Cobalamin-Dependent Methionine Synthase Is a Modular Protein with Distinct Regions for Binding Homocysteine, Methyltetrahydrofolate, Cobalamin, and Adenosylmethionine†

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Received March 6, 1997; Revised Manuscript Received April 25, 1997⊗

ABSTRACT: Methionine synthase (MetH) catalyzes the transfer of a methyl group from bound methylcobalamin to homocysteine, yielding enzyme-bound cob(I)alamin and methionine. The cofactor is then remethylated by methyltetrahydrofolate. We now demonstrate that MetH is able to catalyze methylation of free cob(I)alamin with methyltetrahydrofolate. MetH had previously been shown to catalyze methylation of homocysteine with free methylcobalamin as the methyl donor, in a reaction that is first-order in added methylcobalamin, and we have confirmed this observation using homogenous enzyme. A truncated polypeptide lacking the cobalamin-binding region of the holoenzyme, MetH(2−649), was overexpressed and purified to homogeneity. MetH(2−649) catalyzes the methylation of free cob(I)alamin by methyltetrahydrofolate and the methylation of homocysteine by free methylcobalamin. Furthermore, a protein comprising residues 2−353 of the holoenzyme has now been overexpressed and purified to homogeneity, and this protein catalyzes methyl transfer from free methylcobalamin to homocysteine but not from methyltetrahydrofolate to free cob(I)alamin. The mutations Cys310Ala and Cys311Ala in MetH(2−649) completely abolish methyl transfer from exogenous methylcobalamin to homocysteine but do not affect methyl transfer from methyltetrahydrofolate to exogenous cob(I)alamin, consistent with a modular construction for MetH. We infer that MetH is a modular protein comprising four separate regions: a homocysteine binding region (residues 2−353), a methyltetrahydrofolate binding region (residues 354−649), a region responsible for binding the cobalamin prosthetic group (residues 650−896), and an AdoMet-binding domain (residues 897−1227).

Cobalamin-dependent methionine synthase (MetH) from *Escherichia coli* catalyzes the transfer of a methyl group from enzyme-bound methylcobalamin to homocysteine to generate methionine and cob(I)alamin. The enzyme-bound cob(I)alamin is remethylated by CH_3-H_4folate[1] to regenerate methylcobalamin and H_4folate (*1*). These two half-reactions constitute the primary turnover cycle shown in Figure 1. The cob(I)alamin cofactor is occasionally oxidized to an inactive cob(II)alamin form, which must be reactivated by a reductive methylation using reduced flavodoxin as an electron donor and AdoMet as a methyl donor. The deactivation and reactivation cycle, also shown in Figure 1, occurs once in every 100−2000 turnovers (*2*, *3*). Therefore, the enzyme must catalyze three different methyl transfers to and from its prosthetic group.

Methionine synthase has a molecular mass of 136 kDa and consists of at least three different functional regions. Two of these regions have been prepared by limited proteolysis with trypsin. The 38 kDa C-terminal domain, consisting of amino acids 897−1227, binds AdoMet as demonstrated by covalent modification in the presence of radiolabeled AdoMet following ultraviolet irradiation (*2*). A high-resolution X-ray structure of this fragment, with AdoMet bound, has recently been determined (*4*). If proteolysis is performed on enzyme in the methylcobalamin form, the remaining 98 kDa N-terminal fragment is catalytically active for many rounds of turnover but is gradually inactivated as cob(II)alamin is formed. Addition of AdoMet and a reductant to the 98 kDa fragment does not result in restoration of catalytic activity. These results suggest that the C-terminal 38 kDa fragment is essential for reactivation of the inactive cob(II)alamin form of methionine synthase.

Further tryptic digestion of the N-terminal 98 kDa fragment releases residues 650−896, which lie immediately upstream of the activation domain. This fragment retains the ability to bind the methylcobalamin cofactor (*5*). The X-ray structure of this fragment has been determined with methylcobalamin bound. A surprising feature of the binding of methylcobalamin to the apoenzyme is that the lower axial ligand coordinated to the cobalt atom is Nϵ of His759 rather

† This research has been supported in part by NIH Research Grant R37 GM24908.

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⊗ Abstract published in *Advance ACS Abstracts,* June 15, 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 hydrochloride; H_4MPT, tetrahydromethanopterin; CH_3-H_4MPT, methyltetrahydromethanopterin; H-S-CoM, coenzyme M or 2-mercaptoethanesulfonate; CH_3-S-CoM, methyl-coenzyme M or 2(methylthio)ethanesulfonate; IPTG, isopropyl β-D-thiogalactopyranoside.

S0006-2960(97)00516-3 CCC: $14.00

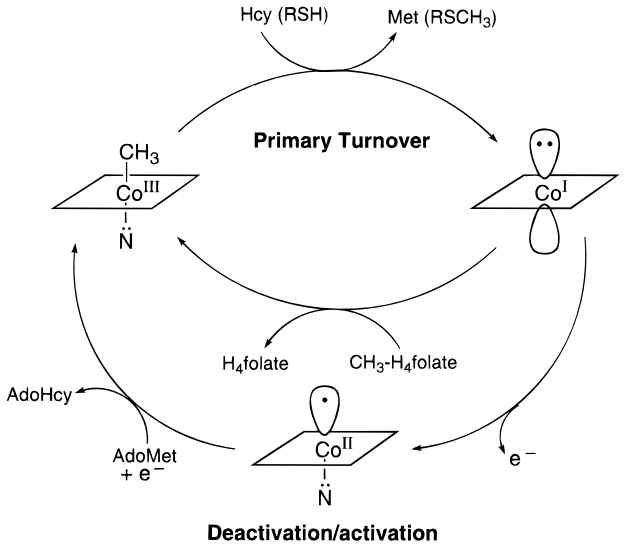
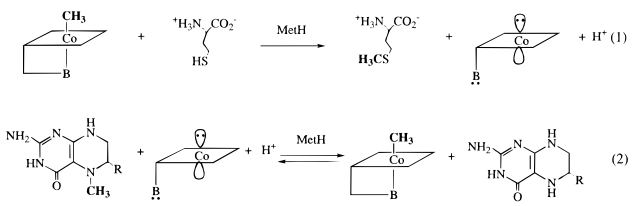


FIGURE 1: Schematic summary of the reactions carried out by methionine synthase. In the catalytic cycle, cobalamin-dependent methionine synthase transfers a methyl group from methylcobalamin to homocysteine, to yield methionine and cob(I)alamin; the cob(I)alamin is remethylated by CH_3-H_4folate to produce 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 (activation), in which an electron is supplied by reduced flavodoxin and a methyl group by AdoMet, to regenerate methylcobalamin.

than a nitrogen from dimethylbenzimidazole, and the dimethylbenzimidazole nucleotide tail of methylcobalamin is embedded in the protein.

By default, the remaining 70 kDa N-terminal region of methionine synthase is thought to control the binding and reactivity of the two substrates, homocysteine and CH_3-H_4folate. However, tryptic digestion of the 98 kDa fragment results in cleavage of the the 70 kDa N-terminal region into multiple smaller fragments (*5*). Evidence to suggest that the 70 kDa region is responsible for substrate binding and activation comes from similarities between the sequence of this region and those of other methyltransferase enzymes. The two half-reactions involved in the primary turnover cycle of methionine synthase are similar to reactions catalyzed by these other methyltransferases. Mammalian betaine−homocysteine methyltransferase catalyzes methyl transfer from betaine to homocysteine to yield dimethylglycine and methionine; this enzyme has limited sequence similarity with the first 364 residues of methionine synthase (*6*), suggesting that the first 364 residues of the N-terminus of methionine synthase include residues responsible for the binding and activation of homocysteine. Residues 350−600 of methionine synthase show limited sequence similarity to a methyl transferase from *Clostridium thermoaceticum* that catalyzes transfer of a methyl group from CH_3-H_4folate to the cobalt of a corrinoid iron-sulfur protein (*7*), strongly suggesting that residues responsible for the binding and activation of CH_3-H_4folate lie in this region of methionine synthase.

