolated as previously described (*19*). Saturation profiles were analyzed by fitting the rates (v) to the Michaelis−Menten equation using the KaleidaGraph software for Macintosh (Synergy Software):

$$v = \frac{VS}{K_m + S} \quad (1)$$

where V is the maximal velocity, S is the substrate concentration, and K_m is the Michaelis−Menten constant.

Hcy:SAM Methyl Transfer Reaction. The discrimination by MetH(649−1227) between methyl groups of AdoMet and CH_3-H_4folate for transfer to Met was measured essentially as described above for turnover assays with MetH(2−649) and MetH(649−1227) except that [*methyl*-^3H]AdoMet replaced unlabeled AdoMet. To reduce the background counts which were present in the commercial preparation of [*methyl*-^3H]AdoMet, AdoMet stock solutions that were to be used in the assays were purified as follows: the stock solutions were loaded onto a Bio-Rex 70 column which had been equilibrated with 1 mM sodium acetate (pH 4.6); the column was washed to remove counts that were not retained by the column, and the AdoMet was eluted with 40 mM HCl. Fractions that contained [*methyl*-^3H]AdoMet were located by liquid scintillation counting. Fractions containing the radioactivity were combined and lyophilized. The resulting residue was dissolved in 1 mM HCl and quantitated using absorbance at 260 nm [$\epsilon_{260\,nm}$ ∼ 15.4 mM^{-1} cm^{-1} at pH 7 (*26*)] in 10 mM KPB. The assays contained 0.1 M KPB, [*methyl*-^3H]AdoMet (0.34 mM), [*methyl*-^{14}C]CH_3-H_4folate, hydroxocobalamin (50 μM), Hcy (∼0.9 mM), 269 nM MetH(649−1227), and variable quantities of MetH(2−649). [*methyl*-^{14}C]CH_3-H_4folate was omitted in experiments where the Hcy:AdoMet methyltransferase reaction was being measured. The reaction mixtures were incubated at 37 °C for 5 min and a sample (50 μL) was withdrawn prior to and after addition of Hcy. Samples (50 μL) were withdrawn and quenched in water (0.5 mL). Radiolabeled Met was separated from [*methyl*-^{14}C]CH_3-H_4folate and [*methyl*-^3H]AdoMet by passing over a Pasteur pipet column that contained a layer (∼3 cm) of AG 50 X8 (Na^+ form) and a layer (∼2 cm) of AG1 X8 (Cl^- form) above the AG 50 resin. The column was washed with 3 mL of water. All of the eluents from this column were collected in a scintillation vial, combined with 10 mL of scintillation fluid, and assayed for ^3H and ^{14}C using the dual label program of a Beckman LS-6500 scintillation counter. The working specific activities of the labels in the assays were determined in reactions that contained an excess of MetH(2−649), MetH(649−1227), and Hcy to ensure complete transfer of methyl groups from AdoMet and CH_3-H_4folate to Hcy.

RESULTS

Expression, Purification, and Preliminary Characterization of MetH(649−1227). Expression of the MetH(649−1227) fragment was accomplished in growth media where ethanolamine replaced glucose and ammonia as a source of carbon and nitrogen. While growth in M9 medium containing glucose led to significantly greater cell mass, the cobalamin prosthetic group of MetH(649−1227) was found to be in multiple states, which behave differently on the hydrophobic interaction and MonoQ chromatographic steps and complicate pooling of the fractions. In addition, the protein had to be returned to the methylcobalamin state by reductive methylation. Following isolation from cells grown in ethanolamine, methylated MetH(649−1227) could be purified to >95% purity as judged by SDS−PAGE analysis. The extinction coefficient of the methylcobalamin bound to MetH(649−1227) (∼$9.2 \times 10^3 \pm 0.6 \times 10^3$ M^{-1} cm^{-1}) was found to be similar to that determined for full-length MetH protein (*18*). N-Terminal sequence analysis of the protein revealed the expected sequence and indicated posttranslational modification to remove the initial methionine. The first amino acid of this fragment corresponds to Ala649 of the full-length protein—hence the designation MetH(649−1227).

