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Methionine Synthase Exists in Two Distinct Conformations That Differ in Reactivity toward Methyltetrahydrofolate, Adenosylmethionine, and Flavodoxin†

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ABSTRACT: Methionine synthase (MetH) from *Escherichia coli* catalyzes the synthesis of methionine from homocysteine and methyltetrahydrofolate via two methyl transfer reactions that are mediated by the endogenous cobalamin cofactor. After binding both substrates in a ternary complex, the enzyme transfers a methyl group from the methylcobalamin cofactor to homocysteine, generating cob(I)alamin enzyme and methionine. The enzyme then catalyzes methyl transfer from methyltetrahydrofolate to the cob(I)-alamin cofactor, forming methylcobalamin cofactor and tetrahydrofolate prior to the release of both products. The cob(I)alamin form of the enzyme occasionally undergoes oxidation to an inactive cob(II)alamin species; the enzyme also catalyzes its own reactivation. Electron transfer from reduced flavodoxin to the cob-(II)alamin cofactor is thought to generate cob(I)alamin enzyme, which is then trapped by methyl transfer from adenosylmethionine to the cobalt, restoring the enzyme to the active methylcobalamin form. Thus the enzyme is potentially able to catalyze two methyl transfers to the cob(I)alamin cofactor: methyl transfer from methyltetrahydrofolate during primary turnover and methyl transfer from adenosylmethionine during activation. It has recently been shown that methionine synthase is constructed from at least four separable regions that are responsible for binding each of the three substrates and the cobalamin cofactor, and it has been proposed that changes in positioning of the substrate binding regions vis-à-vis the cobalamin binding region could allow the enzyme to control which substrate has access to the cofactor. In this paper, we offer evidence that methionine synthase exists in two different conformations that interconvert in the cob(II)alamin oxidation state. In the primary turnover conformation, the enzyme reacts with homocysteine and methyltetrahydrofolate but is unreactive toward adenosylmethionine and flavodoxin. In the reactivation conformation, the enzyme is active toward adenosylmethionine and flavodoxin but unreactive toward methyltetrahydrofolate. The two conformations differ in the susceptibility of the substrate-binding regions to tryptic proteolysis. We propose a model in which conformational changes control access to the cobalamin cofactor and are the primary means of controlling cobalamin reactivity in methionine synthase.

Cobalamin-dependent methionine synthase catalyzes the transfer of a methyl group from CH_3-H_4folate[1] to homocysteine to form H_4folate and methionine, using the tightly bound cobalamin cofactor as the intermediate methyl donor and acceptor (Figure 1). The free methylcobalamin enzyme binds CH_3-H_4folate and homocysteine in a ternary complex and then transfers the methyl group from the methylcobalamin cofactor to homocysteine, forming methionine and generating the cob(I)alamin cofactor as a transient intermediate (*2*). Methyl transfer from CH_3-H_4folate to the cob(I)-alamin cofactor regenerates the methylcobalamin cofactor prior to the release of both H_4folate and methionine (*2*). The cob(I)alamin prosthetic group is a potent reductant and is occasionally oxidized by oxygen and/or protons, generating inactive cob(II)alamin enzyme (*3*, *4*).

In *Escherichia coli* the inactive cob(II)alamin enzyme is returned to the primary turnover cycle by a reductive methylation that involves reduced flavodoxin (*5*) and AdoMet (*6*, *7*). It has been proposed that electron transfer from reduced flavodoxin leads to formation of cob(I)alamin enzyme, which is then methylated by AdoMet (*8*, *9*). However, cob(I)alamin is not observed as an intermediate in this reaction. Electron transfer from flavodoxin hydroquinone or semiquinone to the cob(II)alamin cofactor is thermodynamically unfavorable, since the midpoint potential for the cob(I)alamin/cob(II)alamin couple [$E_{m7} = -490$ mV (*10*, *11*)] is lower than that for either the flavodoxin hydroquinone/semiquinone couple [$E_m = -450$ mV (*12*)]

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[1] Abbreviations: AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; Fld, flavodoxin; Hcy, L-homocysteine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CH_3-H_4Pte(Glu)$_3$, 5-methyltetrahydropteroyltri-γ-L-glutamate; CH_2-H_4folate, 5,10-methylenetetrahydrofolate; CH_3-H_4folate, 5-methyltetrahydrofolate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; PMSF, phenylmethanesulfonyl fluoride; SHE, standard hydrogen electrode; H_4folate, 5,6,7,8-tetrahydrofolate; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

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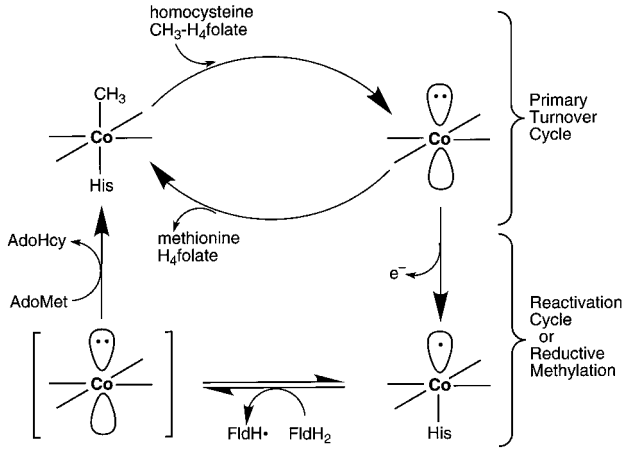

FIGURE 1: Generalized scheme for the primary turnover and reactivation cycles of methionine synthase. Homocysteine and CH_3-H_4folate bind in a ternary complex with methylcobalamin enzyme prior to methyl transfer from methylcobalamin to homocysteine, generating the cob(I)alamin enzyme. Methyl transfer from CH_3-H_4folate to cob(I)alamin regenerates the methylcobalamin enzyme. Occasional oxidation of the cob(I)alamin enzyme results in formation of the inactive cob(II)alamin enzyme. Reactivation of cob(II)alamin enzyme is initiated by electron transfer from reduced flavodoxin, presumably forming cob(I)alamin enzyme, which is then trapped by irreversible methyl transfer from AdoMet, regenerating the active methylcobalamin enzyme. As indicated by brackets, cob(I)alamin is not directly observed as an intermediate in reductive methylation.

or the flavodoxin semiquinone/oxidized couple [$E_m = -250$ mV (*12*)]. Thus electron transfer from reduced flavodoxin to cob(II)alamin may generate an unobservable amount of cob(I)alamin enzyme and methyl transfer from the highly reactive sulfonium, AdoMet, may be used to trap this cob(I)alamin enzyme and regenerate the resting methylcobalamin enzyme (*9*).

The complex requirement for both CH_3-H_4folate and AdoMet for maximal activity has been the subject of several investigations. Early preparations of the enzyme required trace amounts of AdoMet in order to activate the enzyme for turnover with CH_3-H_4folate and homocysteine (*6*, *7*). It was later shown that enzyme was isolated as a mixture of cob(II)alamin and hydroxocob(III)alamin forms and that incubation with [*methyl*-^{14}C]AdoMet and a flavin reducing system leads to formation of [*methyl*-^{14}C]methylcobalamin enzyme (*13*). Incubation with [*methyl*-^{14}C]CH_3-H_4folate and a flavin reducing system, in the absence of AdoMet, did not lead to formation of labeled methylcobalamin enzyme (*14*). However, incubation with [*methyl*-^{3}H]AdoMet and [*methyl*-^{14}C]CH_3-H_4folate (probably with trace H_4folate contamination) in the presence of a reducing system resulted initially in methylation with ^{3}H-methyl from AdoMet, followed by exchange of the ^{3}H-methyl group with ^{14}C-methyl, most likely due to reaction with H_4folate followed by CH_3-H_4folate (*14*). The observation that the enzyme reacts selectively with CH_3-H_4folate or AdoMet under different experimental conditions would seem to be inconsistent with the proposal that both substrates react with the reduced cob(I)alamin cofactor.