Catalytic turnover of methionine synthase occurs in a ternary complex formed by binding homocysteine and CH_3-H_4folate, and reactions of E·cob(I)alamin with CH_3-H_4folate in the absence of homocysteine or of E·methylcobalamin with homocysteine in the absence of CH_3-H_4folate are multiphasic with only small fractions of the reactions occurring at a kinetically competent rate (*1*). We therefore proposed to develop assays using free cobalamin as a methyl acceptor or methyl donor (eqs 1 and 2) to identify residues



involved in substrate binding and activation. In 1962, Guest et al. (*8*) documented the ability of methionine synthase to catalyze methyl transfer from exogenous methylcobalamin to homocysteine. More extensive studies by Taylor (*9*) demonstrated that the rate of this methyl transfer showed first-order dependence on the concentration of free methylcobalamin between 0.1 and 3 mM but exhibited saturation when the homocysteine concentration was increased. Propylation of the endogenous cofactor had no effect on the rate of methyl transfer from exogenous methylcobalamin to homocysteine, although it completely inhibited methyl transfer from CH_3-H_4folate to homocysteine in the absence of added methylcobalamin, suggesting that the endogenous cofactor was not involved in methyl transfer from methylcobalamin to homocysteine. N^5-Methyltetrahydromethanopterin−coenzyme M methytransferase from *Methanobacterium thermoautotrophicum* (*10*) catalyzes the reactions shown in eqs 3 and 4, suggesting by analogy that methionine

$$CH_3\text{-}H_4MPT + \text{cob(I)alamin} + H^+ \rightarrow CH_3\text{-cobalamin} + H_4MPT \quad (3)$$

$$CH_3\text{-cobalamin} + HSCoM \rightarrow CH_3\text{-}SCoM + \text{cob(I)alamin} + H^+ \quad (4)$$

synthase might also catalyze methyl transfer from CH_3-H_4folate to free cob(I)alamin. Similarly, Ragsdale and his colleagues have shown that the CH_3-H_4folate corrinoid iron-sulfur protein methyltransferase from *Clostridium thermoaceticum* catalyzes methyl transfer from CH_3-H_4folate to free cob(I)alamin (*11*).

In the present work we have repeated Taylor's experiments using homogenous methionine synthase to catalyze methyl transfer from free methylcobalamin to homocysteine and demonstrated that the enzyme also catalyzes methyl transfer from CH_3-H_4folate to free cob(I)alamin; both half-reactions are first-order in cobalamin. A truncated methionine synthase protein containing residues 2−649 has been purified to homogeneity and characterized. This protein catalyzes methyl transfer to and from exogenous cobalamin. In contrast, a more drastically truncated methionine synthase containing residues 2−353 only catalyzes methyl transfer from methylcobalamin to homocysteine. We therefore propose that methionine synthase is a modular protein consisting of distinct regions: residues 2−353 are responsible for homocysteine binding and activation, while residues 354−649 are responsible for CH_3-H_4folate binding and activation. Previous studies have shown that residues 650−896 comprise the cobalamin binding region (*5*, *12*), while residues 897−1227 are involved in AdoMet-dependent activation of the enzyme (*2*, *4*).

MATERIALS AND METHODS

Materials. The Geneclean Kit was purchased from Bio 101. Vent polymerase and T4 DNA ligase were purchased from New England Biolabs. Restriction enzymes were obtained from Promega and New England Biolabs. The Sequenase version 2.0 DNA sequencing kit was purchased from U.S. Biochemical Corp. All primers were made at the University of Michigan Protein and Carbohydrate Structure Facility. (6*R*,*S*)-H_4Folate and (6*R*,*S*)-CH_3-H_4Folate (calcium salt) were purchased from Dr. B. Schircks Laboratories (Jona, Switzerland). ^{14}C-(6*R*,*S*)-CH_3-H_4Folate was purchased from Amersham. $TiCl_3$ in 2 M HCl was obtained from Aldrich. L-Homocysteine thiolactone, hydroxocobalamin, AdoMet, and methylcobalamin were purchased from Sigma. AG1-X8 resin and protein dye reagent were purchased from Bio-Rad.

Expression and Purification of Wild-Type and Mutant Methionine Synthase Holoenzymes. The recombinant wild-type methionine synthase, MetH(2−1227), from *Escherichia coli* K-12 strain XL1-Blue/pMMA-07 was overproduced and purified as described previously (*13*). Plasmid pMMA-07 contains a synthetic module, extending between nt 1741 and 2668,[2] that was constructed to introduce new unique restriction sites in the portion of the *metH* gene specifying the cobalamin-binding region to enable mutants to be produced by cassette mutagenesis (*13*). Mutant sequences, constructed as described below, were introduced into this plasmid and expressed and the mutant proteins were purified as previously described for the wild-type enzyme.

Construction of a DNA Fragment Specifying the First 649 Residues of MetH. A plasmid for overexpression of the first 649 residues of MetH [designated MetH(2−649)] was constructed by incorporating a mutant cassette into plasmid pMMA-07 between the unique restriction sites *Sac*I (nt 2131) and *Bgl*II (nt 2187). Oligonucleotides P1 and P′2, whose sequences are shown in Table 1, were synthesized and annealed to generate a double-stranded cassette containing overhanging ends compatible with the *Sac*I and *Bgl*II restriction sites. The sequence of the mutant cassette, which extends from nt 2129 to 2189, incorporates two consecutive TAA stop codons at nt 2170−2176 and deletes the *Bgl*II site to permit screening for the mutant plasmid. Plasmid pMMA-07 was cut with *Bgl*II and *Sac*I and the product was purified by agarose gel electrophoresis and extraction using the Geneclean kit. The cassette was ligated into the vector fragment from pMMA-07, generating plasmid pCWG-02, which was transformed into strain XL1-Blue. The presence of the inserted stop codons at bp 2170−2176 and the absence of unintended alterations were confirmed by DNA sequencing of the region between the *Sac*I and the former *Bgl*II sites (*14*), using the Sequenase kit and primers P3 and P′4 (Table 1).

Subcloning of the Truncated metH Gene Specifying MetH-(2−649). The truncated *metH* gene was subcloned from pCWG-02, a derivative of the expression vector pKK223-3 (Pharmacia), into the high-copy plasmid pUC19 (Life Technologies), with removal of the 3′-end of the *metH* gene that lay downstream of the stop codons introduced into pCWG-02. Plasmid pCWG-02 was digested with *Eco*RI (located upstream of the *metH* gene in the polylinker region) and *Sma*I (nt 2389), and the expression vector pKK223-3, which has both these restriction sites in its polycloning region, was also digested with *Eco*RI and *Sma*I. The truncated *metH* gene and cut vector pKK223-3 were isolated on agarose gels, purified using the Geneclean kit, and ligated to give plasmid pCWG-03. Plasmids pCWG-03 and pUC19 were then digested with *Eco*RI and *Hin*dIII and the truncated *metH* gene was subcloned into pUC19 to give the plasmid pCWG-05.