Methionine Synthesis Activity Is Observed with MetH(2−649) + MetH(649−1227). Separately overexpressed halves of methionine synthase were combined to determine if MetH activity could be reconstituted. Activity was shown to be linearly dependent on the concentration of each fragment (Figure 3). MetH activity was measured at several fixed concentrations of MetH(649−1227) while MetH(2−649) was varied (Figure 3A). Saturation behavior was not observed in these experiments. When the rate constants obtained from the slopes of each curve were plotted against the MetH(649−1227) present in the reactions, a linear correlation was observed (Figure 3B). A second-order rate constant of 47 000 ± 4000 M^{-1} s^{-1} for this reaction was obtained from the slope of the line in Figure 3B.

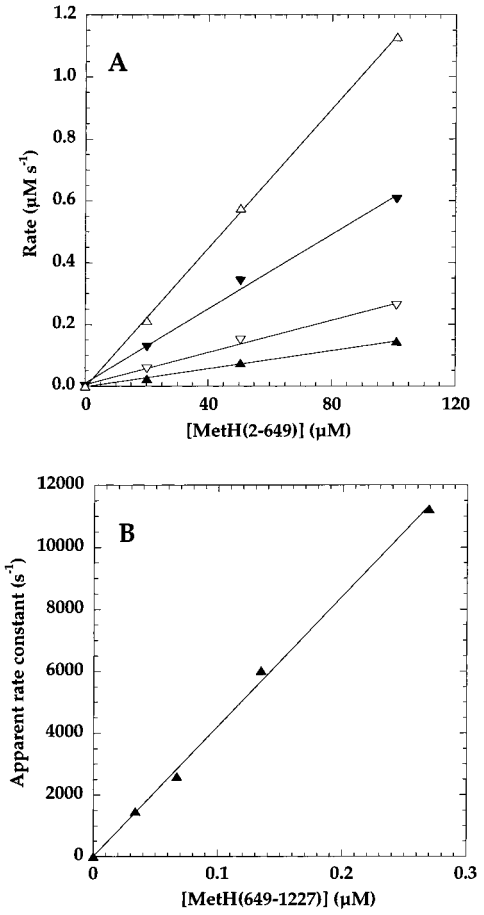

FIGURE 3: Reconstitution of methionine synthase activity with MetH(2−649) and MetH(649−1227). The reactions were measured as described in Experimental Procedures. The data in panel A were obtained in the presence of 34 nM (▲), 67 nM (▽), 135 nM (▼), or 269 nM (△) MetH(649−1227) and the indicated concentrations of MetH(2−649). Panel B shows a replot of the slopes of the curves in panel A against the concentrations of the MetH(649−1227). The second-order rate constant for the methionine synthase activity with MetH(2−649) and MetH(649−1227) was obtained from the slope of the curve in panel B.

Turnover in Two Encounters. Two mechanisms could be envisioned for the methionine synthase activity which is observed when the two halves of the enzyme are mixed. In both scenarios, an initial encounter complex will catalyze the methylation of homocysteine by the methyl group of the methylcobalamin in MetH(649−1227). This complex can have two fates: either the halves will fall apart, rejoin, and carry out a cob(I)alamin:CH_3-H_4folate methyl transfer reaction to complete the catalytic cycle and re-form the methylcobalamin cofactor or a conformational change may juxtapose the CH_3-H_4folate binding domain of MetH(2−649) in the initial encounter complex with the cob(I)alamin in MetH(649−1227) to facilitate the second methyl transfer reaction and complete the catalytic cycle. Therefore, in the latter case, complete turnover requires only a single collision whereas in the former case, two encounters would be required. One can distinguish between the two scenarios using MetH(2−649) variants that can only catalyze *one* of the methyl transfer reactions.