Clearly, methionine synthase can distinguish between CH_3-H_4folate and AdoMet in the production of methionine. Under standard assay conditions, using a chemical reducing system and AdoMet to maintain activity, methionine synthase produces methionine from CH_3-H_4folate and homocysteine with a turnover number of 1600 min^{-1} (*2*). In the absence of CH_3-H_4folate, using only AdoMet as methyl donor, methionine synthase produces methionine with a much slower turnover number of ~1 min^{-1} (*1*).[2] As expected, the enzyme is highly selective in using CH_3-H_4folate for the synthesis of methionine. It is less clear to what extent methionine synthase can distinguish between CH_3-H_4folate and AdoMet in the reductive methylation reaction. It has been reported that both substrates can serve as methyl donors in electrochemical titrations monitored by EPR spectrometry (*9*), although CH_3-H_4folate has a much lower thermodynamic driving force for the coupled electron and methyl transfer reactions. These measurements required long equilibration times, and it is possible that the enzyme is able to distinguish between methyl donors during reductive methylation by reacting with AdoMet at a rate that is much more rapid than with CH_3-H_4folate. When the enzyme is assayed in the absence of AdoMet, starting in its inactive cob(II)alamin form, no activity is detected (*4*), suggesting that CH_3-H_4folate will not substitute for AdoMet in steady-state assays.

Recent structural information may shed some light on the ability of methionine synthase to distinguish between methyl donors. Partial proteolysis of the 136 kDa holoprotein with trypsin results in formation of a 98 kDa fragment that retains bound methylcobalamin and a 38 kDa fragment that binds AdoMet (*4*, *15*). The purified 98 kDa fragment with methylcobalamin bound is initially fully active with CH_3-H_4folate, while the 98 kDa fragment with cob(II)alamin bound is unable to catalyze methyl transfer from CH_3-H_4folate to homocysteine even in the presence of AdoMet, suggesting that the 38 kDa fragment is required for reductive activation of cob(II)alamin enzyme (*4*). A crystal structure of the 38 kDa proteolysis fragment reveals tightly bound AdoMet (*16*). Further proteolysis of the 98 kDa fragment results in degradation of the N-terminal regions, and eventual production of a 28 kDa fragment that retains bound methylcobalamin (*4*, *17*). The N-terminal 70 kDa region has been separately expressed and retains the ability to bind CH_3-H_4folate and homocysteine and activate these substrates toward reactions with exogenous cob(I)alamin and methylcobalamin cofactors, respectively (*18*). These results suggest that methionine synthase contains independently folded regions that bind homocysteine, CH_3-H_4folate, and AdoMet. By controlling the positioning of these regions vis-à-vis the cobalamin binding region, methionine synthase may be able to control which methyl donor is able to react with the cob(I)alamin cofactor.

In this paper, we confirm that methionine synthase maintains a strong kinetic separation between CH_3-H_4folate and AdoMet. We use radiolabeled substrates to demonstrate that methionine synthase does not use AdoMet in primary turnover and does not use CH_3-H_4folate in reductive methylation. We demonstrate using equilibrium dialysis that both CH_3-H_4folate and AdoMet bind with the same affinity to methylcobalamin and cob(II)alamin enzyme, eliminating

[2] The rate measured by Taylor and Weissbach (*1*) for methyl transfer from AdoMet to homocysteine corresponds to a turnover number of 0.4 min^{-1}. They estimated that their enzyme preparation was ~30% pure, but the specific activity in the methyltetrahydrofolate−homocysteine methyltransferase reaction is only 12% that of our homogeneous preparations of methionine synthase.

differential binding as a source of differential reactivity. Using stopped-flow spectroscopy, we demonstrate that cob(I)alamin enzyme generated by demethylation of methylcobalamin enzyme reacts with CH_3-H_4folate at a rate that is 6×10^4 faster than that for reaction with AdoMet. On the other hand, cob(I)alamin that is generated by chemical reduction of cob(II)alamin enzyme reacts with AdoMet at a rate that is $>10^5$ faster than that for reaction with CH_3-H_4folate. Oxidized flavodoxin reacts very slowly with cob(I)alamin generated from methylcobalamin enzyme but reacts rapidly with cob(I)alamin generated from cob(II)alamin enzyme. Finally, we use partial proteolysis of enzyme in the methylcobalamin, cob(II)alamin, and cob(I)alamin oxidation states to demonstrate that the enzyme exists in at least two different conformations which differ in the location of exposed basic residues. We propose a model in which conformational changes that occur in the cob(II)alamin oxidation state control access of AdoMet and flavodoxin to the cobalamin cofactor.

MATERIALS AND METHODS

Materials. Construction of the His759Gly mutation of methionine synthase and the methods for purification of overexpressed wild-type and mutant enzymes have been described (*19*). Flavodoxin was overexpressed using the *E. coli* strain DH01 (*20*), which contains the *E. coli fldA* gene ligated into a pTRC99A plasmid (Pharmacia) and introduced into the *E. coli* strain DH5αF′ (*20*−*22*). The following compounds were obtained from the indicated commercial sources: L-homocysteine thiolactone, *S*-adenosyl-L-methionine (iodide salt), and 5,5′-dithiobis(2-nitrobenzoic acid) from Sigma; methyl viologen and protocatechuic acid from Aldrich; (6*R*,*S*)-CH_3-H_4folate (calcium salt) from Schirks Laboratories; and [*methyl*-^{3}H]-*S*-adenosyl-L-methionine from Amersham. AdoMet was purified by reverse-phase chromatography prior to use in assays or binding experiments. Titanium(III) citrate (80 mM) was prepared from titanium(III) chloride (1.9 M in 2 M HCl, Aldrich) as described previously (*18*). [*methyl*-^{14}C]-(6*S*)-CH_3-H_4PteGlu$_3$ was prepared enzymatically from [^{14}C]formaldehyde (ICN) and pteroyltri-γ-L-glutamic acid (Schirks Laboratories) using *Lactobacillus casei* dihydrofolate reductase and pig liver methylenetetrahydrofolate reductase as previously described (*23*).

Determination of Competition between AdoMet and CH_3-H_4folate in Primary Turnover and Reductive Methylation. To determine the extent to which AdoMet competes with CH_3-H_4folate in the primary turnover cycle, we assayed methylcobalamin enzyme with radiolabeled AdoMet in the presence of unlabeled CH_3-H_4folate. Methylcobalamin enzyme (10 pmol) was incubated with [*methyl*-^{3}H]AdoMet (20 nmol, 35 000 cpm/nmol) and unlabeled CH_3-H_4folate (40 nmol) in 25 mM dithiothreitol and 100 mM potassium phosphate, pH 7.2 (1 mL total volume) under an argon atmosphere for 5 min at 37 °C. Turnover was initiated by adding 500 nmol of homocysteine with a syringe through a rubber septum. After 10 min, the reaction was cooled on ice, and the labeled methionine was isolated by passing the mixture through a 0.5×3 cm column that contained a 1:1 mixture of AG1-X8 and AG50W-X8 ion-exchange resins in water. The column was rinsed with 2 mL of water and the column washes were combined with 8 mL of scintillation fluid and counted. Controls in the absence of enzyme demonstrated that the labeled AdoMet was completely bound to this column.

To determine the extent to which CH_3-H_4folate could compete with AdoMet in reductive methylation using the in vivo reducing system, cob(II)alamin enzyme (5 nmol) was mixed with [*methyl*-^{14}C]-(6*R*,*S*)-CH_3-H_4folate (50 nmol, 11 000 cpm/nmol), unlabeled AdoMet (20 nmol), flavodoxin (0.1 nmol), and NADPH (500 nmol) in 1 mL of 100 mM potassium phosphate, pH 7.2, in an anaerobic cuvette. The mixture was made anaerobic by 20 cycles of evacuation followed by filling with argon. Ferredoxin (flavodoxin) oxidoreductase (0.1 nmol) was added with a syringe and the enzyme incubated at room temperature for 30 min, ensuring complete methylation of the enzyme-bound cofactor. The enzyme was separated from the substrates by gel filtration on a Superose 12 HR 10/30 column (Pharmacia) in 100 mM potassium phosphate, pH 7.2. The enzyme fraction was incubated with 500 nmol of homocysteine for 5 min under aerobic conditions, resulting in the formation of 5 nmol of methionine and complete conversion of the enzyme to the hydroxocob(III)alamin form. Methionine was separated from the enzyme on a 0.5×2 cm AG1-X8 chloride anion-exchange column, which was washed with 2 mL of water. The column eluent was mixed with 8 mL of scintillation fluid and counted.

