Mutations in the B_{12}-Binding Region of Methionine Synthase: How the Protein Controls Methylcobalamin Reactivity†

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ABSTRACT: Vitamin B_{12}-dependent methionine synthase catalyzes the transfer of a methyl group from methyltetrahydrofolate to homocysteine via the enzyme-bound cofactor methylcobalamin. To carry out this reaction, the enzyme must alternately stabilize six-coordinate methylcobalamin and four-coordinate cob(I)alamin oxidation states. The lower axial ligand to the cobalt in free methylcobalamin is the dimethylbenzimidazole nucleotide substituent of the corrin ring; when methylcobalamin binds to methionine synthase, this ligand is replaced by histidine 759, which in turn is linked by hydrogen bonds to aspartate 757 and thence to serine 810. We have proposed that these residues control the reactivity of the enzyme-bound cofactor both by increasing the coordination strength of the imidazole ligand and by allowing stabilization of cob(I)alamin via protonation of the His-Asp-Ser triad. In this paper we report results of mutation studies focusing on these catalytic residues. We have used visible absorbance spectroscopy and electron paramagnetic resonance spectroscopy to probe the coordination state of the cofactor and have used stopped-flow kinetic measurements to explore the reactivity of each mutant. We show that mutation of histidine 759 blocks turnover, while mutations of aspartate 757 or serine 810 decrease the reactivity of the methylcobalamin cofactor. In contrast, we show that mutations of these same residues increase the rate of AdoMet-dependent reactivation of cob(II)alamin enzyme. We propose that the reaction with AdoMet proceeds via a different transition state than the reactions with homocysteine and methyltetrahydrofolate. These results provide a glimpse at how a protein can control the reactivity of methylcobalamin.

The vitamin B_{12}-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, and then from CH_3-H_4folate[1] to enzyme bound cob(I)alamin to regenerate methylcobalamin and H_4folate (Banerjee & Matthews, 1990). The biological forms of vitamin B_{12}, methylcobalamin and adenosylcobalamin, contain carbon−cobalt bonds that can be cleaved through either heterolytic or homolytic mechanisms (Schrauzer, 1976). Methionine synthase is unique among B_{12}-dependent enzymes in *E. coli* in that it uses methylcobalamin as a cofactor and catalysis involves heterolytic carbon−cobalt bond cleavage, while other B_{12}-dependent enzymes contain adenosylcobalamin and catalyze reactions involving homolytic carbon−cobalt bond cleavage.

The binding of methyl- and adenosylcobalamin to their respective enzymes is known to alter the reactivity of these cofactors dramatically. Adenosylcobalamin-dependent enzymes promote the homolysis of the carbon−cobalt bond by a factor of $\sim 10^{10}$ (Hay & Finke, 1987). Binding of methylcobalamin to methionine synthase slows photolytic homolysis of the carbon−cobalt bond in methylcobalamin by ~40-fold (data described below). By comparison of the nonenzymatic rate of reaction of methylcobalamin with 2-mercaptoethanol[2] (Hogenkamp et al., 1985) with the rate of demethylation by homocysteine observed with enzyme-bound methylcobalamin (Banerjee et al., 1990a), we estimate that the enzyme promotes the rate of heterolytic cleavage by a factor of $>10^5$. Studies of model compounds suggest that two factors are primarily responsible for affecting the reactivity of alkylcobalamins. First, the basicity of the *trans* axial ligand controls the strength of the carbon−cobalt bond, with a more basic *trans* ligand resulting in a stronger carbon−cobalt bond (Ng et al., 1982). Second, a bulky *trans* ligand increases the upward folding of the corrin ring (Bresciani-Pahor et al., 1985; Glusker, 1995), and this weakens the carbon−cobalt bond (Ng et al., 1983). However, the mechanisms by which protein binding alters the properties of the cofactor are poorly understood.

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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; CH_2-H_4folate, 5,10-methylenetetrahydrofolate; CH_3-H_4folate, 5-methyltetrahydrofolate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; SHE, standard hydrogen electrode; H_4folate, 5,6,7,8-tetrahydrofolate; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] Hogenkamp et al. (1985) report a rate of $\sim 2 \times 10^{-5}$ s^{-1} in 0.2 M 2-mercaptoethanol at pH 7.1 and 43 °C. Extrapolating this rate to neat 2-mercaptoethanol (14.3 M), we obtain a maximal uncatalyzed rate of $\sim 1.4 \times 10^{-3}$ s^{-1} and compare this to the enzyme-catalyzed rate of 140 s^{-1} at 25 °C and pH 7.2 reported by Banerjee et al. (1990a).