MetH(2−649) variants that are impaired in one of the methyl transfer reactions have been characterized previously (*10*, *13*). MetH contains a catalytically essential zinc divalent metal ion. The Zn^{2+} is coordinated by cysteines 310, 311, and 247 and an exchangeable oxygen/nitrogen ligand that is probably water (*12*). The Cys310Ala, Cys311Ala, or Cys247Ala variants of MetH(2−649) are unable to bind zinc and consequently are impaired in catalyzing methyl transfers between Hcy and exogenous methylcobalamin; by contrast, the cob(I)alamin:CH_3-H_4folate methyl transfer reaction is unimpeded in these variants (*10*). A Cys310Ala variant was chosen for the current studies.

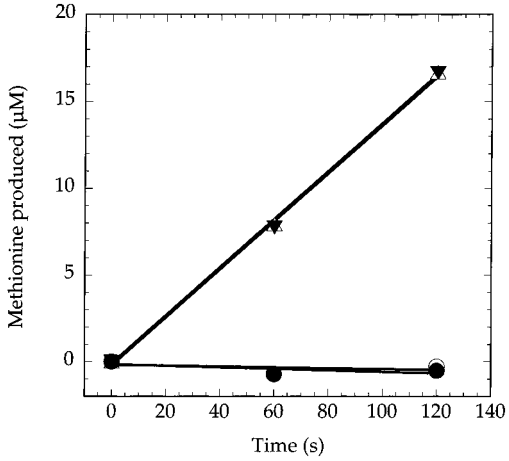

FIGURE 4: Reconstitution of methionine synthase activity with variants of MetH(2−649) and wild-type MetH(649−1227). The reactions were carried out as described in Experimental Procedures and contained 67 nM MetH (649−1227) and either 50 μM wild-type MetH (2−649) (△) or 50 μM each of Cys310Ala MetH(2−649) and Asp522Asn MetH(2−649) (▼). Control experiments where Cys310Ala MetH(2−649) (●) or Asp522Asn MetH(2−649) (○) were omitted are also shown.

The CH_3-H_4folate binding region of MetH shows significant sequence homology to dihydropteroate synthase (*13*) and AcsE (*27*, *28*); these enzymes share a pterin binding site. Dihydropteroate synthase (DHPS) catalyzes the coupling of 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine diphosphate and *p*-aminobenzoate to form dihydropteroate. AcsE from the acetogenic bacterium *Clostridium thermoaceticum* catalyzes the CH_3-H_4folate-dependent methylation of a corrinoid iron−sulfur protein. The X-ray crystal structure of DHPS shows a constellation of aspartate residues (aspartates 45, 84, 160) that interact with the pterin ring (*29*). These aspartate residues are conserved in the sequences of MetH (aspartates 399, 434, 522) and AcsE (aspartates 43, 75, 167), suggesting parallels between the folate binding sites of these enzymes and that of DHPS. Indeed, the X-ray structure of AcsE shows that these conserved Asp residues are located in the active site (*28*). The consequence of site-directed mutations in the corresponding residues in MetH has been investigated (*13*). The Asp522Asn MetH(2−649) protein was chosen for our study since it has been shown to be impaired in binding CH_3-H_4folate and lacks detectable CH_3-H_4folate:cob(I)alamin methyltransferase activity. This variant, however, catalyzes Hcy:methylcobalamin methyl transfer with the same efficiency as the wild-type protein.

Methionine synthesis activity could be reconstituted by mixing equal quantities of *both* the Cys310Ala and Asp522Asn variants of MetH(2−649) with MetH(649−1227) (Figure 4). The observed activity is equivalent to that seen with an equimolar amount of wild-type MetH(2−649). By contrast, no enzymatic activity is observed when either variant of