Determination of AdoMet and CH_3-H_4folate Binding Constants by Equilibrium Dialysis. The method for determining binding using a microdialysis setup has been described (*11*). For experiments to measure AdoMet binding to MetH, tightly bound AdoMet was removed prior to equilibrium dialysis. MetH in the cob(II)alamin form (∼100−200 μM) was incubated with 25 mM dithiothreitol in 100 mM potassium phosphate buffer, pH 7.2, and equilibrated with argon in a test tube with a small stir bar and a rubber septum. After 15 min, aquocobalamin was added with a syringe to a final concentration of 5 μM and homocysteine was added to a final concentration of 500 μM. After 1 h of incubation at room temperature, the enzyme was repurified by gel filtration. ^{3}H-Labeled AdoMet was purchased from Amersham and diluted with HPLC-purified unlabeled AdoMet to a final specific activity of 35 000 cpm/nmol. ^{14}C-Labeled (6*R*)-CH_3-H_4Pte(Glu)$_3$ was prepared from ^{14}C-labeled formaldehyde (Amersham) and pteroyltriglutamate (Schirks Laboratories) using an enzymatic procedure (*23*) and was used undiluted at a specific activity of 22 200 dpm/nmol. The buffer for all of the dialysis experiments was 8 mM sodium ascorbate, 1 mM EDTA, and 100 mM potassium phosphate, pH 7.2. The enzyme solution (60 μL) was placed in the cap of a 0.5 mL Eppendorf tube, the substrate solution (200 μL) was placed in the tube, and the solutions were separated by a dialysis membrane. The samples were equilibrated ∼15 h, and the upper solution containing free substrate was separated by cutting a small hole in the bottom of the tube, inverting the tube in a 1.5 mL microfuge tube, and spinning for 2 min at 500 rpm in a benchtop microfuge. The enzyme solution with bound substrate was removed by puncturing the membrane with a syringe. Each sample (50 μL) was mixed with scintillation fluid and counted. The concentration of free substrate was calculated from the counts in the upper solution, while the concentration of bound substrate was calculated by subtract-

ing counts in the upper solution from counts in the lower enzyme solution. Binding constants were calculated by fitting a plot of [bound substrate] vs [free substrate] to a quadratic binding equation. No attempt was made to correct the data for spontaneous decomposition of the substrates over the long incubation times required for dialysis; thus the binding constants reported should be considered upper limits.

Reactivity of Cob(I)alamin Enzyme with AdoMet, CH_3-H_4folate, and Flavodoxin. Cob(I)alamin enzyme can be prepared by two routes: demethylation of methylcobalamin enzyme with homocysteine to generate cob(I)alamin enzyme that is normally formed during the primary turnover cycle, and reduction of cob(II)alamin enzyme to form cob(I)alamin enzyme that is presumably an intermediate during reductive methylation (*24*). In the first protocol, methylcobalamin enzyme (30 μM) in 25 mM Hepes and 25 mM KCl, pH 7.2, was placed in a glass tonometer and the solution was made anaerobic by repeated cycles of evacuation and filling with argon over 20 min. An anaerobic solution of homocysteine (27 μM final concentration) was added with a syringe through a stopcock, and the solution was allowed to react for ~5 min. A spectrum of the enzyme measured in the stopped-flow spectrophotometer prior to mixing indicated the formation of ~20 μM cob(I)alamin enzyme, in addition to the presence of ~10 μM residual methylcobalamin enzyme. This enzyme sample was rapidly mixed with an anaerobic solution of (6*R*,*S*)-CH_3-H_4folate (1 mM), and the formation of methylcobalamin enzyme was monitored at 390 and 525 nm. Since the cob(I)alamin enzyme is prone to oxidation within the glass tonometer over 1−2 h, the much slower reactions with AdoMet and with flavodoxin were determined in a slightly different manner. In this case, methylcobalamin enzyme (30 μM) was mixed within the stopped-flow spectrophotometer with a solution that contained homocysteine (27 μM) plus AdoMet (500 μM) or flavodoxin (150 μM). Formation of the cob(I)alamin enzyme is essentially complete by ~1 s; after this time spectral changes are due to remethylation or oxidation of the cofactor. Remethylation of cob(I)alamin enzyme was monitored at 390 and 525 nm and by measuring spectra of the reaction at intervals. Electron transfer from cob(I)alamin to flavodoxin was monitored by measuring the formation of flavodoxin semiquinone at 580 nm. Since changes in the cobalamin spectra are negligible at this wavelength, changes at this wavelength predominantly reflect oxidation of cob(I)alamin by electron transfer from methionine synthase to flavodoxin.

In the second protocol for the production of cob(I)amin enzyme, cob(II)alamin enzyme (30 μM) in 25 mM Hepes and 25 mM KCl, pH 7.2, was placed in an anaerobic tonometer and the solution was made anaerobic by repeated cycles of evacuation and filling with argon over 20 min. Titanium(III) citrate (4 mM final concentration) was added with a syringe and the solution incubated for 10 min at 25 °C to ensure complete equilibration. Control experiments showed that cob(I)alamin enzyme was formed under these conditions with an apparent rate constant of ~0.008 s^{-1}. Spectra of the enzyme solution before and after addition of titanium(III) citrate suggested ~20−25 μM cob(I)alamin was formed at equilibrium. The enzyme solution was mixed in the stopped-flow instrument with anaerobic solutions of AdoMet (500 μM), (6*R*,*S*)-CH_3-H_4folate (1 mM), or flavodoxin (150 μM). When the cob(I)alamin enzyme was rapidly mixed with AdoMet or CH_3-H_4folate, formation of methylcobalamin enzyme was monitored at 390 and 525 nm. When the cob(I)alamin enzyme was rapidly mixed with flavodoxin, electron transfer was monitored at 390 and 580 nm. The rate of electron transfer from titanium(III) citrate to flavodoxin was also measured. Titanium(III) citrate (4 mM) was rapidly mixed with flavodoxin (150 μM) in a stopped-flow spectrometer at 25 °C, and flavodoxin semiquinone formation was monitored at 580 nm.

Partial Proteolysis of Methionine Synthase Holoprotein. The methionine synthase holoprotein can be cleaved by partial proteolysis with trypsin into a series of stable fragments (*4*, *15*) that have been proposed to correspond to functional regions of the protein (*18*). The original procedure (*4*) was modified slightly for these studies. Wild-type methylcobalamin enzyme was purified to homogeneity as previously described (*25*) and exchanged into a buffer containing 25 mM Hepes, 25 mM KCl, and 0.1 mM EDTA, pH 7.2, by gel filtration on a Superdex 200 column (Pharmacia). The enzyme was diluted to 3.5 μM (~0.5 mg/mL) in the same buffer and proteolysis was initiated by the addition of trypsin (0.1−3% w/w). The mixture was incubated at 23 °C, samples were removed, and further proteolysis stopped by adding the protease inhibitor TLCK (1 μg). Cob(II)alamin enzyme was generated by aerobic photolysis (*24*) ~10 min prior to proteolysis. Where present, flavodoxin (100 μM) was added prior to proteolysis. Cob(I)alamin enzyme was generated under argon from methylcobalamin enzyme by the addition of homocysteine (500 μM) 10 min prior to proteolysis. Cob(I)alamin enzyme was generated from cob(II)alamin enzyme by aerobic photolysis of the initial methylcobalamin enzyme, followed by anaerobic reduction with titanium(III) citrate 10 min prior to proteolysis. Purification and proteolysis of the His759Gly cob(II)alamin enzyme was carried out in a manner similar to that described for the wild-type methylcobalamin enzyme. Titanium(III) citrate has a general inhibitory effect on proteolysis that is seen as an increase in the stability of the intact holoenzyme and all of the fragments. Proteolysis fragments were separated by SDS−polyacrylamide gel electrophoresis on 12% acrylamide gels and visualized by staining with Coomassie Brilliant Blue G (Sigma). To purify each major proteolytic fragment, the fragments were separated by FPLC chromatography on a MonoQ anion-exchange column employing phosphate buffer gradients. The identity of major fragments was established for the wild-type methylcobalamin enzyme and the His759Gly cob(II)alamin enzymes by a combination of electrospray mass spectrometry and N-terminal sequence analysis by Edman degradation. Analyses were performed by the Protein and Carbohydrate Structure Laboratory at the Biomedical Research Core Facility of the University of Michigan Medical School.

RESULTS

AdoMet Is Not Competitive with CH_3-H_4folate during Primary Turnover. We devised a protocol to test competition between AdoMet and CH_3-H_4folate during primary turnover in the absence of reductive methylation. For this protocol, we omitted aquocobalamin from the components used in the standard in vitro assay of methionine synthase, which include dithiothreitol as a source of reducing equivalents for reductive methylation. Aquocobalamin is essential in this chemical

reducing system, as it mediates the transfer of electrons between the two-electron reductant, dithiothreitol, and the one-electron acceptor, cob(II)alamin enzyme. By starting our assay with methylcobalamin enzyme and by leaving aquocobalamin out of the assay mix, we could measure incorporation of methyl groups from radiolabeled AdoMet into the product methionine during the primary turnover cycle only. The assay system was designed to produce ~20 nmol of methionine in the presence of 40 nmol of CH_3-H_4folate and 20 nmol of AdoMet. If AdoMet were incorporated into every molecule of methionine, ~700 000 cpm would have been found in the product. We found that the product methionine contained only ~70 cpm of tritium, indicating that ≤0.01% of the methionine produced incorporated tritium label from AdoMet. Thus AdoMet does not compete directly with CH_3-H_4folate during the primary turnover cycle.