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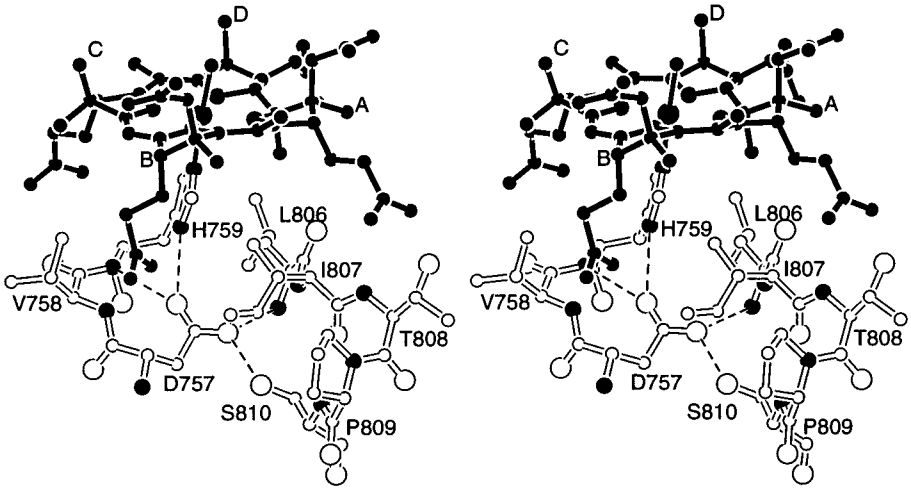


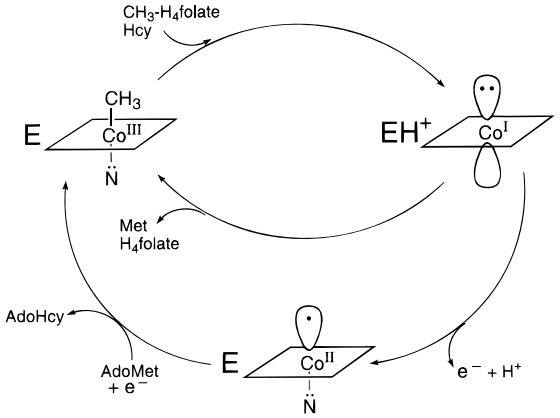
FIGURE 1: Stereoview showing a hydrogen-bonding network involving His759, the lower ligand to the methylcobalamin in methionine synthase. The hydrogen bonds connecting Asp757 to His759, Ser810, and the backbone amide nitrogens of His759 and Leu806 are shown as dashed lines. The methylcobalamin is in black. The protein residues are shown as open bonds, with oxygen atoms enlarged and nitrogen atoms in black. The A, B, C, and D rings of the corrin macrocycle are labeled, and the dimethylbenzimidazole nucleotide tail has been omitted for clarity.

The structure of a 27 kDa fragment of *E. coli* methionine synthase with methylcobalamin bound has recently been solved by X-ray crystallography (Drennan et al., 1994a). The B_{12}-binding region is composed of two domains. The lower face of the corrin ring interacts with an α/β domain similar to the Rossmann nucleotide-binding domain; the dimethylbenzimidazole nucleotide is no longer coordinated to cobalt but is inserted deep within a hydrophobic binding pocket formed between the β-sheet and α-helices of this domain. Histidine 759 is the lower ligand to the cobalt in the enzyme-bound cofactor and is connected by hydrogen bonds to aspartate 757 and serine 810, forming a ligand triad (Figure 1) (Drennan et al., 1994a,b). These residues are likely to play a role in modifying the ligation strength of the histidine ϵ-nitrogen. The top face of the corrin ring is covered by the hydrophobic residues of an α-helical domain reminiscent of the globin core of a heme binding protein. This cap region forms a hydrophobic protein cage around the methyl group, limiting access to and from the active site, and may be important in protecting the resting methylcobalamin form of the enzyme.