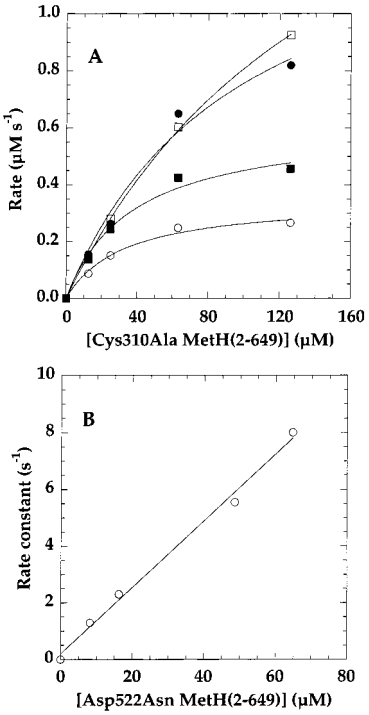

FIGURE 5: Steady-state kinetic assays to determine the second-order rate constant for demethylation of endogenous methylcobalamin with Hcy. The reactions in panel A were carried out as described in Experimental Procedures. The assays contained 269 nM MetH(649−1227) and 8.8 μM (○), 17.5 μM (■), 52.5 μM (●), or 70.0 μM (□) Asp522Asn MetH(2−649) and the indicated concentrations of Cys310Ala MetH(2−649). The maximal velocities from the Michaelis−Menten fits to the experimental data were divided by the concentration of MetH(649−1227) and plotted against the concentration of Asp522Asn MetH(2−649) in each series. The slope of the curve in panel B is the second-order rate constant for the demethylation of endogenous methylcobalamin with Hcy.

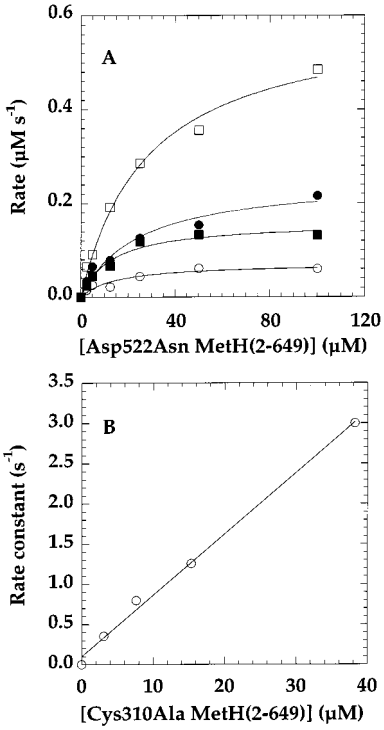

FIGURE 6: Steady-state kinetic assays to determine the second-order rate constant for methylation of endogenous cob(I)alamin with CH_3-H_4folate. The reactions in panel A were carried out as described in Experimental Procedures. The assays contained 206 nM MetH(649−1227) and 3.1 μM (○), 7.6 μM (■), 15.3 μM (●) or 38.2 μM (□) Cys310Ala MetH(2−649) and the indicated concentrations of Asp522Asn MetH(2−649). The maximal velocities from the Michaelis−Menten fits to the experimental data were divided by the concentration of MetH(649−1227) and plotted against the concentration of Cys310Ala MetH(2−649), which was present in each series. The slope of the curve in panel B is the second-order rate constant for the methylation of endogenous cob(I)alamin with CH_3-H_4folate.

MetH(2−649) is omitted. These results distinguish between the single and the double encounter scenarios which were discussed earlier. Since each of the Cys310Ala and Asp522Asn variants of MetH(2−649) is impaired in one of two methyl transfers that is required for complete turnover, MetH activity observed must arise from two encounters. Moreover, the rate observed with the variants is equivalent to that observed with an equimolar quantity of wild-type MetH(2−649); therefore, one can conclude that the activity observed with the wild-type MetH(2−649) also requires the formation of two encounter complexes.