CH_3-H_4folate Is Not Competitive with AdoMet during Reductive Methylation. Cob(II)alamin enzyme is normally returned to the methylcobalamin form by electron transfer from reduced flavodoxin and methyl transfer from AdoMet. Flavodoxin is reduced by ferredoxin (flavodoxin):$NADP^+$ oxidoreductase (*8*). In the absence of homocysteine, the reductive methylation reaction produces stable methylcobalamin enzyme, which can be separated from flavodoxin and AdoMet by gel-filtration chromatography. Reaction of this enzyme with homocysteine produces 1 equiv of methionine, which can be separated from the enzyme by ion-exchange chromatography. We reductively methylated 5 nmol of cob(II)alamin enzyme in the presence of 20 nmol of unlabeled AdoMet and 50 nmol of radiolabeled CH_3-H_4folate using flavodoxin, ferredoxin (flavodoxin):$NADP^+$ oxidoreductase, and NADPH as the reducing system. The resulting methylcobalamin enzyme was reacted with homocysteine under aerobic conditions, producing 1 equiv of methionine and hydroxocob(III)alamin enzyme. Under these conditions, if CH_3-H_4folate were used for reductive methylation instead of AdoMet, ~55 000 cpm would be detected in the product methionine. The product methionine incorporated undetectable levels of ^{14}C-methyl (<50 cpm), suggesting that CH_3-H_4folate is used in ≤0.1% of reductive methylation reactions. Thus CH_3-H_4folate does not directly compete with AdoMet during reductive methylation of cob(II)alamin enzyme.

CH_3-H_4folate and AdoMet Bind to both Methylcobalamin and Cob(II)alamin Enzyme Forms. One possible means of discriminating between CH_3-H_4folate and AdoMet during primary turnover and reductive methylation might be for the enzyme to modulate the binding affinity for each substrate in response to the oxidation state of the enzyme. If this were the case, CH_3-H_4folate would be expected to bind selectively to methylcobalamin enzyme, while AdoMet would bind selectively to cob(II)alamin enzyme. We devised a microdialysis method that allowed determination of the binding affinities of CH_3-H_4folate and AdoMet for methylcobalamin and cob(II)alamin enzyme (*11*). Commercially available CH_3-H_4folate is a mixture of diastereomers and, due to its monoglutamate side chain, binds relatively weakly to methionine synthase [K_d = 5 μM (*11*, *26*)]. To observe tight binding, we prepared radiolabeled (6*S*)-CH_3-H_4Pte$(Glu)_3$, which is chemically similar to CH_3-H_4folate except that it has three glutamates in the side-chain, using an enzymatic procedure that produces only the natural 6*S* isomer (*23*). (6*S*)-CH_3-H_4Pte$(Glu)_3$ binds to methylcobalamin and cob(II)alamin methionine synthase with equal affinity, giving a K_d of 0.4 ± 0.2 μM. The affinity for cob(II)alamin enzyme was unaffected by the presence or absence of 100 μM AdoMet. AdoMet binds to methylcobalamin and cob(II)alamin enzyme with similar affinities, giving a K_d of 1.2 ± 0.3 μM. The affinity for methylcobalamin enzyme was unaffected by the presence or absence of 500 μM (6*R*,*S*)-CH_3-H_4folate. These data suggest that both (6*S*)-CH_3-H_4Pte$(Glu)_3$ and AdoMet bind tightly to active methylcobalamin enzyme and to inactive cob(II)alamin enzyme.

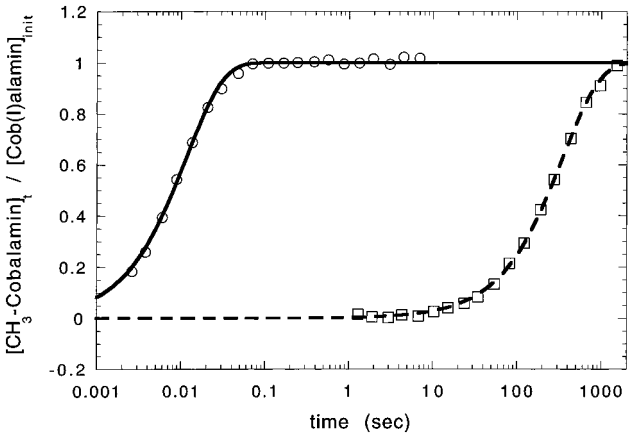

FIGURE 2: Reactivity of cob(I)alamin enzyme produced from methylcobalamin enzyme (30 μM) by demethylation with substoichiometric homocysteine (27 μM). Cob(I)alamin enzyme (~20 μM) was mixed with (6*R*,*S*)-CH_3-H_4folate (1 mM, ○, solid curve) in 25 mM Hepes and 25 mM KCl, pH 7.2, in an anaerobic stopped-flow spectrophotometer at 25 °C, and the formation of methylcobalamin enzyme was monitored at 525 nm. Methylcobalamin enzyme was mixed with AdoMet (500 μM, □, dashed curve) and homocysteine (27 μM) in 25 mM Hepes and 25 mM KCl, pH 7.2, in an anaerobic stopped-flow spectrophotometer at 25 °C, and the formation of methylcobalamin enzyme was monitored at 525 nm. Solid and dashed curves correspond to single-exponential fits using rate constants of 90 and 0.003 s^{-1}, respectively.

Cob(I)alamin Enzyme Produced by Demethylation of Methylcobalamin Enzyme Reacts Selectively with CH_3-H_4folate. In vitro steady-state assays described above suggested that AdoMet does not compete with CH_3-H_4folate in primary turnover. However, it had been reported that methionine synthase displays AdoMet:homocysteine methyltransferase activity in the absence of CH_3-H_4folate (*1*). We decided to measure directly the rates at which CH_3-H_4folate and AdoMet react with cob(I)alamin enzyme under strictly anaerobic conditions in a stopped-flow spectrophotometer. Methylcobalamin enzyme was placed in a tonometer and made anaerobic by repeated cycles of evacuation and equilibration with argon. Homocysteine (0.9 equiv) was added with a syringe to the solution in the tonometer and the enzyme was incubated 5 min at room temperature, generating ~70% of the enzyme in the cob(I)alamin oxidation state. The enzyme was rapidly mixed with CH_3-H_4folate in the stopped-flow spectrophotometer at 25 °C, and the formation of methylcobalamin enzyme was monitored at 525 nm (Figure 2). CH_3-H_4folate reacted with cob(I)alamin enzyme with an observed rate constant of 90 s^{-1}, comparable to the rate constant of 50 s^{-1} that had been previously reported (*2*). This rate observed in a binary reaction with cob(I)alamin enzyme is ~50% slower than the estimated rate constant of 180 s^{-1} for methylation of cob(I)alamin enzyme in a ternary complex with CH_3-H_4folate and methionine (*2*).

In a separate experiment, methylcobalamin enzyme was reacted in situ with homocysteine (0.9 equiv) in the presence of excess AdoMet at 25 °C. Cob(I)alamin enzyme (~25 μM) is formed within the first second of the reaction with homocysteine, and this cob(I)alamin enzyme is subsequently remethylated by AdoMet. AdoMet reacted with cob(I)alamin enzyme with a rate constant of ~0.003 s^{-1}. These results suggest that cob(I)alamin enzyme that is produced by reacting methylcobalamin enzyme with homocysteine exists in a conformation that is optimized to react rapidly with CH_3-H_4folate but in which AdoMet has limited access to the cob(I)alamin cofactor.