Construction of a DNA Fragment Specifying Residues 2−353 of MetH. Site-directed mutagenesis was carried out using overlap extension PCR (*15*); the changes incorporated a TAG stop codon at nt 1282 and also introduced an extra *Ssp*I restriction site at nt 1280 into the truncated *metH* gene in pCWG-05. For construction of the mutation, four oligonucleotide primers, P5, P′6, P7, and P′8, were used; their sequences are shown in Table 1. Two separate PCR reactions were carried out in a Perkin-Elmer DNA thermal cycler 480, using Vent polymerase to minimize the possibility of errors during amplification. The first reaction contained primers P5 and P′8, and the second reaction contained primers P′6 and P7; In both reactions, the plasmid pCWG-05 was used as the template. The resulting products of amplification were purified by electrophoresis on a 1.5% agarose gel; the 250 and 350 bp bands were excised and purified using the Geneclean kit. We next used the amplified products from the two reactions described above as templates and P5 and P′6 as primers in a single PCR reaction. The product was run on a 1.5% agarose gel and the 536 bp band was excised and purified using the Geneclean kit. The PCR product was digested with *Apa*I (nt 974) and *Cla*I (nt 1415), and the plasmid pCWG-05 was also digested with same

Table 1: Sequences of Primers Used in Construction of Mutant Forms of *metH*

primer	sequence[a]	nt[b]
P1	5′ CGCCGAGAAA TATCGCGGCA CGAAAACCGA CGACACCGCC AACGCC**TAAT AA**GCGGAGTG GC	2133-2185
P′2	5′ GATCGCCACT CCGC**TTATTA** GGCGTTGGCG GTGTCGTCGG TTTTCGTGCC GCGATATTTC TCGGCGAGCT	2189-2129
P3	5′ ATCGTTGGTGTGGTGCTGCA	2515 - 2534
P′4	5′ GGCGCGTAGCCTGCTGGC	2270 - 2287
P5	5′ GCG CCA CGC CGA AGC TC	927 - 943
P′6	5′ ACC ATC GCC CGT TCG GC	1463 - 1447
P7	5′ GCTGAACATTGGCGAAG**ACTAG**TTGTTTGTGAACG	1263 - 1297
P′8	5′ CGTTCACAAACAA**CTAGT**CTTCGCCAATGTTCAGC	1297 - 1263
P9	5′ CAATATCGTCGGCGGC**GC**CTGTGGCACCACGCC	1134 - 1166
P′10	5′ GGCGTGGTGCCACAG**GC**CGCCGCCGACGATATTG	1166 - 1134
P11	5′ CGTCGGCGGCTGC**GC**TGGCACCACGCC	1140 - 1166
P′12	5′ GGCGTGGTGCCA**GC**CGCAGCCGCCGACG	1166 - 1140
P13	5′ CAATATCGTCGGCGG**GTC**CTGTGGCACCAC	1134 - 1163
P′14	5′ GTGGTGCCA**CAG**GACCCGCCGACGATATTG	1163 - 1134
P-15	5′ CGTCGGCGGCTGCT**CG**GGCACCACGCC	1140 - 1166
P-16	5′ GGCGTGGTGCC**CG**AGCAGCCGCCGACG	1166 - 1140

[a] Changes in sequence introduced using primers are shown in boldface type. [b] The numbering used is that shown in the GenBank sequence J04975 of *metH*. Translation of the *metH* gene begins at base pair 223 in the GenBank sequence. Primers without primes have sequences corresponding to the noncoding strand of the *metH* gene, while those shown with primes are complementary to the noncoding strand.

[2] Numbering of nucleotides in the *metH* gene is based on the sequence deposited as GenBank Accession Number J04975 in which nt 223 is the first nucleotide in the coding sequence for the protein.

restriction enzymes. The PCR product was then ligated into pCWG-05 to yield plasmid pCWG-06. The presence of a stop codon at residue 1282 was confirmed by DNA sequencing between the *Apa*I and *Cla*I sites, using the Sequenase kit and primers P5 and P′6. To overexpress MetH(2-353), an *Eco*RI−*Sac*I fragment containing the truncated *metH* gene was subcloned into the *Eco*RI and *Sac*I sites of plasmid pCWG-02 to form plasmid pCWG-07, which was transformed into XL1-Blue cells.

Construction of Truncated metH Genes for Mutants Cys310Ala, Cys310Ser, Cys311Ala, and Cys311Ser. Mutations Cys310Ala(Ser) and Cys311Ala(Ser) were constructed in MetH(2−649) using overlap extension PCR by methods identical to those used to produce MetH(2−353) and using the same restriction sites and outside primers. The primers used to introduce the mutations are listed in Table 1: P9 and P′10 were used to construct the Cys310Ala mutant, P11 and P′12 to construct the Cys310Ser mutant, P13 and P′14 to construct the Cys311Ala mutant, and P15 and P′16 to construct the Cys311Ser mutant. After construction and sequencing of the mutant plasmids in pCWG-05, they were subcloned into pMMA-07 and p-CWG-02 between the *Eco*RI and *Sac*I sites, so that the mutant proteins could be overexpressed both as holoenzymes and in MetH(2−649).

Overexpression and Purification of the MetH(2−649) Wild-Type and Mutant Proteins and Wild-Type MetH(2−353). For purification of MetH(2−649), cells of strain XL1-Blue containing pCWG-02 were grown aerobically at 37 °C in LB medium (*16*) containing 100 μg/mL ampicillin. Protein expression was induced by addition of 100 mM IPTG at an A_{420} of ∼1.0 and the cells from 2 L of culture were harvested just before stationary phase was reached (A_{420} ∼ 7.0), approximately 7 h after inoculation and 5 h after addition of IPTG. The cells were centrifuged at 12000*g* for 10 min, washed with 50 mM potassium phosphate buffer, pH 7.2, and stored at −80 °C prior to protein purification. The purification procedure was a modification of the method for holoenzyme purification (*13*). After disruption of the cells by sonication and ultracentrifugation to remove cell debris, the enzyme was purified by chromatography at 4 °C on DEAE-Sepharose (Sigma) using a 10 × 2 cm column equilibrated with 50 mM potassium phosphate buffer, pH 7.2. The enzyme was eluted with a 300 mL linear gradient from 50 to 500 mM potassium phosphate buffer, pH 7.2. Fractions were monitored by measuring the absorbance at 285 nm; the fractions with the highest absorbance, eluting at ∼200 mM buffer concentration, were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE). Fractions containing protein of the correct 71 kDa mass were concentrated to 20 mL in a pressure cell (Amicon) with a PM30 membrane (Amicon), dialyzed against 50 mM potassium phosphate buffer, and applied to a Mono Q 16/10 FPLC column (Pharmacia) equilibrated at 20 °C with the same buffer. The column was washed with 100 mL of 50 mM potassium phosphate buffer, then with 120 mL of 118 mM potassium phosphate buffer, and finally with a 150 mL linear gradient between 188 and 320 mM potassium phosphate buffer. The fractions were monitored at 276 nm and the fractions with the highest absorbance, eluting at ∼200 mM potassium phosphate, were collected and analyzed by SDS−PAGE. Fractions containing homogenous protein of the expected 71 kDa mass for MetH(2−649) were pooled and exchanged into 50 mM potassium phosphate for storage at −80 °C. The MetH(2−353) wild-type protein was purified by the same method from cells containing pCWG-07, but the cells were harvested at an A_{420} ∼ 6.0, after about 6 h of inoculation, and the desired fractions were identified by the presence of a protein of 38 kDa mass on analysis by SDS−PAGE.

Methyltetrahydrofolate−Homocysteine Methyltransferase Assay. Methionine synthase activity was determined using a nonradioactive assay that measures the conversion of CH_3-H_4folate to H_4folate by derivatization of the latter to form CH^+═H_4folate (*17*). Briefly, the enzyme was activated by incubating for 5 min at 37 °C in 800 μL of 100 mM potassium phosphate buffer, pH 7.2, containing 250 μM (6-*R,S*)-CH_3-H_4folate, 19 μM AdoMet, 50 μM hydroxocobalamin, and 25 mM dithiothreitol. Turnover was initiated by the addition of homocysteine (500 μM final concentration). After 10 min at 37 °C, the reaction was quenched with 200 μl of formic acid/hydrochloric acid (5 N HCl in aqueous 60% formic acid), and the mixture was incubated at 80 °C to convert H_4folate to CH^+═H_4folate. The derivatized product was quantitated by measuring its absorbance at 350 nm (ϵ = 26 000 M^{-1} cm^{-1}). One unit of enzyme activity is defined as one micromole of product formed per minute.