Steady-State Method To Determine Rate Constants for the Half-Reactions. Steady-state kinetics were used to extract the second-order rate constants for the two methyl transfer reactions occurring in the course of turnover with MetH-(2−649) + MetH(649−1227). In these experiments, we employed reaction mixtures which contained MetH(649−1227) and varied concentrations of Asp522Asn and Cys310Ala MetH(2−649). We reasoned that each methyl transfer reaction is dependent on the concentration of one of the variants. For instance, the rate of the Hcy:methylcobalamin methyl transfer reaction will be proportional to the concentration of the Asp522Asn MetH(2−649) variant, whereas the CH_3-H_4folate:cob(I)alamin methyl transfer reaction will be dependent on the concentration of the Cys310Ala MetH-(2−649) protein. However, as the concentration of one of the variants is raised, saturation behavior may be observed. For instance, if the concentration of Cys310Ala MetH(2−649) is raised while the concentration of Asp522Asn MetH-(2−649) is kept constant, methionine synthesis will become limited by the rate of the Hcy:methylcobalamin methyl transfer reaction and vice versa. This type of experiment would allow the two methyl transfer reactions to be uncoupled and the second-order rate constant for the reaction catalyzed by the limiting component of the assay to be measured. The results of these experiments are shown in Figures 5 and 6. In the first set of experiments, rates of methionine synthesis were measured in the presence of constant MetH(649−1227) and Asp522Asn MetH(2−649), while Cys310Ala MetH(2−649) was varied (Figure 5). In successive experiments, the concentration of Asp522Asn MetH(2−649) was varied, and the maximal velocities determined from the family of fits shown in Figure 5A were replotted against the concentration of the Asp522Asn fragment (Figure 5B). Since Asp522Asn MetH(649−1227) can only catalyze the Hcy:methylcobalamin methyl transfer reaction, the slope of the replot is the second-order rate constant for the demethylation (k_{pong}) of *endogenous* methylcobalamin by MetH(2−649). The second-order rate constant for the remethylation (k_{ping}) of cob(I)alamin by CH_3-H_4folate was determined exactly as described above for k_{pong} (see Figure 6). The second-order rate constants for the

Table 1: Second-Order Rate Constants Obtained for Demethylation of Methylcobalamin by Hcy and for Methylation of Cob(I)alamin by CH_3-H_4folate with Exogenous and Endogenous Cobalamin

	second-order rate constants (M^{-1} s^{-1})		
reaction	exogenous[a]	endogenous	fold rate enhancement[c]
Hcy:methylcobalamin methyl group transfer	2300	135 000 ± 17 000	60
CH_3-H_4folate:cob(I)alamin methyl group transfer	600	70 000 ± 6 000	120
turnover	500[b]	47 000 ± 4 000	100

[a] From ref *10*. [b] From eq 2 as described in the text. [c] Calculated as the ratio of second-order rate constants for reaction with endogenous versus exogenous cobalamin.

two methyl transfer reactions and the second-order rate constants for the analogous reaction with exogenous cobalamin (*10*) and the resulting rate enhancements are summarized in Table 1.

The rate constants determined for the individual reactions can be used to estimate the rate of the overall reaction using the equation:

$$k_{cat} = \frac{k_1 k_2}{k_1 + k_2} \quad (2)$$

where k_1 and k_2 are the second-order rate constants for the individual reactions and k_{cat} is a second-order rate constant. The estimate obtained from eq 2 (46 000 M^{-1} s^{-1}) agrees well with the experimentally measured value of 47 000 M^{-1} s^{-1}.

Hcy:SAM Methyl Transfer Reaction. During the course of the catalytic reaction, the cob(I)alamin form of the cofactor is occasionally oxidized to the catalytically inactive cob(II)alamin state. In vivo, electrons supplied by flavodoxin:NADPH flavodoxin reductase and a methyl group supplied by AdoMet return cob(II)alamin to the catalytically active methylcobalamin form. In vitro, DTT, hydroxocobalamin, and AdoMet serve to reductively methylate oxidized cobalamin formed in the course of the assays. The almost complete exclusion of AdoMet-derived methyl groups during primary turnover with MetH(2−1227) is documented (*30*). Since, in the assays described thus far, transfer of radioactive methyl groups between CH_3-H_4folate and Hcy is monitored, we were concerned that a significant Hcy:AdoMet methyl transfer reaction would lead to an underestimate of the true rate constants for the Hcy:CH_3-H_4folate methyl transfer step. Therefore, experiments were carried out with [*methyl*-^{3}H]-AdoMet and [*methyl*-^{14}C]CH_3-H_4folate to allow direct comparison of the contribution of each reaction to the overall reaction. The results of these experiments are shown in Figure 7. As shown previously, the rate of the Hcy:CH_3-H_4-folate methyl transfer reaction is first order with respect to the concentration of MetH(2−649). Interestingly, the rate of the Hcy:AdoMet methyl transfer reaction saturates with respect to MetH(2−649). In addition, the rate of consumption of AdoMet decreases in the presence of CH_3-H_4folate, indicating that CH_3-H_4folate and AdoMet compete for a common intermediate. While exclusion of AdoMet from primary turnover is no longer complete during catalysis *in trans*, the rate of AdoMet consumption has little effect on the rate of conversion of Hcy to Met measured under the conditions described above.