Cob(I)alamin Enzyme Produced by Reduction of Cob(II)alamin Enzyme Reacts Selectively with AdoMet. Reductive methylation of cob(II)alamin enzyme has been proposed to involve reduction of the cofactor to cob(I)alamin followed by methyl transfer from AdoMet (*9*), generating the active methylcobalamin enzyme. Radiolabeling experiments described above suggest that CH_3-H_4folate does not compete with AdoMet in reductive methylation using the enzymatic reducing system. However, experiments in which CH_3-H_4folate was incubated with cob(II)alamin enzyme in an electrochemical cell in the absence of AdoMet suggest that CH_3-H_4folate is used for reductive methylation, at least over very long incubation times (*9*). When flavodoxin is used as the reductant, cob(I)alamin enzyme is not directly observed as an intermediate in reductive methylation and therefore the observed rate of methylcobalamin formation may not reflect the actual rate of methyl transfer from AdoMet to cobalamin. Recent introduction of the powerful reductant titanium(III) citrate (*18*, *27*) now allows reduction of cob(II)alamin enzyme to cob(I)alamin enzyme. We used this reduction method to measure directly the rate at which AdoMet and CH_3-H_4folate react with cob(I)alamin enzyme formed by reduction of cob(II)alamin enzyme. When an excess of titanium(III) citrate was added to an anaerobic solution of cob(II)alamin enzyme, the enzyme was slowly reduced to cob(I)alamin over ~10 min. This cob(I)alamin enzyme was then rapidly mixed with an anaerobic solution of AdoMet or CH_3-H_4folate in a stopped-flow spectrophotometer at 25 °C, and the formation of methylcobalamin enzyme was monitored at 525 nm (Figure 3). AdoMet methylates cob(I)alamin enzyme under these conditions in a biphasic reaction with rate constants of 25 and 10 s^{-1}. This is the first direct observation of a kinetically competent reaction between wild-type cob(I)alamin methionine synthase and AdoMet, and it supports the proposal that cob(I)alamin is an intermediate in reductive methylation (*9*). CH_3-H_4folate reacts very slowly with cob(I)alamin enzyme formed by reduction of cob(II)alamin enzyme with a rate constant of $\leq 10^{-4}$ s^{-1}. The wide discrepancy in the reactivity of AdoMet and CH_3-H_4folate with cob(I)alamin formed by reduction suggests that the reduced enzyme exists in a conformation that is optimized to react with AdoMet and in which CH_3-H_4folate has very limited access to the cob(I)alamin cofactor.

The UV/visible spectra of cob(I)alamin formed by demethylation of methylcobalamin enzyme and cob(I)alamin formed by reduction of cob(II)alamin enzyme are indistinguishable (*11*). The wide variation in reactivity between these two forms of cob(I)alamin enzyme suggests that these forms differ in their protein conformation. Since it is unlikely that these conformations have identical stabilities, one might expect that the higher energy conformation would slowly convert to the more thermodynamically stable conformation and that the ability to maintain a large difference in reactivities between AdoMet and CH_3-H_4folate would be lost over time. This was not observed. When methylcobalamin enzyme was converted to cob(I)alamin enzyme with homocysteine and then incubated for 2 h at room temperature, the enzyme still reacted selectively with CH_3-H_4folate with rates similar to those reported above. Conversely, when cob(II)alamin enzyme was converted to cob(I)alamin enzyme by incubation with titanium(III) citrate for 2 h, the enzyme still reacted selectively with AdoMet, again with rates similar to those reported above. The failure to observe conformational interconversion suggests that the cob(I)alamin enzymes are "locked" in different conformations that cannot interconvert. The only route to conformational interconversion may be through methylation to methylcobalamin enzyme or oxidation to cob(II)alamin enzyme.

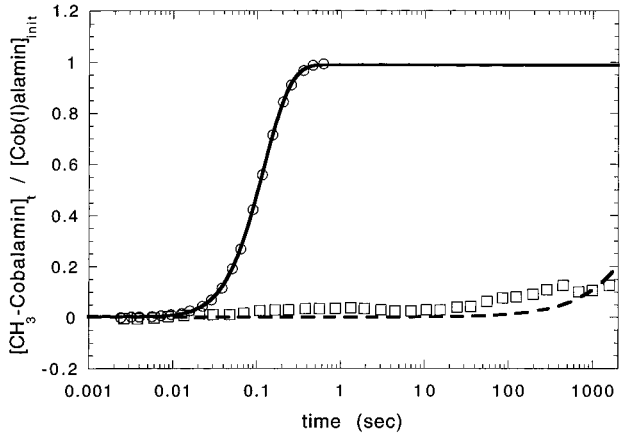

FIGURE 3: Reactivity of cob(I)alamin enzyme produced from cob(II)alamin enzyme by reduction with excess titanium(III) citrate. Cob(I)alamin enzyme (~23 μM) was mixed with AdoMet (500 μM, ○, solid curve) or CH_3-H_4folate (1 mM, □, dashed curve) in 25 mM Hepes and 25 mM KCl, pH 7.2, in an anaerobic stopped-flow spectrophotometer at 25 °C, and the formation of methylcobalamin enzyme was monitored at 525 nm. The reaction with AdoMet was monitored at many wavelengths from 370 to 650 nm, and spectral deconvolution suggests two exponential phases with rate constants of 10 and 25 s^{-1} (solid curve). We propose that these correspond to methyl transfer from AdoMet to cob(I)alamin followed by slower religation of His759 to the cobalt. The reaction with CH_3-H_4folate is not fit by a simple function, although a curve assuming complete methyl transfer at a rate of 10^{-4} s^{-1} is shown for comparison (dashed curve). Spectra of the enzyme before and after the reaction suggest ~1 μM methylcobalamin enzyme was formed in 32 min.

Flavodoxin Oxidizes Cob(I)alamin Formed by Reduction of Cob(II)alamin Enzyme More Rapidly Than It Oxidizes Cob(I)alamin Formed by Demethylation of Methylcobalamin Enzyme. The midpoint potential required for reduction of cob(II)alamin enzyme to cob(I)alamin enzyme ($E_{m7} = -490$ mV) is significantly lower than the midpoint potential for the flavodoxin semiquinone/oxidized couple (−250 mV), suggesting that cob(I)alamin methionine synthase should be oxidized by flavodoxin present in the cell. However, this would result in oxidation of cob(I)alamin formed during primary turnover and would result in inhibition of enzyme activity in the presence of excess flavodoxin. Thus, methionine synthase may have evolved to limit the interaction

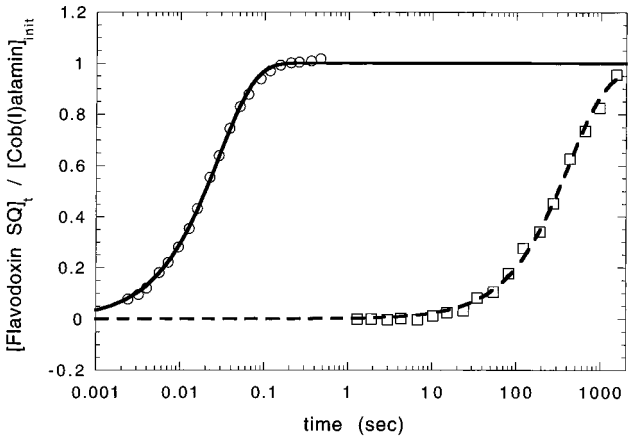

FIGURE 4: Electron transfer from cob(I)alamin enzyme (~10 μM) to oxidized flavodoxin (75 μM). Cob(I)alamin enzyme was produced by reduction of cob(II)alamin enzyme (○) or demethylation of methylcobalamin enzyme (□) as described in Figures 2 and 3 and in the Materials and Methods. Oxidized flavodoxin was rapidly mixed with the cob(I)alamin enzyme in an anaerobic stopped-flow spectrophotometer, and electron transfer was monitored by following the formation of flavodoxin semiquinone at 580 nm. Solid and dashed curves correspond to single-exponential fits using rate constants of 35 and 0.002 s^{-1}, respectively. Electron transfer from titanium(III) citrate to flavodoxin was much slower than from cob(I)alamin; by ignoring the spectral changes after 0.5 s (○), this chemical reduction could be ignored.

between flavodoxin and cob(I)alamin formed during primary turnover.

We measured the rate of electron transfer between cob(I)alamin and oxidized flavodoxin by monitoring the formation of flavodoxin semiquinone at 580 nm (Figure 4). When cob(I)alamin enzyme is produced by reduction of cob(II)alamin enzyme with titanium(III) citrate, electron transfer from cob(I)alamin to flavodoxin occurs with a net rate constant of ~35 s^{-1}. This rate may be somewhat limited by the relatively weak binding between cob(I)alamin enzyme and flavodoxin (*12*); the estimated K_d is ~10−100 μM (*12*) and suggests that under these reaction conditions only ~40−80% of the cob(I)alamin enzyme is complexed with flavodoxin. In a separate control experiment, titanium(III) citrate (4 mM) was shown to reduce oxidized flavodoxin (150 μM) to the semiquinone with a rate constant of 0.05 s^{-1} and flavodoxin semiquinone to the hydroquinone with a rate constant of 0.005 s^{-1}. The rate constants for reduction of flavodoxin by titanium citrate are significantly smaller than the rate constant for reduction of flavodoxin by the cob(I)alamin form of methionine synthase. When cob(I)alamin enzyme is formed by demethylation of methylcobalamin enzyme with homocysteine, flavodoxin reacts very slowly, with an observed rate constant of ~0.002 s^{-1}. While methionine synthase must, by necessity, allow flavodoxin access to the cobalamin cofactor in the conformation that exists during reductive methylation, the enzyme appears to be very effective at protecting cob(I)alamin formed during primary turnover from oxidation by flavodoxin.