Methyltetrahydrofolate−Cob(I)alamin Methyltransferase Assay. A solution of titanium(III) citrate was prepared anaerobically from titanium(III) chloride by a modification of a previous method (*18*) that was communicated to us by Stephen Ragsdale. The synthesis was carried out under anaerobic conditions in a vessel equipped with a syringe port. Nine milliliters of 0.5 M sodium citrate was added to 1 mL of 1.9 M titanium(III) chloride in 2 M HCl, and this mixture was stirred at room temperature for 30 min. The solution was neutralized by the slow addition of 3 mL of saturated sodium bicarbonate solution, followed by 7 mL of 100 mM Tris-chloride buffer, pH 7.3, and stirred overnight. The final pH of the solution should be checked and adjusted to pH 7.3 if necessary and the final color of titanium(III) citrate should be greenish-brown at pH 7.3. The final concentration of the solution is 95 mM titanium(III) citrate with ∼70% of the titanium in the titanium(III) form. The concentration of titanium in the titanium(III) form was determined by titrating benzyl viologen with titanium(III) citrate (*19*).

Assays were carried out under anaerobic conditions in the dark in an anaerobic vessel equipped with a side arm and syringe port. The reaction was carried out at pH 7.2 in a volume of 1 mL. The reaction mixture contained 0.125−3.5 mM hydroxocobalamin, 0.5 mM ^{14}C-CH_3-H_4folate (1185 dpm/nmol), 50 mM Tris-chloride buffer, pH 7.2, and 100 mM KCl, with 160 μg of enzyme in the side arm. The reaction mixture was taken through seven cycles of partial evacuation followed by replacement with argon. Titanium(III) citrate solution, 150 μL, was then added to a final concentration of 12 mM and the reaction mixture was incubated at 37 °C for 10 min; at this point one should observe a change in color of the cob(III)alamin solution from pink to greenish-black. The assay was initiated by mixing the enzyme and reaction mixture. At intervals, 100 μL aliquots of the reaction mixture were removed with a syringe and placed directly on ice to terminate the reaction. AG1-X8 (Bio-Rad) columns were prepared for each time point. Portions of the reaction mixture (50 μL) from each time point were applied to the column, and the ^{14}C-methylcobalamin and cob(III)alamin were eluted with H_2O (2 × 1 mL). Any

unreacted ^{14}C-CH_3-H_4folate, which is anionic, remained bound to the column. Scintillation fluid (10 mL) was added to the pooled washes and the samples were counted to determine the total methylcobalamin produced. A zero time point was determined that contains all the reaction components except the enzyme. To quantify the total number of counts associated with complete conversion of (6*S*)-CH_3-H_4folate to H_4folate, 50 μL of the reaction mixture was added to 2 mL of H_2O and 10 mL of scintillation fluid. The number of counts was halved due to the presence of the unreactive 6*R* isomer of 5-CH_3-H_4folate. The rate of reaction was found to be linear for the first 3−20 min; the extent of linearity depended on the initial concentration of cob(I)alamin. The initial rate of reaction is dependent on the concentrations of cob(I)alamin, 5-CH_3-H_4folate, and enzyme. The concentration of radiolabeled methylcobalamin produced is calculated from

$$[\text{methylcobalamin produced}]_t = [(6S)\text{-}CH_3\text{-}H_4\text{folate}]_0\left[\frac{\text{cpm}_t - \text{cpm}_{\text{blank}}}{(\text{cpm}_{\text{total}}/2)}\right] \quad (5)$$

Homocysteine−Methylcobalamin Methyltransferase Assay. The reaction rate was monitored spectrophotometrically in the dark at 37 °C using a procedure modified from that described by Taylor (*20*). The assay was carried out under anaerobic conditions using a 0.4 cm path length cuvette with a side arm. The initial reaction mixture contained 0.125−1.0 mM methylcobalamin, 50 mM Tris-chloride buffer, pH 7.2, 100 mM KCl, and 160 μg of enzyme, with enough homocysteine in the side arm to reach a final concentration of 0.5 mM after addition. The reaction mixture was exchanged with argon as described above. The cuvette was incubated at 37 °C for 10 min and then the assay was initiated by the addition of homocysteine. The reaction was followed by observing the disappearance of methylcobalamin at 525 nm. At each methylcobalamin concentration, the period during which product formation was linear with time (∼3−7 min) was determined. The absorbance at 525 nm was measured before the reaction was initiated. To determine the change in $A_{525\text{nm}}$ when all the methylcobalamin was consumed, once the reaction appeared to have reached completion the cuvette was immersed in an ice bath and was exposed to a tungsten/halogen lamp (650 W) at a distance of 10 cm. Two exposures of 30 s were sufficient for complete photolytic demethylation of the remaining methylcobalamin. The absorbance at 525 nm was measured after all the methylcobalamin had been converted to cob(II)alamin.

Under conditions where cob(II)alamin is the predominant product formed during the course of the reaction, the concentration of methylcobalamin consumed can be calculated from the absorbance change at 525 nm as shown:

$$[\text{methylcobalamin}]_t = \Delta\,\text{abs}_t[\text{methylcobalamin}]_0/\Delta\,\text{abs}_\infty \quad (6)$$

Tetrahydrofolate−Methylcobalamin Methyltransferase Assay. The tetrahydrofolate solution was prepared from solid tetrahydrofolate in degassed H_2O at pH 8.0 and then neutralized with degassed dilute HCl. The reaction is monitored at 525 nm, using procedures that are the same as in the homocysteine−methylcobalamin methyltransferase assay. The assay was carried out under anaerobic conditions using a 0.4 cm path length cuvette with a side arm. The initial reaction mixture contained 0.125−1.0 mM methylcobalamin, 50 mM Tris-chloride buffer, pH 7.2, 100 mM KCl, and 160 μg of enzyme, with enough tetrahydrofolate in the side arm to reach a final concentration of 0.5 mM after addition. The assay was carried out under the same conditions as the methylcobalamin−homocysteine methyltransferase assay described above, except that tetrahydrofolate rather than homocysteine was added to initiate the reaction. The final absorbance at 525 nm was measured after all the methylcobalamin had been converted to cob(II)alamin. Under conditions where cob(II)alamin is the predominant product formed during the course of the reaction, the concentration of methylcobalamin consumed can be calculated from the absorbance change at 525 nm as shown in eq 6.

RESULTS

MetH(2−1227) Catalyzes Methyl Transfer from CH_3-H_4Folate to Free Cob(I)alamin. We wished to determine whether methionine synthase holoenzyme could catalyze methyl transfer from CH_3-H_4folate to free cob(I)alamin. To produce free cob(I)alamin we reduced hydroxocob(III)alamin with a 10-fold excess of titanium(III) citrate; the complete conversion of cob(III)alamin to cob(I)alamin was confirmed by UV/visible spectroscopy using a $\Delta\epsilon_{388}$ of 20 000 M^{-1} cm^{-1}. The MetH holoenzyme was initially in the inactive cob(II)alamin form but is reduced to the endogenous cob(I)alamin form by titanium(III) citrate; since no homocysteine is present in the reaction mixture, the endogenous cobalamin can only undergo a single cycle of methylation. We followed the methylation of free cob(I)alamin with (6*S*)-CH_3-H_4folate catalyzed by 1.2 μM MetH holoenzyme using the radioactive assay described in the Materials and Methods section. To ensure that methyl transfer was being catalyzed by MetH holoenzyme, we varied the concentration of enzyme while keeping the substrate concentrations constant, and we obtained a linear relationship between the measured initial rate and enzyme concentration (data not shown). The initial rate of methylation was then determined with concentrations of free cob(I)alamin ranging from 0.25 to 3.5 mM, while keeping the concentration of titanium(III) citrate (12 mM) and (6*S*)-CH_3-H_4folate (0.25 mM) constant in all reactions (Figure 2). The reaction rate increased as the concentration of cob(I)alamin increased, and even at 3.5 mM exogenous cob(I)alamin no saturation was observed (Figure 2, inset). The reaction was found to be first-order in exogenous cob(I)alamin at concentrations greater than 1.5 mM and the calculated second-order rate constant is 429 M^{-1} s^{-1}. As shown in the inset to Figure 2, at the lower concentrations of exogenous cob(I)alamin we observed apparent inhibition of the rate of methyl transfer, possibly due to the high ratio of titanium(III) citrate to cob(I)alamin. To confirm that the methyl transfer from CH_3-H_4folate to cob(I)alamin formed methylcobalamin we followed the methylation of free cob(I)alamin (0.1 mM) with (6*S*)-CH_3-H_4folate (0.25 mM) catalyzed by MetH holoenzyme using UV/visible spectroscopy. Cob(I)alamin has an absorption peak at 388 nm (ϵ = 26 000 cm^{-1} M^{-1}) and methylcobalamin has an absorption peak at 525 nm (ϵ = 9100 cm^{-1} M^{-1}); one can monitor the disappearance of cob(I)alamin at 388 nm and the appearance of methylcobalamin at 525 nm. Such an experiment is shown in Figure 3; the experiment was carried out with a