Methionine synthase catalyzes the formation of methionine by a double-displacement mechanism associated with net retention of stereochemistry at the transferred methyl group (Zydowsky et al., 1986). During catalysis, the enzyme-bound prosthetic group cycles between methylcobalamin and cob(I)alamin (Scheme 1). The cobalt of enzyme-bound methylcobalamin is six-coordinate, with four equatorial ligands donated by the pyrrole nitrogens of the corrin ring, a methyl group in the upper axial position, and histidine in the lower axial position, while cob(I)alamin is preferentially four-coordinate due to electron density in the d_z^2 orbital perpendicular to the plane of the corrin ring (Lexa & Savéant, 1983). During catalytic turnover, homocysteine demethylates methylcobalamin in a nucleophilic displacement reaction, and cleavage of the carbon−cobalt bond in the upper axial position must be synchronized with removal of the nitrogen ligand in the lower axial position. A similar removal of the nitrogen ligand during reduction of five-coordinate cob(II)alamin enzyme to four-coordinate cob(I)alamin is associated with proton uptake (Drummond & Matthews, 1994). We have proposed that the ligand triad, His759-Asp757-Ser810, is also protonated during cob(I)alamin formation from methylcobalamin enzyme (Drennan et al., 1994a). The transient cob(I)alamin intermediate is a potent nucleophile and demethylates CH_3-H_4folate in a second nucleophilic displacement. This requires deprotonation and recoordination of the ligand triad to re-form the six-coordinate methylcobalamin enzyme (Drennan et al., 1994a). The cob(I)alamin intermediate is occasionally oxidized to the inactive cob(II)alamin state. EPR spectroscopy has established that histidine 759 is coordinated to the lower axial position of enzyme-bound cob(II)alamin (Drennan et al., 1994a). Reactivation of enzyme in the cob(II)alamin form requires a reductive methylation in which AdoMet serves as the methyl donor and reduced *E. coli* flavodoxin as the electron donor, yielding the six-coordinate methylcobalamin enzyme (Banerjee et al., 1990b; Fujii & Huennekens, 1974). The mechanism of this reaction is not known, and it is not clear what role the ligand triad might play in this reaction.

We have undertaken a characterization of the properties of the ligand triad mutants His759Gly, Asp757Glu,

Scheme 1. Catalytic Turnover and Oxidation/Reactivation of Methionine Synthase[a]



[a] Methylcobalamin enzyme binds Hcy and CH_3-H_4folate and reacts in a ternary complex forming cob(I)alamin enzyme, which is remethylated by CH_3-H_4folate prior to release of both substrates (Banerjee et al., 1990a). Cob(I)alamin formation requires removal of the axial nitrogen ligand, and is presumably accompanied by protonation of the ligand triad, signified by E to EH^+ (see text). Cob(I)alamin is occasionally oxidized to cob(II)alamin enzyme. Reactivation is accomplished by electron transfer from reduced flavodoxin and methyl transfer from AdoMet.

Asp757Asn, and Ser810Ala. The construction of a semisynthetic *metH* gene specifying methionine synthase, the expression of these mutant proteins in *E. coli* using this semisynthetic gene, and the characterization of the steady-state activities of each mutant protein are described in the accompanying paper (Amaratunga et al., 1996). In this paper we have extended the physical and kinetic characterization of these mutant proteins. We suspected that each residue in the ligand triad contributes to the strength of the cobalt−nitrogen bond and have used visible absorbance and EPR spectroscopy to detect changes in ligation strength. It is difficult to measure the rates of the individual methyl transfer reactions because these occur in a ternary complex of the enzyme with both substrates, and we have instead used the initial rate of approach to steady state as a measure of the relative rates of heterolytic methyl transfer for each mutant (Banerjee et al., 1990a). The rate of reactivation of enzyme in the cob(II)alamin form with AdoMet and flavodoxin hydroquinone was also measured. These studies point to the importance of ligand triad residues in determining cobalamin reactivity.

MATERIALS AND METHODS

Materials. The following were obtained from the indicated commercial sources and used without further purification: L-homocysteine thiolactone, *S*-adenosylmethionine (iodide salt), dithiothreitol, hydroxocobalamin, and methylcobalamin from Sigma, methyl viologen, protocatechuic acid, and TEMPO from Aldrich, and CH_3-H_4folate (calcium salt) from Schirks Laboratories. Phosphate buffer refers to potassium phosphate buffer at pH 7.2 unless otherwise specified. Methylcobinamide was synthesized from methylcobalamin by $Ce(OH)_3$ hydrolysis as described by Hay and Finke for the synthesis of adenosylcobinamide from adenosylcobalamin (Hay & Finke, 1987; Renz, 1971). Flavodoxin was overexpressed and purified as described previously (Bianchi et al., 1993a; Osborne et al., 1991). Ferredoxin (flavodoxin)-$NADP^+$ oxidoreductase was expressed from overproducing *E. coli* K-12 strain C600/pEE1010 (Bianchi et al., 1993b) and purified by an adaptation of the previously described method (Eliasson et al., 1992). 5-Deazaflavin was a gift from Prof. Vincent Massey (University of Michigan). Protocatechuate dioxygenase was a gift from Prof. David Ballou (University of Michigan). The construction of synthetic wild-type and mutant methionine synthase genes, as well as the overexpression and purification of the resulting proteins, is described in the accompanying paper (Amaratunga et al., 1996).