Partial Proteolysis Suggests That Flavodoxin Binding Induces a Conformational Change That Is Mimicked by the Mutation of Histidine 759 to Glycine. Methionine synthase is folded into a modular structure in which individual regions of the peptide sequence correspond to functional units that bind homocysteine, CH_3-H_4folate, cobalamin, and AdoMet (*18*). These functional regions can be separated by partial

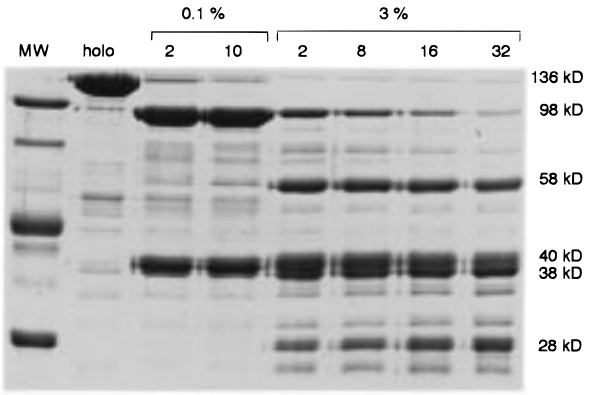

FIGURE 5: Time course of the proteolysis of wild-type methylcobalamin enzyme (0.5 mg/mL) with 0.1% and 3% (w/w) trypsin in 25 mM Hepes and 25 mM KCl, pH 7.2 at 23 °C. A sample of the enzyme was removed prior to the addition of trypsin and used as the $t = 0$ lane. The subsequent lanes correspond to samples removed at various times (in minutes) after the addition of trypsin. Proteolysis was stopped by addition of TLCK and proteolytic fragments were separated by denaturing gel electrophoresis on a 12% polyacrylamide gel.

proteolysis of the native enzyme with trypsin followed by gel electrophoresis under denaturing conditions. Partial proteolysis of the methylcobalamin holoprotein with 0.1% (w/w) trypsin results in rapid cleavage between arginine 896 and lysine 897 (Figure 5), generating 98 and 38 kDa fragments (*4*, *15*). The fragments were identified by electrospray mass spectrometry and N-terminal sequencing as described in Materials and Methods. The 98 kDa fragment retains bound methylcobalamin and is active for methyl transfer from CH_3-H_4folate to homocysteine in anaerobic assays in the absence of AdoMet or a reducing system (*4*). The 38 kDa fragment is very stable to proteolysis and retains bound AdoMet but has no known activity (*4*, *16*). Further proteolysis with 0.1% trypsin results in much slower cleavage of the 98 kDa fragment at several sites, resulting in fragments that range in size from 27 to 70 kDa. Proteolysis with higher concentrations of trypsin (3% w/w) reveals that some of these fragments are relatively stable to further proteolysis (Figure 5). When the initial wild-type holoprotein is in the active methylcobalamin state, cleavage between arginine 373 and leucine 374 generates fragments with sizes of 40 kDa and 58 kDa. As diagrammed in Figure 6, the 40 kDa fragment corresponds approximately to the homocysteine binding region, as expressed and assayed by Goulding et al. (*18*), while the 58 kDa fragment corresponds to a fusion of the CH_3-H_4folate and cobalamin-binding regions. Prolonged proteolysis of these fragments results in destruction of the 40 kDa fragment, while only the CH_3-H_4folate binding region of the 58 kDa fragment is destroyed, ultimately yielding the proteolytically stable 28 kDa cobalamin binding region. This cleavage pattern suggests that the active methylcobalamin enzyme exists in a conformation in which there is a relatively exposed loop or hinge between the AdoMet- and cobalamin-binding regions and between the homocysteine- and CH_3-H_4folate-binding regions, but in which the CH_3-H_4folate binding region is closely associated with the cobalamin binding region. Interestingly, the inactive cob(II)alamin and hydroxocob(III)alamin enzymes display a similar proteolysis pattern, suggesting that the conformation that gives rise to this pattern may be a stable conformation that is shared by all oxidized forms of methionine synthase.

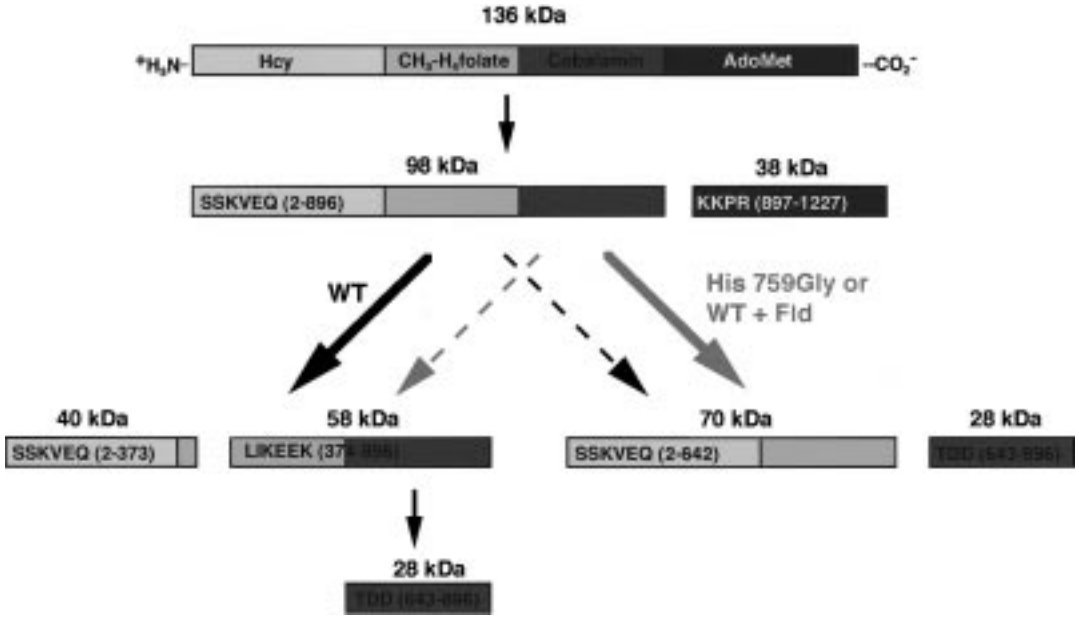

FIGURE 6: Scheme describing the primary proteolysis pathways shown in Figures 5 and 7. Both pathways start with cleavage of holoprotein to generate the 38 kDa AdoMet-binding domain. The 98 kDa fragment formed from wild-type methylcobalamin and cob(II)alamin enzymes, as well as from cob(I)alamin enzyme produced by demethylation of methylcobalamin enzyme, cleaves to generate 40 and 58 kDa fragments, which correspond to the homocysteine binding region and the CH_3-H_4folate + cobalamin-binding regions, respectively. The 98 kDa fragment formed from wild-type cob(II)alamin enzyme with flavodoxin bound, from His759Gly cob(II)alamin enzyme, and from cob(I)alamin enzyme produced by reduction of cob(II)alamin enzyme cleaves to generate 70 and 28 kDa fragments that correspond to the homocysteine + CH_3-H_4folate-binding regions and the cobalamin-binding region, respectively. As indicated by the dashed arrows, the separation is not complete and some fragments corresponding to the other pathway are observed in each case. Masses and N-terminal sequences shown in this figure were determined as described in Materials and Methods, except for those of the 98 and 38 kDa fragments, which were previously reported (*15*, *34*).

Addition of flavodoxin to the inactive cob(II)alamin enzyme results in a shift in the primary proteolysis pathway. The initial cleavage occurs at the same point in the sequence, generating 98 and 38 kDa fragments identical to those observed in the absence of flavodoxin. Subsequent proteolysis in the presence of flavodoxin results in cleavage between lysine 642 and threonine 643, resulting in the formation of a 70 kDa fragment that corresponds to the homocysteine- and CH_3-H_4folate-binding regions and a 28 kDa fragment that corresponds to the cobalamin-binding region (Figures 6 and 7). These fragments are stable to further proteolysis. As suggested in Figure 6, while the two primary cleavage pathways are as described above, the separation between these proteolysis pathways is not complete. A minor cleavage product in the absence of flavodoxin is ~70 kDa, while a significant amount of 58 kDa fragment is observed in the presence of flavodoxin. One possible explanation is that the cob(II)alamin enzyme form exists in an equilibrium distribution of two conformations in which the primary turnover conformation dominates in the absence of flavodoxin and the reactivation conformation dominates in the presence of bound flavodoxin.