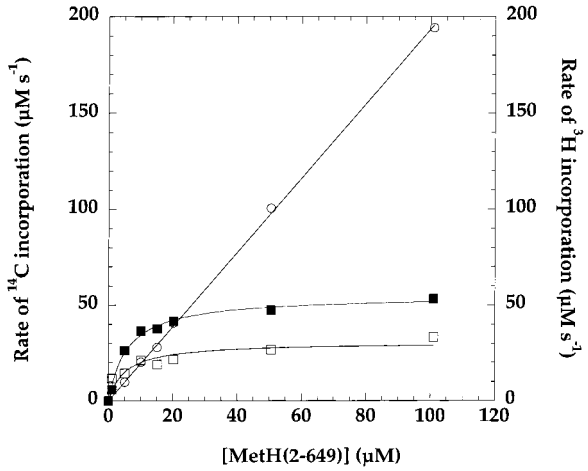

FIGURE 7: Competition between AdoMet and CH_3-H_4folate for cob(I)alamin. To determine the extent of the Hcy:AdoMet methyl transfer reaction in the absence of CH_3-H_4folate, MetH(2−649) and MetH(649−1227) were incubated with [*methyl*-^{3}H]-*S*-adenosyl-L-methionine (■) and Hcy. The competition between CH_3-H_4folate and AdoMet for cob(I)alamin was measured by incubating the two halves of MetH with Hcy, [*methyl*-^{3}H]-*S*-adenosyl-L-methionine, and [*methyl*-^{14}C]CH_3-H_4folate. The rates of the Hcy:SAM (□) and Hcy:CH_3-H_4folate (○) methyltransferase reactions were measured by monitoring the ^{3}H/^{14}C content of the methionine isolated from reactions containing both labels. The dependence of the Hcy:SAM methyltransferase reactions on the concentration of MetH(2−649) was fitted to the Michaelis−Menten equation; the Hcy:CH_3-H_4-folate reaction had a linear dependence with respect to MetH-(2−649), as had been observed previously (see Figure 3).

DISCUSSION

MetH catalyzes three methyl transfer reactions:

$$\text{Hcy} + \text{methylcobalamin} \rightarrow \text{Met} + \text{cob(I)alamin} \quad (4)$$

$$\text{cob(I)alamin} + CH_3\text{-}H_4\text{folate} \rightarrow \text{methylcobalamin} + H_4\text{folate} \quad (5)$$

$$\text{cob(I)alamin} + \text{AdoMet} \rightarrow \text{methylcobalamin} + \text{AdoHcy} \quad (6)$$

The first two reactions constitute the two halves of the catalytic cycle. The third reaction reaction is involved in the return of oxidized cobalamin to its catalytically active form. Enhancements in the methyl transfer rates, due to the binding of cobalamin to the protein, were measured in this study for the two reactions in the catalytic cycle (eqs 4 and 5). The $\sim 10^2$ rate enhancements we observe are similar to those measured in the reactions of other cobalamin-dependent methyltransferases (*31*). It is noteworthy that all cobalamin-dependent methyltransferases that have been examined retain the ability to react with exogenous cobalamin, even though their physiological substrates are protein-bound corrinoids[3] (*31*−*34*). While substitution of the lower axial ligand to the cobalt is a common theme among methyltransferases, a histidine-containing catalytic triad is not always the successor to the dimethylbenzimidazole. For instance, the corrinoid iron−sulfur protein of *C. thermoaceticum* and the related

[3] Cobalamin-dependent methyltransferases from some sources utilize modified analogues of cobalamin where the dimethylbenzimidazole lower axial is replaced with 5-hydroxybenzimidazolylcobamide (*3*) or 5-methoxybenzimidazolylcobamide (*4* and references therein).

proteins from *Methanosarcina barkeri* and *Methanosarcina thermophila* have been shown to lack coordination to a histidine residue from the protein (*3*, *35*). In a further twist, sequence alignment of CmuA, a methylcorrinoid-dependent chloromethane dehalogenase from *Methylobacterium* sp., with the cobalamin binding region of MetH suggests that this enzyme harbors a glutamine instead of a histidine (*17*).