Conversion of Methionine Synthase to Methylcobalamin and Cob(II)alamin. Enzyme isolated from the preparations contained B_{12} in methylcobalamin, cob(II)alamin, and cob(III)alamin forms. Each protein was converted to the methylcobalamin form by reductive methylation in the presence of AdoMet (Luschinsky et al., 1992). The enzyme (50−200 μM in 0.1 M phosphate buffer, pH 7.2, containing 0.2 M KCl) was mixed with AdoMet (500 μM) and methyl viologen (500 μM) in an electrochemical cell with a gold working electrode (Harder et al., 1989). The cell was made anaerobic and poised at −450 mV (vs SHE) for 45 min. The methylated protein was purified in the dark by gel filtration on a Superose 12 column (30 × 1 cm, Pharmacia) equilibrated with 10 mM phosphate buffer/1 mM EDTA, pH 7.2, and then concentrated in a centricon 30 concentrator (Amicon). This procedure was shown to remove AdoMet.

Cob(II)alamin enzyme was prepared by two previously described methods (Chen & Chance, 1993; Drummond et al., 1993). In the first method (Drummond et al., 1993), homocysteine (1 mM final concentration) was added to methylcobalamin enzyme (∼50 μM) in the presence of dithiothreitol (25 mM), and the system was made anaerobic by flushing with argon. Conversion to cob(II)alamin, monitored spectrally, was complete within 15 min to 2 h, depending on which mutant was used. Excess reagents were removed by gel filtration and the samples concentrated as described above. After removal of dithiothreitol, the Asp757Asn and Asp757Glu mutant proteins slowly oxidize to cob(III)alamin enzyme, while the other proteins remain in the cob(II)alamin state. Alternatively, cob(II)alamin enzyme was produced by anaerobic photolysis of methylcobalamin enzyme (Chen & Chance, 1993). A solution of methylcobalamin enzyme (10−100 μM) and TEMPO (500 μM) in phosphate buffer (50 mM) was made anaerobic by repeated cycles of equilibration with argon and evacuation over 30 min. The enzyme was immersed in an ice water bath and exposed to visible light (650 W tungsten/halogen lamp at 90 V rheostat setting) for 10−20 s, or until no further spectral changes were detected.

Visible Spectra and Extinction Coefficients of Mutant Methionine Synthase. Spectra were recorded from 300−700 nm on a Perkin-Elmer λ4c spectrophotometer. The instrument has a spectral resolution of ±1 nm, and spectra are reproducible to ±0.005 absorbance units. In each case, spectra of controls containing all ingredients but the enzyme were recorded separately in the same cell and used to correct spectra of samples containing the enzyme.

Extinction coefficients were determined for each protein in the methylcobalamin and cob(II)alamin forms and, for all proteins but His759Gly, in the cob(III)alamin form. Using an extinction coefficient at 520 nm of 9100 M^{-1} cm^{-1} for methylcobalamin at pH 7.0 (Hill et al., 1964), the extinction coefficient of free methylcobalamin in 80 mM Tris chloride buffer, pH 8.0, containing 6 M guanidine hydrochloride was found to be 9180 M^{-1} cm^{-1} at 520 nm. The following procedure was repeated for each mutant: a stock solution of methylcobalamin enzyme (∼100 μM in 50 mM phosphate buffer at pH 7.2) was prepared and a spectrum recorded. A portion of this stock was diluted 5-fold in guanidine hydrochloride (6 M in 80 mM Tris chloride buffer, pH 8.0, after dilution), and a spectrum of the released methylcobalamin was recorded and used to determine the exact concentration of the protein stock solution. The native methylcobalamin protein was also diluted 5-fold in 500 μM TEMPO/50 mM phosphate buffer, pH 7.2, and the sample made anaerobic by purging with argon. The spectrum of the methylcobalamin enzyme was recorded, the enzyme was converted to cob(II)alamin by photolysis as described above, and the spectrum of the resulting cob(II)alamin enzyme was recorded. Another sample of enzyme in the methylcobalamin form was diluted 5-fold in 50 μM potassium ferricyanide/50 mM phosphate buffer and the sample photolyzed to produce the aquocob(III)alamin enzyme.