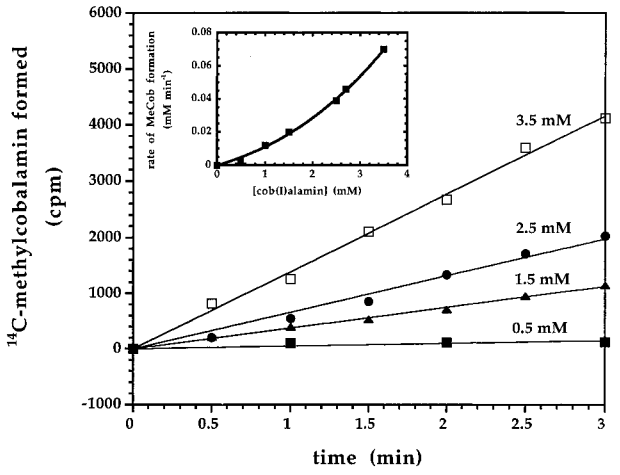


FIGURE 2: Formation of radiolabeled methylcobalamin from exogenous cob(I)alamin and CH_3-H_4folate catalyzed by MetH holoenzyme. The initial concentrations of exogenous cob(I)alamin were 0.5 mM (■), 1.5 mM (▲), 2.5 mM (●), and 3.5 mM (□). Cob(I)alamin was generated from hydroxocobalamin by reduction with 12 mM titanium(III) citrate in the presence of (6*R*,*S*)-(^{14}C)-CH_3-H_4folate (0.5 mM) in 50 mM Tris-chloride buffer (pH 7.2) at 37 °C; the reaction was initiated by addition of 1.2 μM MetH holoenzyme. Inset: The initial rate of methylcobalamin formation against cob(I)alamin concentration catalyzed by MetH holoenzyme is plotted as a function of the concentration of exogenous cob(I)-alamin.

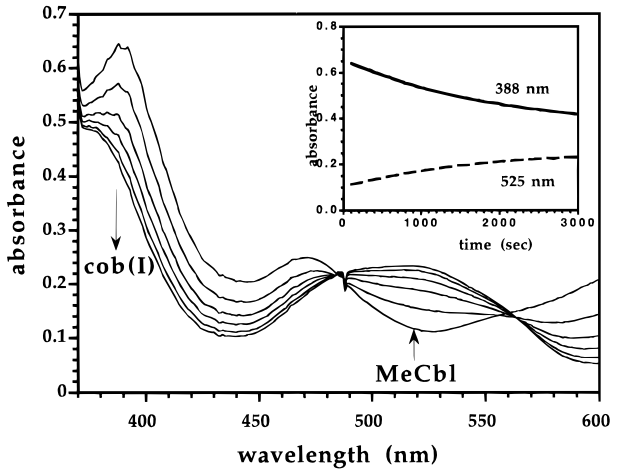


FIGURE 3: Absorbance changes associated with methyl transfer from (6*R*,*S*)-CH_3-H_4folate (0.5 mM) to exogenous cob(I)alamin (0.1 mM). Two equivalents of titanium(III) citrate were used to reduce hydroxocobalamin to cob(I)alamin. The spectra shown are taken at 0, 500, 1000, 1500, 2000, and 3500 s after addition of enzyme to initiate the reaction. The change in absorbance at 600 nm is due largely to oxidation of titanium(III) citrate to titanium(IV) citrate. Inset: The increase in absorbance with time at 525 nm correlates with the formation of methylcobalamin, and the decrease in absorbance at 388 nm correlates with disappearance of cob(I)alamin as it becomes methylated.

1:2 molar ratio of exogenous cob(III)alamin to titanium(III) citrate and one can see a small amount of cob(II)alamin (λ_{max} at 474 nm, $\epsilon = 9470$ cm^{-1} M^{-1}).

Methyl Transfer from Free Methylcobalamin to H_4folate Is Also Catalyzed by MetH Holoenzyme. We looked for catalysis of methyl transfer from methylcobalamin to H_4folate, the reverse of the reaction demonstated in the previous section. The holoenzyme was in the inactive endogenous cob(II)alamin form throughout the reaction, as no reducing agent was present. The reaction of H_4folate and free methylcobalamin catalyzed by MetH holoenzyme was followed by visible spectroscopy, observing the spectral change at 525 nm, which is associated with the demethylation of methylcobalamin. Even under anaerobic conditions, in the presence of H_4folate, cob(I)alamin is oxidized to cob(II)-alamin. The initial rate of reaction plotted against initial concentration of exogenous methylcobalamin yielded a linear relationship; no saturation was observed up to a concentration of 1.5 mM methylcobalamin. The second-order rate constant for methyl transfer is 256 M^{-1} s^{-1}, which is about half as fast as the rate constant for the forward reaction with CH_3-H_4folate and exogenous cob(I)alamin.

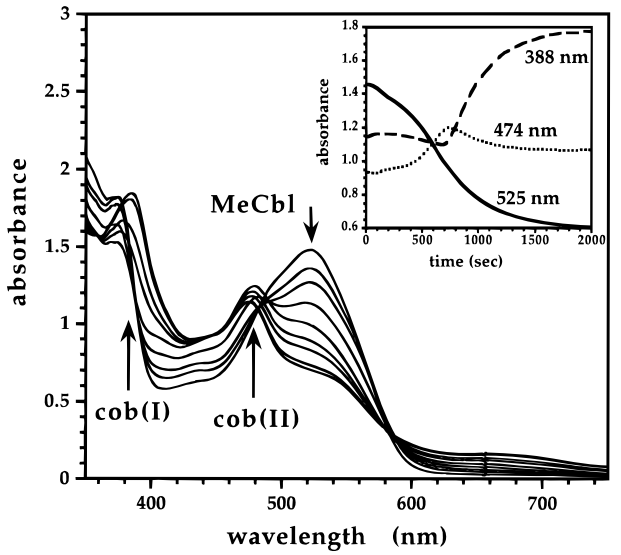


FIGURE 4: Absorbance changes associated with methyl transfer from exogenous methylcobalamin (0.125 mM) to homocysteine (0.5 mM). The reaction was carried out in 50 mM Tris-chloride buffer, pH 7.2, containing 100 mM KCl. The products formed are a combination of cob(I)alamin (λ_{max} at 388 nm) and cob(II)alamin (λ_{max} at 474 nm). The spectra shown are taken at 0, 200, 400, 600, 800, 1000, 1200, 1400, and 1600 s after addition of homocysteine to initiate the reaction. Inset: The rate of demethylation of methylcobalamin by homocysteine monitored at 525 nm is compared with the rate of formation of cob(I)alamin, monitored at 388 nm, and cob(II)alamin, monitored at 474 nm.