When cob(II)alamin is bound to methionine synthase, the intramolecular dimethylbenzimidazole ligand that coordinates to cobalt in the free cofactor is replaced by a histidine ligand donated by the protein, His 759 (*17*). The cobalt in the enzyme-bound cob(II)alamin is 5-coordinate, with four equatorial ligands contributed by the corrin heterocycle as well as the axial nitrogen ligand contributed by His759. Flavodoxin binding to the cob(II)alamin holoprotein has been shown to be associated with displacement of histidine 759 from the cobalt, resulting in formation of a 4-coordinate cob-

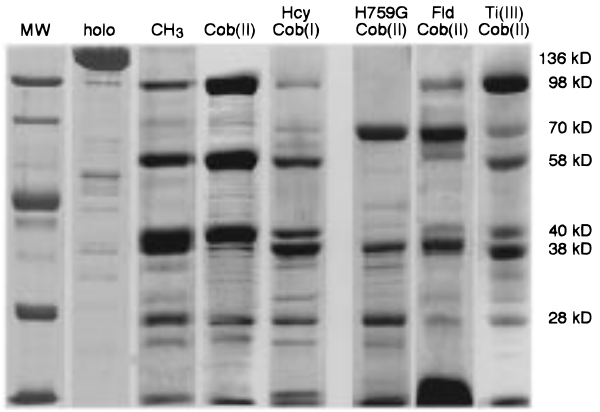

FIGURE 7: Fragments resulting from the treatment of methionine synthase (0.5 mg/mL) with 3% trypsin for 8 or 32 min. Lane 1, molecular weight ladder; lane 2, unproteolyzed enzyme; lane 3, wild-type methylcobalamin enzyme (8 min); lane 4, wild-type cob(II)alamin enzyme (8 min); lane 5, cob(I)alamin enzyme produced by demethylation of methylcobalamin enzyme with homocysteine (8 min); lane 6, His759Gly cob(II)alamin enzyme (8 min); lane 7, wild-type cob(II)alamin enzyme + 100 μM flavodoxin (8 min); lane 8, cob(I)alamin enzyme produced by reduction of cob(II)alamin enzyme with titanium(III) citrate (32 min). Proteolysis of all fragments was significantly slowed in the presence of titanium(III) citrate, and the longer incubation time for this sample reflects our attempt to compare the sizes and amounts of the 58−70 kDa fragments. All experiments were performed in 25 mM Hepes and 25 mM KCl, pH 7.2 at 23 °C. This figure is a composite of lanes scanned from several different polyacrylamide gels, and therefore the various fragments do not perfectly align.

(II)alamin cofactor (*12*). This reorganization of the cobalt ligand environment may be a result of the conformational change that is favored by flavodoxin binding. We have generated a mutant enzyme in which histidine 759 is replaced

by glycine; this enzyme is isolated as a stable 4-coordinate cob(II)alamin enzyme (*19*). The His759Gly mutant enzyme has both UV/visible and EPR spectra that resemble those of the wild-type cob(II)alamin enzyme in the presence of excess flavodoxin (*12*, *28*). The His759Gly mutant enzyme binds with a 20-fold higher affinity to flavodoxin than the wild-type enzyme, suggesting that it exists in a conformation that is preorganized for binding to flavodoxin (*12*). Partial proteolysis of the His759Gly mutant enzyme with trypsin results in formation of a stable 70 kDa fragment (Figure 7), similar to that observed as the predominant cleavage product for the wild-type enzyme in the presence of flavodoxin. We propose that the dissociation of His759 from the cobalt in the cob(II)alamin oxidation state, as a result of flavodoxin binding or mutagenesis, is linked to a conformational change that results in increased exposure of lysine 642 and protection of arginine 373.

Cob(I)alamin Formed by Reduction of Cob(II)alamin Enzyme and Cob(I)alamin Formed by Demethylation of Methylcobalamin Enzyme Differ in Proteolytic Susceptibility. The altered proteolysis patterns described above suggest that methylcobalamin enzyme and 4-coordinate cob(II)alamin enzyme exist in different conformations. However, both reaction of methylcobalamin enzyme with homocysteine and reduction of cob(II)alamin enzyme with titanium(III) citrate lead to formation of 4-coordinate cob(I)alamin enzyme. We have already shown that cob(I)alamin enzyme produced by these two different routes differs dramatically in reactivity toward CH_3-H_4folate, AdoMet, and flavodoxin, and we have suggested that this may be due to differences in the conformation of the enzyme. If this were the case, then partial proteolysis of cob(I)alamin enzyme produced from methylcobalamin enzyme vs cob(I)alamin enzyme produced from cob(II)alamin enzyme should reflect these conformational differences.

We generated cob(I)alamin enzyme from methylcobalamin enzyme by incubating with excess homocysteine under an argon atmosphere for 10 min. A spectrum of the enzyme demonstrated complete conversion to cob(I)alamin enzyme. A small amount of argon-purged trypsin was added with a syringe through a rubber septum, and then samples were removed at intervals and proteolysis was quenched with TLCK. The proteolysis pattern observed was similar to the pattern that was observed for wild-type methylcobalamin enzyme (Figure 7). In particular, the 98 kDa fragment is cleaved to yield a 58 kDa fragment, while little of the 70 kDa fragment is observed. The similarity between this pattern and the proteolysis pattern of methylcobalamin enzyme suggests that cob(I)alamin enzyme produced during the primary turnover cycle remains in the same gross conformation as the initial methylcobalamin enzyme.

In contrast, we generated cob(I)alamin enzyme by reduction of cob(II)alamin enzyme with excess titanium(III) citrate and found that the proteolysis pattern more resembled that observed for wild-type cob(II)alamin enzyme in the presence of flavodoxin. The 98 kDa fragment is cleaved slowly and produces a mixture of 70 kDa and 58 kDa fragments (Figure 7). Remaining production of the 58 kDa fragment may be due to incomplete reduction of the enzyme under the conditions of this experiment. The finding that reduction of the cofactor increases production of the 70 kDa fragment can be rationalized if the redox process is assumed to be ordered. Cob(II)alamin enzyme exists in an equilibrium between 5-coordinate and 4-coordinate cofactors (*28*), which differ in bonding between the cobalt and histidine 759 and also differ in the conformational state of the enzyme. If histidine dissociation and the accompanying conformational change must precede electron transfer to the cobalt, then the cob(I)alamin enzyme that results from electron transfer will have the same conformation as the 4-coordinate cob(II)alamin enzyme.

DISCUSSION

Cobalamin-dependent methionine synthase catalyzes three methyl transfer reactions to or from the cobalamin cofactor: methyl transfer from methylcobalamin enzyme to homocysteine, from CH_3-H_4folate to cob(I)alamin enzyme, and from AdoMet to the cob(I)alamin enzyme (Figure 1). The two consecutive methyl transfers from CH_3-H_4folate to cob(I)alamin enzyme, and thence from methylcobalamin enzyme to homocysteine, proceed with net retention of stereochemistry and have been interpreted as involving two consecutive S_N2 reactions (*29*). The methyl transfer from AdoMet to cob(I)alamin is also presumed to be an S_N2 reaction. An S_N2 methyl transfer requires that the nucleophile, methyl group, and leaving group form a linear transition state in which bond formation between the nucleophile and methyl group and bond cleavage between the leaving group and methyl group are concerted. Thus, methionine synthase must position three different substrates directly over the cobalt atom of the cobalamin cofactor at various times during catalytic turnover, requiring that methionine synthase undergo conformational changes that alter the relative positions of the substrates vis-à-vis the cobalamin cofactor.

Methionine synthase has evolved to use CH_3-H_4folate and AdoMet as methyl donors at different times during the catalytic cycle. In this paper, we demonstrate that during primary turnover, when the enzyme starts in the methylcobalamin form, methionine synthase reacts with CH_3-H_4folate at a rate that is 6×10^4 faster than the rate of reaction with AdoMet. Conversely, during reductive methylation, when the enzyme starts in the cob(II)alamin form, methionine synthase reacts with AdoMet at a rate that is $>10^5$ faster than the rate of reaction with CH_3-H_4folate. It has been previously proposed that these differences in reactivity could be due to differences in chemical mechanism between primary turnover and reductive methylation (*12*). Cob(I)alamin was already known to be a kinetically competent intermediate during primary turnover with CH_3-H_4folate (*2*); in this paper we demonstrate that cob(I)alamin is also a kinetically competent intermediate during reductive methylation with AdoMet. Cob(I)alamin is a potent nucleophile that is reactive with most alkylating agents that can approach the reactive face of the cofactor (*30*, *31*). The lack of reactivity with either AdoMet or CH_3-H_4folate under certain conditions is presumably due to the inability to access the cob(I)alamin cofactor. Thus, not only does methionine synthase undergo conformational changes that allow each substrate access to the cobalamin cofactor, but it also maintains exquisitely tight control over these conformational states, presumably in response to the initial oxidation state of the cofactor.