Electron Paramagnetic Resonance of Mutant Methionine Synthase. EPR spectra were recorded on a Century Series Varian EPR spectrometer operating at 9.173 GHz and 3 mW power. Spectra were composed of an average of eight scans that were collected from 220 to 420 mT and digitized to 1024 points on a TRACOR-Northern NS-900 Signal Averager and processed with software developed by W. Dunham

(University of Michigan). To prepare the samples, methylcobalamin enzyme (400 μL, ~150 μM) in 50 mM phosphate buffer, pH 7.2, was made anaerobic by passing argon over the sample for 45 min, and then the sample was tipped into an attached EPR tube. The sample was converted to cob(II)alamin enzyme by photolyzing as described above (Chen & Chance, 1993), but in the absence of TEMPO. Free cob(II)alamin and cob(II)inamide were prepared in the same manner in 1:1 (v/v) 50 mM phosphate buffer/methanol. Methanol as a cosolvent improves resolution in these samples (Bayston et al., 1970). The cob(II)alamin samples were equilibrated at 25 °C for 2 min and then frozen in liquid nitrogen. Samples were cooled to 50 K for data collection.

Stability of the Mutant Methylcobalamin Enzymes. The rate of photolysis of methylcobalamin enzyme was measured by stopped-flow spectroscopy on a Hi-Tech diode-array instrument. The light source was a xenon arc lamp (80 W, 78 V), which was used simultaneously for continuous-wave photolysis and for spectral observation. The light from the xenon lamp was channeled directly to the sample by a fiber optic cable. After passing through the sample, the light was directed into a diode-array spectrophotometer, and spectra were recorded every 0.1−10 s with 10 ms averaging and 2 nm resolution. Alternatively, the light was passed through a monochromator (2 nm resolution) and detected at the wavelength of interest with a photomultiplier tube operating at −450 V. To initiate the reaction, a fresh sample of methylcobalamin enzyme (10 μM final) was mixed with aerobic phosphate buffer and passed into the flow cell, where exposure to the xenon lamp produced photolysis. The temperature was maintained at 25 ± 1 °C throughout the experiment. The rates were determined by fitting a single exponential to an average of 4−6 consecutive trials.

Stopped-Flow Studies of Turnover and Reactivation. The rate of approach to steady state and the turnover number were determined by stopped-flow spectroscopy at 25 °C on a Hi-Tech instrument equipped with a tungsten lamp, a presample monochromator, and a photomultiplier tube operating at 580 V. The instrument was equilibrated with 1 mM protocatechuate and 0.5 μM protocatechuate dioxygenase for 12 h to remove absorbed oxygen. All solutions were kept rigorously anaerobic by flushing with argon/vacuum cycles for 15 min in an anaerobic tonometer, followed by addition of 1 mM protocatechuate and 0.5 μM protocatechuate dioxygenase as an oxygen scrubbing system. The methylcobalamin enzyme (10 μM final) was reacted with homocysteine (100 μM) and (6-*R*,*S*)-CH_3-H_4folate (1 mM) at 25 °C, and the reaction observed at 390 nm to follow the formation of cob(I)alamin, and at 525 nm to follow the demethylation of methylcobalamin (Banerjee et al., 1990a). The His759Gly mutant protein was followed at 390 and 470 nm. Because a substantial amount of demethylation of the enzyme occurs in the 3 ms dead time of the stopped-flow, we first assumed that the final remethylated enzyme has the same spectrum as the initial methylcobalamin enzyme. We then fit the approach to steady-state with a single exponential and used this curve to estimate the initial rate of cob(I)alamin formation. Division of this rate by the enzyme concentration provides an estimate of the rate constant for demethylation of methylcobalamin by 100 μM homocysteine. For the wild-type enzyme, where the most demethylation occurs in the dead time of the instrument, this procedure gave an estimated rate constant of 108 s^{-1}, in good agreement with the previous estimate of the rate constant for homocysteine demethylation (Banerjee et al., 1990a). Turnover numbers were calculated from the enzyme-monitored turnover curve by numerically integrating the area under the kinetic curve. The fractional amount of homocysteine consumed at any time t is then the area from time = 0 to t divided by the total area (Gibson et al., 1964). In this way a plot of rate vs [homocysteine] was extracted from the stopped-flow data and the maximal turnover number (under saturating substrate conditions) was determined by extrapolating to infinite homocysteine concentration.

The rate of reductive methylation of cob(II)alamin enzyme was also determined by stopped-flow spectroscopy at 25 °C. Again, all samples were maintained under anaerobic conditions. Flavodoxin hydroquinone was generated by incubating oxidized flavodoxin (100 μM) with 5-deazaflavin (10 μM) and EDTA (1 mM) in phosphate buffer (25 mM) under anaerobic conditions. The tonometer was immersed in a cold water bath and repeatedly exposed for 10 s to light from a 650 W tungsten/halogen lamp (Massey & Hemmerich, 1978). The progress of the reduction was monitored spectrally with an attached cuvette. Typically, the resulting sample was ~90 μM hydroquinone and ~10 μM semiquinone as determined by spectral deconvolution. Cob(II)alamin enzyme (10 μM final concentration) was preincubated with AdoMet (200 μM) and then reacted with flavodoxin hydroquinone (50 μM), and the spectral changes were followed for 16 min.