MetH Holoenzyme Catalyzes Methyl Transfer from Free Methylcobalamin to Homocysteine. Methyl transfer from methylcobalamin to homocysteine was monitored at 525 nm by UV/visible spectrometry. The reaction was catalyzed by MetH holoenzyme with its prothestic group in the inactive cob(II)alamin form. The rate of methyl transfer is first-order in methylcobalamin as previously observed (*9*), yielding a second-order rate constant of 2430 M^{-1} s^{-1}. Under the conditions of these experiments, cob(II)alamin was the major product formed, as described previously (*9*). One would expect cob(I)alamin to be the initial product of demethylation of methylcobalamin by homocysteine (*1*) but it has been reported that cob(I)alamin is rapidly oxidized to cob(II)-alamin in the presence of disulfides (*21*). In Figure 4, we show an experiment in which homocysteine was freshly prepared by hydrolysis of the thiolactone (*17*). It can be seen that cob(II)alamin is the major product observed initially, although some cob(I)alamin appears to be formed rapidly, which then decays to cob(II)alamin. Cob(I)alamin is the predominant product formed later in the reaction. This experiment supports the formation of cob(I)alamin as the initial product of the reaction, if we assume rapid oxidation to cob(II)alamin results from the presence of substoichiometric homocystine in the homocysteine.

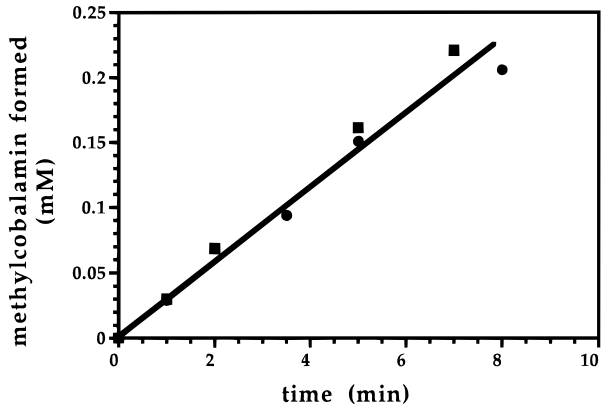


FIGURE 5: Comparison of the rate of methyl transfer from (6R,S)-CH_3-H_4folate (0.5 mM) to exogenous cob(I)alamin (0.5 mM) catalyzed by (a) 1.2 μM MetH holoenzyme (●) and (b) 1.2 μM His759Gly mutant protein (■). The reaction was carried out at 37 °C in 50 mM Tris-chloride buffer (pH 7.2).

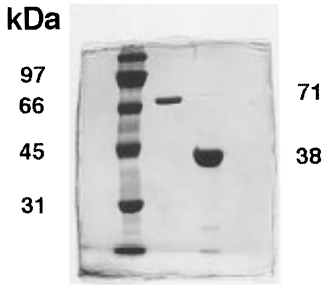


FIGURE 6: Overexpression and purification to homogeneity of MetH(2−649) and MetH(2−353). Analysis by SDS−PAGE is shown: lane 1, standards; lane 2, purified MetH(2−649) protein; lane 3, purified MetH(2−353) protein.

His759Gly Mutant Holoenzyme Catalyzes Methyl Transfer from CH_3-H_4Folate to Free Cob(I)alamin at the Same Rate as MetH Holoenzyme. We have shown in the previous sections that MetH holoenzyme can utilize exogenous methylcobalamin as a methyl donor and exogenous cob(I)-alamin as a methyl acceptor as well its endogenous cobalamin cofactor. The question arises whether or not the endogenous cobalamin cofactor is required for the exogenous cobalamin methyl transfer reactions. His759 is coordinated to the lower axial position of the endogenous cobalamin in the wild-type enzyme; mutation of His759 to glycine results in an enzyme in which endogenous cofactor is unreactive toward homocysteine and methyltetrahydrofolate (*12*). The His759Gly mutant holoenzyme was found to catalyze all three methyl transfers with exogenous cobalamin as a methyl acceptor or a methyl donor. As shown in Figure 5, the initial rate of the CH_3-H_4folate−cob(I)alamin methyltransferase reaction was the same whether MetH holoenzyme or His759Gly holoenzyme was used as catalyst. This result suggests that the endogenous cobalamin cofactor plays no role in the exogenous cobalamin reactions, as demonstrated by Taylor (*20*). Therefore, these exogenous cobalamin methyl transfer reactions can be used as a tool to define residues responsible for substrate binding and activation of the isolated MetH(2−649) region, which lacks the endogenous cobalamin binding region.

Preliminary Characterization of the MetH(2−649) Protein. MetH(2−649) was overexpressed and purified to homogeneity as seen on the SDS−PAGE gel shown in Figure 6. The N-terminal sequence of MetH(2−649) was determined by Edman degradation and yielded amino acids corresponding to residues 2−7 of wild-type methionine synthase, indicating that the N-terminal methionine is removed after translation. The observed mass of 70 949 ± 32 Da determined by electrospray mass spectrometry is in good agreement with the predicted mass of 70 982 for residues 2−649 of wild-type MetH holoenzyme. MetH(2−649) was found to lack catalytic activity in the CH_3-H_4folate−homocysteine methyltransferase assay, as expected for a protein that lacks endogenous cobalamin.

MetH(2−649) Protein Retains Catalytic Activity with Exogenous Cobalamin. As with MetH holoenzyme, methylation of exogenous cob(I)alamin with CH_3-H_4folate is catalyzed by MetH(2−649) protein. The initial rate of reaction is first-order with respect to cob(I)alamin concentration between 1.5 and 3.5 mM, as observed with MetH holoenzyme (data not shown). The second-order rate constant of 593 M^{-1} s^{-1} is slightly higher than that for the MetH holoenzyme (429 M^{-1} s^{-1}). MetH(2−649) protein also catalyzed the methylation of homocysteine by exogenous methylcobalamin and the reaction rate was first-order with respect to exogenous methylcobalamin concentration (data not shown). The second-order rate constant for MetH(2−649) protein, 2320 M^{-1} s^{-1}, was identical to the second-order rate constant calculated for MetH holoenzyme.

Expression of MetH(2−353) Protein. MetH(2−353) protein was overexpressed and purified to homogeneity (Figure 6). N-Terminal sequencing showed a sequence corresponding to residues 2−7 of the intact holoenzyme. Electrospray mass spectrometry indicated a mass of 38 082.4 ± 15 Da, in good agreement with the predicted mass of 38 128 Da. As with the MetH(2−649) protein, MetH(2−353) protein was found to lack catalytic activity in the CH_3-H_4folate−homocysteine methyltransferase assay. The reactions with exogenous cobalamin were then carried out using MetH(2−353) protein as the catalyst. Methyl transfer from methyltetrahydrofolate to exogenous cob(I)alamin was not catalyzed by MetH(2−353) protein. However, the MetH-(2−353) protein did catalyze methyl transfer from exogenous methylcobalamin to homocysteine, with a second-order rate constant of 2430 M^{-1} s^{-1}, similar to that of MetH(2−649) protein and MetH holoenzyme (data not shown). One can conclude that the determinants for homocysteine binding and reactivity lie within residues of 2−353 of MetH, while at least some of the determinants for methyltetrahydrofolate binding and reactivity do not. By elimination, the region encompassing residues 354−649 of MetH is likely to be responsible for CH_3-H_4folate binding and activation, as previously suggested by Roberts et al. (*7*). We also constructed a plasmid for overexpression of MetH(353−649) protein by deleting the DNA sequence that specifies the first 353 residues of MetH in plasmid pCWG-02. Unfortunately, the MetH(353−649) protein did not overexpress under normal conditions of expression for the MetH holoenzyme, MetH(2−649) protein, or MetH(2−353) protein.