The second-order rate constants for model reactions in which enzyme-bound homocysteine and CH_3-H_4folate were replaced with free thiolates and *N*-methylamines have been measured. The demethylation of methylcobalamin by a thiol, measured at pH values where the thiol is fully ionized, exhibits second-order rate constants[4] of 2.2×10^{-2} to 1×10^{-6} M^{-1} s^{-1} (*5*, *6*). Hcy bound to MetH(2−649) reacts $\sim 10^5$−10^9 fold more rapidly than the thiolates in these model reactions (*10*). When $PhNMe_3^+$ was used to model CH_3-H_4folate (*36*) in the remethylation of cob(I)alamin, second-order rate constants of 2×10^{-3} $M^{-1}s^{-1}$ were measured; the model reaction is $\sim 3 \times 10^5$ fold slower than the reaction of enzyme-bound CH_3-H_4folate with MetH(2−649). The second-order rate constants reported in this paper afford direct comparisons to the second-order rate constants obtained in model reactions. The binding of cobalamin to the protein [MetH(649−1227)] appears to have led to an $\sim 10^2$-fold enhancement in rate relative to the rates measured with MetH(2−649) and exogenous cobalamin (*10*). The bulk of the catalytic enhancements in the overall reaction are realized in the presence of the region of the protein that binds the Hcy and CH_3-H_4folate substrates. If one can exclude the possibility that the Hcy and CH_3-H_4folate binding regions of MetH(2−649) modulate the reactivity of the cobalamin, the binding of cobalamin to the protein only serves to fine-tune the reactivity of the cobalamin.

The most conspicuous difference between the enzyme-bound and free forms of cobalamin is the substitution of the dimethylbenzimidazole ligand in the solution structure with the imidazole side chain of His759 (*14*). The imidazole is hydrogen bonded to the carboxylate of Asp757, which in turn interacts with the hydroxyl group of Ser810. This "triad" of residues has been proposed to modulate the reactivity of the cobalamin. During catalysis, the cobalamin has to shuttle between the base-on/off forms: the base-off form is preferred in the cob(I)alamin state, and the base-on geometry is observed in the methylcobalamin form (*37*, *38*). The ligand triad may be involved in facilitating the interconversion of cobalamin between the two states. Site-directed mutations in the catalytic triad residues have been prepared (*18*). Most notably, the His759Gly variant of methionine synthase appears to be completely inactive in overall turnover ($<10^5$ of wild type). The His759Gly variant of MetH(649−1227) is also inactive in turnover (V. Bandarian and R. G. Matthews, unpublished results). The lack of detectable activity in this mutant, however, is in stark contrast to the 10^3 rate enhancement observed in model reactions where the demethylation of methylcob*inamide* relative to methylcob*alamin* (*6*) by Hcy was measured.[5] Recent X-ray crystallographic studies of the His759Gly variant of MetH(649−1227) are consistent with the notion that the severe phenotype of the His759Gly variant may result from locking of the protein in a conformation which excludes the regions of the protein that bind the substrates, Hcy and CH_3-H_4folate, which are used in primary turnover (V. Bandarian, K. Pattridge, B. L. Lennon, M. L. Ludwig, and R. G. Matthews, unpublished results). The direct determination of the rate enhancements in the current study leads us to conclude that, at most, the catalytic triad is responsible for a rate enhancement of $\sim 10^2$; the contributions from the individual amino acids of the triad remain to be established.