RESULTS

Mutations in the Ligand Triad Affect the Rates of Methyl Transfer to and from the Substrates. We have previously proposed that the residues in the ligand triad may be important in promoting methyl transfer reactions to and from enzyme-bound methylcobalamin (Drennan et al., 1994a,b). The steady-state activities of the ligand triad mutants reported in the preceding paper suggested that there were differences in the reactivity of the prosthetic group in these enzymes (Amaratunga et al., 1996). To study this more directly, we turned to enzyme-monitored turnover experiments, using a stopped-flow spectrophotometer equilibrated at 25 °C. In these experiments, turnover is initiated by mixing methylated enzyme with 10 equiv of homocysteine and an excess of CH_3-H_4folate and is monitored by observing the spectrum of the enzyme-bound cobalamin. Under anaerobic conditions in the stopped-flow spectrophotometer, the wild-type enzyme-bound cob(I)alamin does not oxidize to cob(II)alamin and no reducing system or AdoMet is required to sustain turnover. Furthermore, because these experiments monitor turnover by measuring absorbance changes of the enzyme-bound cobalamin, the activities of mutant enzymes can be assessed even in the presence of traces of wild-type enzyme.

The primary turnover reactions involve methyl transfers that occur in a ternary complex of the enzyme with both of its substrates (Figure 2) (Banerjee et al., 1990a). Under anaerobic conditions, the reaction of wild-type methylcobalamin enzyme with saturating amounts of homocysteine and CH_3-H_4folate results in the rapid demethylation of methylcobalamin enzyme, a slight overshoot of steady-state cob(I)alamin levels, and, finally, the formation of ~30% steady-state cob(I)alamin (Banerjee et al., 1990a). Cob(I)alamin has a strong absorbance maximum at ~390 nm and has a lower absorbance than methylcobalamin at 525 nm. The reaction mechanism involves methyl transfer reactions from methylcobalamin to homocysteine and between CH_3-

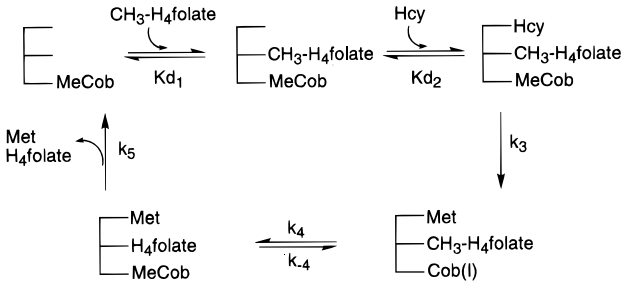


FIGURE 2: Kinetic scheme for the primary turnover reactions of methionine synthase. This minimal scheme is based on stopped-flow experiments with wild-type enzyme and simulations using rate constants of $k_3 = 140\ s^{-1}$, $k_4 = 180\ s^{-1}$, $k_{-4} = 60\ s^{-1}$, and $k_5 = 50\ s^{-1}$ (Banerjee et al., 1990a).

H_4folate and the enzyme-bound cob(I)alamin (Figure 2). The rate of approach to steady-state involves contributions from each of these rates:

$$\begin{aligned} d[\text{cob(I)alamin}]/dt = {} & k_3 \\ & [E{\cdot}CH_3\text{-cobalamin}{\cdot}CH_3\text{-}H_4\text{folate}{\cdot}Hcy] - \\ & k_4[E{\cdot}\text{cob(I)alamin}{\cdot}CH_3\text{-}H_4\text{folate}{\cdot}Met] + \\ & k_{-4}[E{\cdot}CH_3\text{-cobalamin}{\cdot}H_4\text{folate}{\cdot}Met] \quad (1) \end{aligned}$$

Since $E{\cdot}CH_3$-cobalamin$\cdot CH_3$-H_4folate$\cdot$Hcy is the only species present immediately after mixing, the initial rate depends primarily on the rate constant for demethylation of enzyme-bound methylcobalamin by homocysteine (k_3). Mutations in the ligand triad affect the initial rate of approach to steady state as shown in Figure 3A and Table 1. The initial rate of approach to steady state is decreased 39% by the Ser810Ala mutation, 46% by the Asp757Asn mutation, and 67% by the Asp757Glu mutation. Wild-type enzyme and the mutant proteins Ser810Ala, Asp757Asn, and Asp757Glu each undergo turnover, and when homocysteine is exhausted, the enzyme accumulates in the methylcobalamin form (Figure 3B). In contrast, while the His759Gly mutant demethylates to form cob(I)alamin (Figure 3C), the reaction occurs at a rate that is 100 000-fold slower than wild-type enzyme and the cob(I)alamin oxidizes to cob(II)alamin rather than being remethylated by CH_3-H_4folate. Thus the His759Gly mutant appears to be unable to catalyze the overall reaction. Since methyl transfers to homocysteine and from CH_3-H_4folate involve nucleophilic substitutions and cob(I)alamin is a kinetically competent intermediate in these transfers (Banerjee et al., 1990a), these observations suggest that residues in the ligand triad play a role in heterolytic carbon−cobalt bond cleavage.