Effects of the Mutations Cys310Ala, Cys310Ser, Cys311Ala, and Cys311Ser in MetH Holoenzyme and MetH(2−649) Protein. All the cobalamin-dependent methionine synthase sequences thus far obtained show a conserved region between residues 308 and 320, GGCCGTxPxHI, and this sequence is also conserved in betaine−homocysteine methyltransferase (*6*), suggesting that determinants for homocysteine binding and/or activation might be contained in this region. Within this motif the two consecutive conserved cysteines, flanked

Table 2: Specific Activities of Wild-Type and Mutant MetH Proteins

form of MetH	specific activity (μmol min^{-1} mg^{-1})	activity as compared to wild-type MetH (%)
wild type	11.81	100
Cys310Ala	0.011	< 0.1[a]
Cys310Ser	0.018	< 0.1[a]
Cys311Ala	0.014	< 0.1[a]
Cys311Ser	0.021	< 0.1[a]

[a] Strain XL1-Blue, in which our MetH proteins are expressed, contains a wild-type chromosomal copy of the *metH* gene in addition to the overexpression plasmids. Thus crude extracts contain approximately 0.1% (relative to the plasmid-specified protein) wild-type MetH, which copurifies with the desired mutant enzymes.

Table 3: Comparison of the Second-Order Rate Constants for Reaction of MetH and Its Truncated Derivatives with Exogenous Cobalamin

form of MetH	cob(I)alamin + CH_3-H_4folate (M^{-1} s^{-1})	methylcobalamin + Hcy (M^{-1} s^{-1})
wild type	594	2320
Cys310Ala	593	< 10
Cys311Ala	590	< 10

by three glycines, are of particular interest, as studies of cobalamin-independent methionine synthase show that a conserved cysteine, Cys726, is required for the activation of homocysteine (*22*). Therefore Cys310Ala, Cys310Ser, Cys311Ala, and Cys311Ser mutant MetH holoenzyme proteins were overexpressed and purified to homogeneity. The wild-type and mutant methionine synthase proteins were assayed for their ability to catalyze methyltetrahydrofolate−homocysteine methyl transfer, and it was found that the Cys310 and Cys311 mutants all had background activity as compared to wild-type MetH holoenzyme protein[3] (Table 2). This result indicated that the cysteine mutations have a dramatic effect on primary turnover of MetH holoenzyme, so the corresponding mutant MetH(2−649) proteins were overexpressed and purified to homogeneity. Methyl transfer reactions with exogenous cob(I)alamin and CH_3-H_4folate, or exogenous methylcobalamin and homocysteine, were carried out with mutant and wild-type MetH(2−649) proteins, to monitor the ability of the mutant proteins to bind and activate homocysteine and methyltetrahydrofolate. We observed that the cysteine mutations had no effect on methyltetrahydrofolate−cob(I)alamin methyltransferase activity (Table 3). On the other hand, the cysteine mutant proteins were unable to catalyze the methylation of homocysteine by exogenous methylcobalamin (Table 3).

DISCUSSION

These studies suggest that methionine synthase is a modular protein that presents methyl donors and/or acceptors to endogenous cobalamin from three distinct regions of the polypeptide. The N-terminal module, comprising residues 2−353, binds and activates homocysteine independently of the remaining downstream residues, while the fragment containing residues 2−649 binds and activates both methyltetrahydrofolate and homocysteine. Our results do not address the issue of whether the second module of methionine synthase, residues 354−649, is completely responsible for the binding and activation of methyltetrahydrofolate or whether the first 353 amino acids of MetH contain determinants required for this activation. However, the sequence similarities between residues 354−649 of methionine synthase and the corrinoid iron-sulfur protein methyltransferase from *Clostridium thermoaceticum* (*7*) lead us to postulate that the second module of methionine synthase does contain all the determinants necessary to bind and activate methyltetrahydrofolate for methyl transfer to cob(I)alamin. The third module, containing residues 650-896, binds the cobalamin cofactor, while the fourth module, comprising residues 897−1227, binds adenosylmethionine.

The reactions catalyzed by the homocysteine and methyltetrahydrofolate binding/activation modules have analogies in acetogens and the methanogenic archaebacteria, although thus far cobalamin-dependent methionine synthase has not been identified in any archaebacterium. Acetogens such as *Clostridium thermoaceticum* convert carbon dioxide and molecular hydrogen to acetate (*23, 24*). In this pathway a cobamide-dependent methyltransferase plays an essential role in providing methyl groups for the synthesis of acetyl CoA by carbon monoxide dehydrogenase. The transfer of the methyl group of methyltetrahydrofolate to the corrinoid iron-sulfur protein of *C. thermoaceticum*, catalyzed by a methyltransferase that lacks any organic cofactor (*7*), is similar to the methyltetrahydrofolate half-reaction of methionine synthase. Exogenous cob(I)alamin can replace the protein-bound corrinoid that is the methyl acceptor *in vivo* (*11*).

For the production of methane from carbon dioxide and hydrogen in methanogens, methyltetrahydromethanopterin, which is an analogue of methyltetrahydrofolate, provides methyl groups for the methylation of coenzyme M, and this methyl group is then reduced to form methane. Methane can also be generated from acetate, methanol, or methylamines, and these pathways involve other cobamide-dependent methyltransferases that provide methyl groups for coenzyme M. The corrinoid-containing subunit associated with the methyltetrahydromethanopterin:coenzyme M methyltransferase complex in *Methanobacterium thermoautotrophicum* has been shown to have a histidine ligand to the cobamide cofactor (*25*). In *Methanosarcina barkeri*, depending on the substrate used as a source of methyl groups, different pathways lead to the production of methylcoenzyme M. The corrinoid-binding β subunit of one such 480 kDa coenzyme M methyltransferase complex in *M. barkeri* has a signature sequence that strongly suggests that the cobamide cofactor has a histidine ligand (*26*). The 480 kDa complex is an octamer of $\alpha\beta$ heterodimers. Grahame and his colleagues have studied two of the isozymes responsible for transfer of methyl groups from methylcobamide-containing proteins to coenzyme M in *M. barkeri* and have shown that they are both zinc-containing metalloenzymes (*27*). These proteins show sequence homology with the 40 kDa α component of the 480 kDa complex, suggesting that the α peptide is responsible for methylcobamide−coenyzme M methyl transfer in the complex (*26*). They contain a conserved T/LV/ILHICG sequence near their C-terminus that is a potential candidate for the zinc-binding site (*27*). Although the sequences of these polypeptides are homologous with each other, they do not show sequence homology

[3] *E. coli* strain XL1-Blue, in which our MetH proteins are expressed, contains a wild-type chromosomal copy of the *metH* gene, in addition to the overexpression plasmids. Thus crude extracts contain approximately 0.1% (relative to the plasmid-specified protein) wild-type MetH, which copurifies with the intact holoenzyme.

with the homocysteine-binding region of methionine synthase or with betaine−homocysteine methyltransferase.

The reactions of the cobamide-dependent methyltransferases associated with methanogenic metabolism are chemically similar to the half-reactions of methionine synthase utilizing homocysteine and methyltetrahydrofolate. Each of these enzymes contains a bound corrinoid that must be methylated by a donor substrate bound to another polypeptide or domain, and each has a separate polypeptide or domain responsible for catalyzing transfer to a sulfur-containing substrate. Furthermore, each enzyme can catalyze methyl transfer to or from exogenous cobalamin as well as to or from the enzyme-bound cobamide, consistent with a modular protein construction in which the methyltransferase activities are localized on domains or polypeptides distinct from the cobalamin-binding region. As noted above, the cobalamin-binding region of methionine synthase shows sequence homology with the β-subunit of a coenzyme M methyltransferase complex from *M. barkeri* (*26*), and even the AdoMet-binding activation domain of methionine synthase has an analogue in the ATP-binding methyltransferase activator protein involved in the activation of the corrinoid subunit of the methanol:cobamide methyltransferase complex from *M. barkeri* (*28*, *29*).