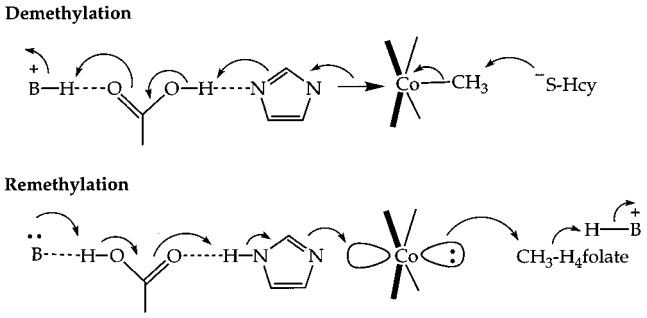

FIGURE 8: Schematic representation of the role of the catalytic triad in modulating the reactivity of the endogenous cofactor by altering the electron density at the cobalt.

The contribution of the ligand triad to the rate enhancement is in line with the $\sim 10^3$ differential in reactivity between methylcobalamin and methylcobinamide in the model studies, which may be considered the upper limit on the rate enhancements that can be garnered by the ligand triad. A plausible role for the ligand triad is diagrammed in Figure 8. In this model, the triad serves to pull electrons away from, or push electrons toward, the cobalt. Removal of electron density from the cobalt can favor demethylation, whereas during remethylation pushing electron density toward the cobalt will increase the nucleophility of the cob(I)alamin. Alternatively, one may envision that, during demethylation, pulling away of electrons preorganizes the cobalamin ligand environment for transition to 4-coordinate Co^{1+}; pumping of electrons (delivered by the imidazole ligand) back into the cobalt stabilizes the methylcobalamin form. Recently, Smith and Matthews (*13*) have suggested that methyl transfer between methylcobalamin and Hcy, or methyltetrahydrofolate and cob(I)alamin (and the reverse reaction), by MetH may be catalyzed by an oxidative addition pathway, which would involve the formation of cis complexes (*13*). The push−pull mechanisms outlined above can also be useful in the oxidative addition mechanisms where formation and breakdown of the complexes may be facilitated by interaction with the imidazole; removal of the lower ligand would facilitate the formation of complex between methylcobalamin and homocysteine, whereas general acid catalyzed breakdown of the complex of tetrahydrofolate and methylcobalamin would be aided by ligation of the imidazole in the lower

[4] The second-order rate constants quoted in this paper for the demethylation of methylcobalamin were measured under different conditions. Hogenkamp and co-workers (*5*) utilized β-mercaptoethanol at 43 °C, whereas Norris and Pratt (*6*) utilized ethanethiol, homocysteine, or cysteine at 25 °C. In the latter study, demethylation of methylcobalamin by the thiols tested could not be observed, and only an upper limit for the second-order rate constants ($<1 \times 10^{-6}$ M^{-1} s^{-1}) was reported.

[5] The model studies were carried out in aqueous milieu, and one cannot assume that the lower axial position of the cobalamin was empty; rather, it is likely that a weak ligand (such as H_2O) was occupying this position.

axial position of the cobalamin. In principle, the removal and religation of the lower axial base of cobalamin should not require the elaborate machinery that MetH has supplied, and indeed, the reactions proceed at significant rates with unbound cobalamin (*10*). Interestingly, methylation of exogenous cob(I)*inamide* with methanol by methanol:coenzyme M methyltransferase from *M. barkeri* only proceeds in the presence of added imidazole (*33*).

The MetH reaction requires that the region of the protein that binds the cobalamin alternately face the Hcy and CH_3-H_4folate binding modules during primary turnover and the binding domain for SAM during reductive methylation. However, while MetH is unique among methyltransferases in that all protein regions which are involved in the production of methionine are located on a single polypeptide, thus far, all proteins that have been shown to catalyze cobalamin-dependent methyl transfer reactions dispense with the dimethylbenzimidazole as the lower axial ligand to the cobalt.[3] At the present, one cannot exclude the possibility that a role of the triad may be to transmit information about the oxidation or alkylation state of the cobalamin to the substrate or reactivation modules and, perhaps, be responsible for excluding AdoMet from interacting with cob(I)alamin generated during primary turnover. Future work will focus on deconvoluting the contributions of the residues in the catalytic triad to the 100-fold rate enhancement that was measured in this study.

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