After the initial demethylation by homocysteine, the wild-type enzyme and the Ser810Ala, Asp757Asn, and Asp757Glu mutant enzymes each reach a steady state in which 35−55% of the enzyme is in the cob(I)alamin form (Figure 3B). The level of cob(I)alamin in the steady state depends on a balance between the rates of three methyl transfer reactions (k_3, k_4, and k_{-4} in Figure 2), and a change in the rate of any single step would result in a change in the steady-state level of cob(I)alamin. Since the cob(I)alamin level was largely unaffected by the Ser810 and Asp757 mutations, this suggests that the rates of each methyl transfer to homocysteine (k_3) and between CH_3-H_4folate and cobalamin (k_4 and k_{-4}) were affected approximately to the same extent, and that these reactions have similar transition states. The level of cob(I)alamin in the steady state gradually rises after several turnovers in the Asp757 mutant enzymes (Figure 3B). We

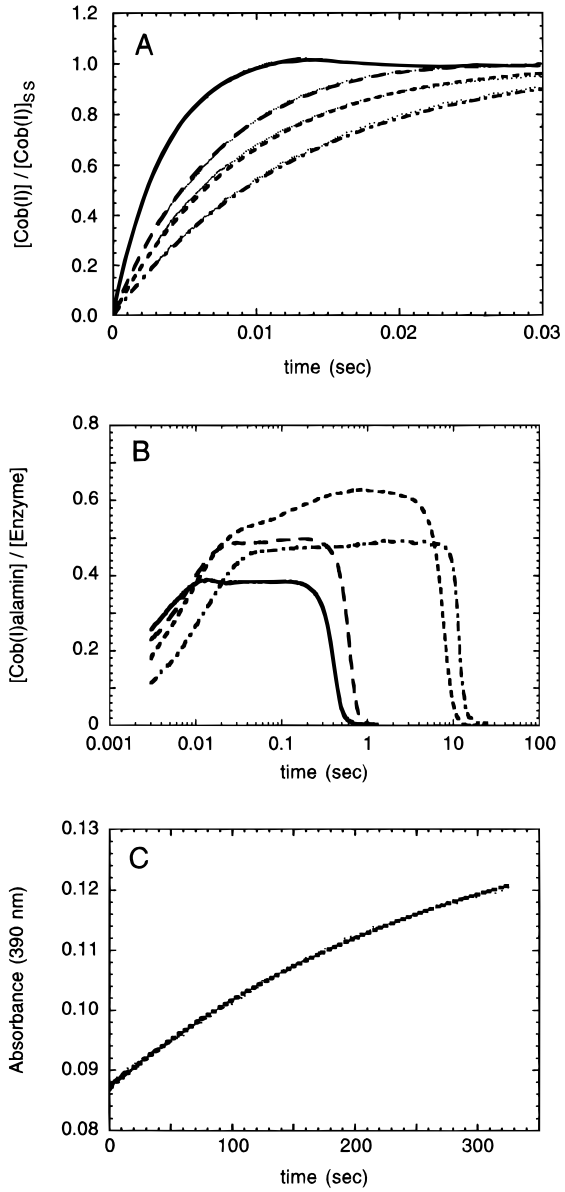


FIGURE 3: (A) Pre-steady-state rate of demethylation of methylcobalamin enzyme observed under anaerobic conditions in a stopped-flow spectrophotometer at 25 °C. Enzyme in the methylcobalamin form (~10 μM) was mixed with 10 equiv of homocysteine and 50 equiv of CH_3-H_4folate, as described in Materials and Methods, and the reaction was monitored at 525 nm. The rate of approach to steady state is shown for wild-type methionine synthase (—), Ser810Ala (− −), Asp757Asn (- - -), and Asp757Glu (− - −) proteins. The absorbance data for each experiment were normalized to the steady-state cob(I)alamin concentration. The wild-type data were fit to a single-exponential curve for the first 13 ms (solid line); the fit to the remainder of the data is an extrapolation between the maximum concentration of cob(I)alamin and the steady-state concentration. For the other mutants, the data collected over the 30 ms time course were fit to a single-exponential curve. Values for the rate constants were estimated by calculating the slope of the initial 20% of the exponential curve (prior to normalization) and dividing this slope by the enzyme concentration; these rate constants are given in Table 1. (B) The complete enzyme-monitored turnover curves for wild-type (—), Ser810Ala (− − −), Asp757Asn (- - -), Asp757Glu (− - −) proteins. The cob(I)alamin concentration was determined by monitoring the absorbance at 525 nm and normalized using the absorbance decrease at 525 nm determined by complete demethylation of the methylated enzyme to cob(I)-alamin enzyme using excess homocysteine. (C) Demethylation of His759Gly (− - −) by homocysteine under the conditions described above. The reaction was monitored at 390 nm. Spectra taken at various time points indicated that cob(I)alamin is the initial product of demethylation but is converted to cob(II)alamin at a rate comparable to the demethylation rate.

Table 1: Kinetics of stopped-flow turnover, AdoMet-dependent reactivation, and photolysis

mutant protein	stopped-flow turnover number[a] (s^{-1})	rate constant for intial approach to steady state[b] k_{init} (s^{-1})	rate constant for AdoMet-dependent reactivation $1/t_{1/2}$[c] (s^{-1})	rate constant for aerobic photolysis $k_{apparent}$ (s^{-1})