The ability of each of these methyltransferases to use exogenous cob(I)alamin as a methyl acceptor and exogenous methylcobalamin as a methyl donor shows that ligand replacement of dimethylbenzimidazole by histidine is not essential for methyl transfer to and from cobalamin. The second-order rate constants in exogenous cobalamin reactions cannot be directly compared with first-order rate constants with endogenous cobalamin reactions. However, one can estimate the concentration of exogenous methylcobalamin required to attain the observed rate of methyl transfer from endogenous methylcobalamin to homocysteine, which is 140 s^{-1} (*1*); the concentration of methylcobalamin required to achieve this rate is 65 mM. We calculate that the maximal concentration of methylcobalamin that could be achieved at the enzyme active site, where the reactants have been taken out of dilute solution and are held in close proximity to each other, would be about 1 M. This comparison implies that exogenous methylcobalamin is an effective substrate in the methyl transfer reaction to homocysteine and suggests that the observed rate of methyl transfer from endogenous methylcobalamin to homocysteine may be limited by factors other than the intrinsic chemical reactivity.

The reaction between exogenous methylcobalamin and the free thiolate of 2-mercaptoethanol is 10^6 times slower than the enzyme-catalyzed methyl transfer from exogenous methylcobalamin to bound homocysteine, even though both thiols have microscopic p*K* values of ~10. We propose that the factors which contribute to the considerable rate enhancement of the enzyme-catalyzed methyl transfer cannot be limited to the formation of enzyme-bound thiolate (*21*).

We have shown that the methyl transfer reactions using exogenous cobalamin as a substrate are catalyzed at the same rate when either the holoenzyme or MetH(2−649) of methionine synthase is the catalyst. From these observations we can conclude that the C-terminal half of methionine synthase, including the bound cobalamin, does not block access of exogenous cobalamin to the bound homocysteine. As the methyl transfer reactions are first-order in exogenous cobalamin over the range of concentrations examined, there

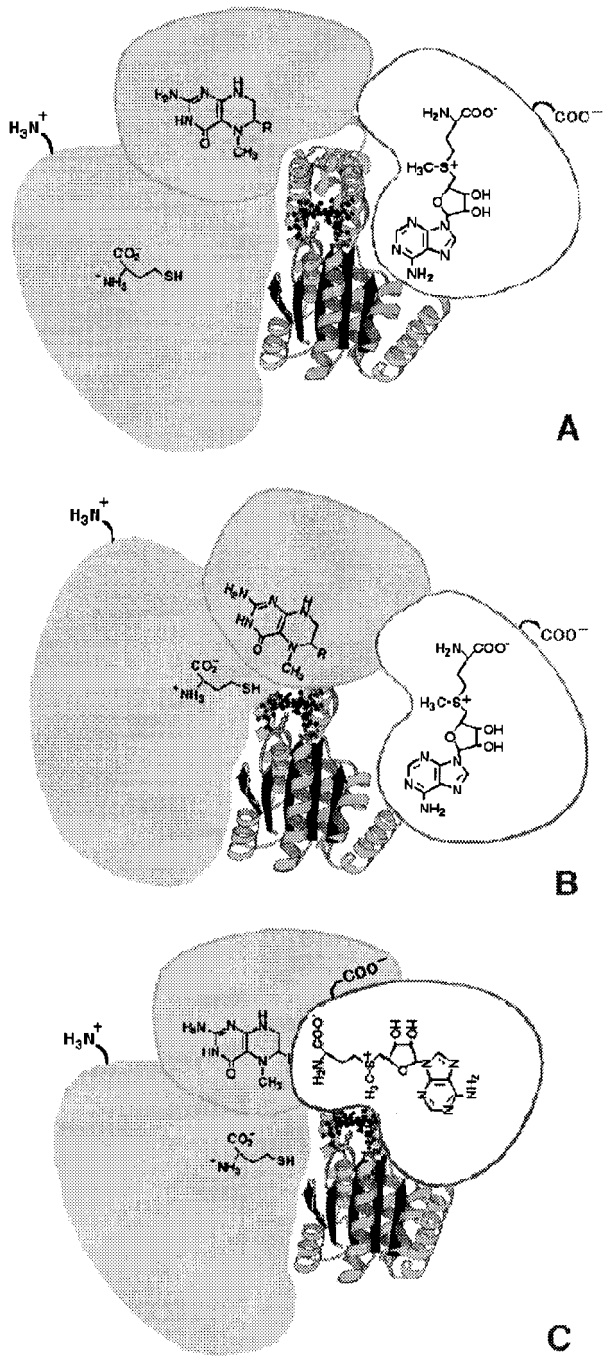


FIGURE 7: Representation of the three postulated conformational states: (A) the resting conformational state; (B) turnover conformation; and (C) the activation conformation.

is no evidence that exogenous cobalamin binds to methionine synthase, so one may assume that the substrates homocysteine and methyltetrahydrofolate are not deeply buried within the protein structure but lie on the surface of their respective modules. Because mutations of cysteine residues in the homocysteine-binding region abolish activity with the endogenous cofactor, it is likely that this region of the protein also presents homocysteine to the cobalamin, suggesting that exogenous cobalamin reacts with an enzyme conformation that differs from the conformation catalyzing methyl transfer to and from endogenous cobalamin. This raises the question of how endogenous cobalamin performs the three different methyl transfer reactions within the catalytic cycle. The two methyl transfers in primary turnover from endogenous methylcobalamin to homocysteine and from methyltetrahy-

drofolate to endogenous cobalamin requires the substrates to be bound to the N-terminal 70 kDa region, which must interact with the central cobalamin binding region; and the third methyl transfer from adenosylmethionine to endogenous cobalamin involves the interaction of the C-terminal 38 kDa region with the central cobalamin binding region. To accommodate the interactions of the central cobalamin binding region with both the N-terminal and C-terminal regions of methionine synthase to perform three different methyl transfers, we postulate that methionine synthase has three different conformational states (Figure 7). The X-ray crystal structure of the cobalamin binding region of methionine synthase consists of two separate domains, a cobalamin binding domain that interacts with the lower face of the prosthetic group and a helical "cap" that covers the upper face (*12*); in the resting conformational state of methionine synthase, the cap domain protects the methyl group of methylcobalamin from interactions with substrates and solvent (*30*). During primary turnover, we postulate that the position of the cap domain is shifted to allow the N-terminal 70 kDa substrate binding/activation region access to the cobalamin cofactor to perform methyl transfers that require the substrates to be positioned directly above the cobalamin cofactor; this we designate as the turnover conformation. When endogenous cob(I)alamin becomes oxidized to the inactive cob(II)alamin form, we propose that the N-terminal substrate binding region returns to its resting position and the C-terminal adenosylmethionine binding domain is now positioned over the endogenous cobalamin cofactor to allow adenosylmethionine to remethylate the prosthetic group (activation conformation).

Efforts are underway to probe the mechanism of binding and activation of homocysteine and will be the topic of a subsequent paper. We note, however, that a cysteine residue is essential for the activity of cobalamin-independent methionine synthase, which also catalyzes methyl transfer from methyltetrahydrofolate to homocysteine (*22, 31)*. In cobalamin-independent methionine synthase, the essential cysteine is thought to be a ligand to a zinc ion, which binds homocysteine, activating it for nucleophilic attack on the methyl group of methyltetrahydrofolate (*22*). Zinc has also been detected in two of the methylcobamide:coenzyme M methyltransferases from *M. barkeri*, and treatment with EDTA results in complete loss of enzyme activity (*27*). Alignment of the deduced amino acid sequences of the three methylcobamide:coenzyme M methyltransferase isozymes thus far sequenced indicates three conserved cysteine residues (*26, 32*), and three cysteines are conserved in alignments of the homocysteine-binding regions of cobalamin-dependent methionine synthase from diverse sources.

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BI9705164