Several previous results have hinted at conformational differences between the enzyme during primary turnover and

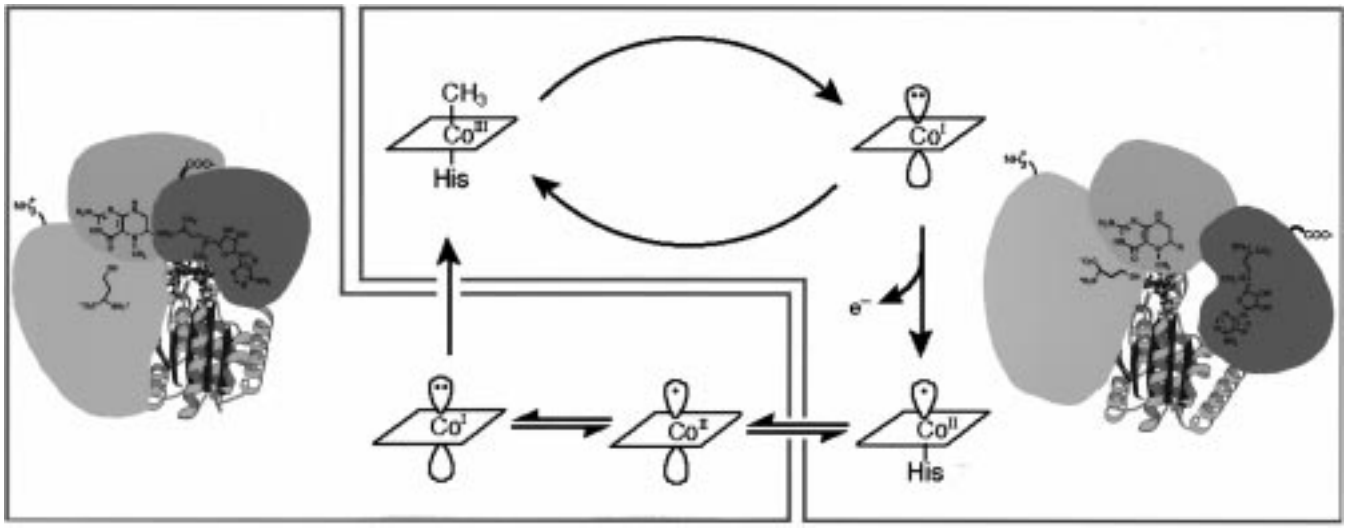

FIGURE 8: Cartoon depicting the proposed conformational interconversions that occur during methionine synthase reactivation. Primary turnover occurs within a conformation in which CH_3-H_4folate and homocysteine have access to the cobalamin cofactor. Oxidation of the cob(I)alamin enzyme results in formation of an inactive 5-coordinate cob(II)alamin enzyme which remains in the same conformation. The 5-coordinate cob(II)alamin enzyme is in equilibrium with a conformation that contains 4-coordinate cob(II)alamin enzyme; flavodoxin binding drives this equilibrium toward the 4-coordinate reactivation conformation. In this conformation, AdoMet has access to the cobalamin cofactor. Irreversible methyl transfer from AdoMet and recoordination of His759 to the cobalt trigger conversion back to the active primary turnover conformation.

during reductive methylation. Methionine synthase is inactivated by nitrous oxide due to electron transfer from cob(I)alamin enzyme to N_2O, generating cob(II)alamin enzyme, N_2, and a hydroxyl radical (*10*, *32*). However, no permanent damage to the enzyme or cofactor occurs when this reaction takes place during primary turnover, despite the production of a hydroxyl radical, and the resulting cob(II)alamin enzyme can be easily reactivated with AdoMet and a reducing system. When cob(I)alamin enzyme is produced during reductive methylation in an electrochemical cell, the enzyme is irreversibly inactivated by 1 equiv of N_2O due to hydrogen atom abstraction from Val 1177, followed by alkylation of Val 1177 by the electrochemical mediator triquat. Val1177 is now known to be located near the AdoMet-binding pocket in the C-terminal 38 kDa domain (*16*). These experiments suggested that when cob(I)alamin is produced during reductive methylation, Val 1177 is located near the cobalamin cofactor. However, when cob(I)alamin is produced during primary turnover, Val 1177 and the AdoMet-binding pocket are presumably much farther away.

More recently, studies of the interaction of flavodoxin with methionine synthase suggested that conformational changes accompanied flavodoxin binding (*12*). Cob(II)alamin enzyme is primarily 5-coordinate, with His759 providing the lower axial nitrogen ligand. When flavodoxin binds to cob(II)alamin enzyme, the histidine dissociates from the cobalt, resulting in formation of a 4-coordinate cob(II)alamin enzyme. This is observed by EPR spectrometry as a loss of the superhyperfine coupling associated with the axial Co−N bond and by UV/visible spectroscopy as a shift in the visible absorption band at 477 nm in the 5-coordinate enzyme to 465 nm in the 4-coordinate enzyme. Reorganization of the cobalt ligand environment is presumably the result of a conformational change upon flavodoxin binding. This is further supported by experiments that show that the methylcobalamin enzyme, which has a strong bond between His759 and the cobalt, does not bind flavodoxin, while the His759Gly mutant enzyme, which presumably mimics the 4-coordinate wild-type enzyme, binds tightly to flavodoxin (*12*).

Direct evidence for conformational changes is difficult to obtain. The ideal situation is to obtain a structure of each conformation and a thorough understanding of the equilibria and kinetics of conformational interconversion. For example, X-ray crystal structures of the R and T states of hemoglobin have been obtained, and studies have probed the conditions that favor conformational interconversion (*33*). Although X-ray structures of various fragments of methionine synthase have been solved (*16*, *17*) or are in progress, the way these fragments fit together and undergo conformational changes is unknown. We have found that partial proteolysis of methionine synthase with trypsin leads to the formation of various stable fragments (*4*, *15*), and we presume that highly solvent-exposed basic residues are most susceptible to proteolysis. Conformational changes within methionine synthase lead to a change in the susceptibility of certain residues within the N-terminal substrate binding region to proteolysis (summarized in Figure 6).

Control of these conformational interconversions is presumably linked to the oxidation state and ligand geometry of the cofactor; the oxidation states that are associated with the primary turnover and reactivation conformations are depicted in Figure 8. Primary turnover is associated with a conformation in which the N-terminal 70 kDa region is near the methylcobalamin or cob(I)alamin cofactor (Figure 8, right panel). Upon oxidation of cob(I)alamin to the 5-coordinate cob(II)alamin cofactor, the enzyme remains in this conformation. The cobalt is weakly bonded to His759 in the cob(II)alamin oxidation state and this oxidation state is in an equilibrium between 4-coordinate and 5-coordinate cofactors (*12*, *28*). This equilibrium is accompanied by the conformational change that we observe in proteolysis experiments reported above; the 4-coordinate cob(II)alamin enzyme assumes the structure we have labeled the reactivation

conformation (Figure 8, left panel). Upon reduction of 4-coordinate cob(II)alamin enzyme to cob(I)alamin, the enzyme remains in the altered conformation of 4-coordinate cob(II)alamin enzyme. In this conformation, the C-terminal 38 kDa AdoMet-binding region is near the cob(I)alamin cofactor. Methyl transfer from AdoMet to the cobalt is accompanied by recoordination of His759 to the cobalt; this is likely to be the point at which the reactivation conformation is converted back to the primary turnover conformation.

These experiments raise many questions concerning conformational changes that may remain unanswered until more structural information is available. The apparent modular construction of the methionine synthase holoenzyme suggests that the respective substrate and cofactor binding regions may be acting as independent units that are tethered together by relatively flexible linker regions (*18*). Repositioning of these units may involve large conformational changes. However, major repositioning is not necessarily a requirement; movements of only a few ångstroms could dramatically decrease the reactivity with one substrate and increase the reactivity with another substrate, without large changes in the structure of the protein. Further work will be needed to probe the magnitude of these conformational changes and to understand how the enzyme uses the oxidation state of the cofactor to control the positioning of the substrates.

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