wild type	27.1 (1)	108 (1)	0.13 (1)	0.023 (1)
Ser810Ala	18.5 (0.68)	66 (0.61)	0.31 (2.4)	0.044 (1.9)
Asp757Asn	1.5 (0.056)	58 (0.54)	0.27 (2.1)	0.055 (2.4)
Asp757Glu	0.99 (0.037)	36 (0.33)	1.87 (14.4)	0.076 (3.3)
His759Gly	0	0.003 (1×10^{-5})	1.86 (14.3)	0.53 (23)
CH_3-cobalamin				0.95 (41)
CH_3-cobinamide				0.88 (38)

[a] Stopped-flow turnover experiments were conducted at 25 °C as described in Materials and Methods, and the calculation of the turnover number is described there. The mutant enzyme preparations contain ~0.5% wild-type enzyme. The consumption of substrate by this contaminating wild-type enzyme results in a small error in the measured turnover number, which we have not attempted to correct. [b] Initial rates of approach to steady state were converted to rate constants by division with the enzyme concentration (see Materials and Methods). [c] Reactivation of cob(II)alamin enzyme is a complex reaction with at least four kinetic phases (unpublished data). The rate shown ($1/t_{1/2}$) is an attempt to simplify the data shown in Figure 4 for comparison with the other data shown above.

suspect that this is due to the accumulation of catalytically incompetent cob(I)alamin as an off-pathway intermediate, a proposal that future experiments will probe more carefully.

Since the enzymes were reacted with ~10 equiv of homocysteine and an excess of CH_3-H_4folate, the steady-state level of cob(I)alamin was maintained for a few seconds and then the enzyme returned to the original methylcobalamin state (Figure 3B). The time required to return the enzyme to the methylcobalamin state was used to calculate a turnover number under V_{max} conditions for anaerobic catalysis, in the absence of oxidation or reactivation reactions (see Materials and Methods). This turnover number was measured at 25 °C and is shown in Table 1. The turnover number contains contributions from each step in Figure 2:

$$\text{turnover number} = V_{max}/E_T = k_3k_4k_5/(k_3k_4 + k_3k_{-4} + k_3k_5 + k_4k_5) \quad (2)$$

The slowest step in the turnover of wild-type enzyme has been shown to be product release (Banerjee et al., 1990a) and changes in the turnover number primarily reflect changes in this rate-limiting step (k_5). The Asp757 mutants are 15−30-fold impaired with respect to turnover, consistent with the observed steady-state activities (Amaratunga et al., 1996). Since the pre-steady-state rates are only 2−3-fold impaired (step 3 in Figure 2) and the steady-state concentration of cob(I)alamin is not greatly altered, product release (k_5) is likely to be seriously impaired in the Asp757Glu and Asp757Asn mutant proteins.

The enzyme-monitored turnover experiments can be analyzed in terms of a specific kinetic model and will provide estimates of the rates of individual steps in the reaction; a fit of the approach to steady state of the wild-type enzyme using the model in Figure 2 was described by Banerjee et al. (1990a). Since one of the substrates (homocysteine) is completely consumed over the course of the reaction, a complete simulation of both pre-steady-state and steady-state data requires that one can model the binding behavior of the limiting substrate. Preliminary experiments with wild-type and mutant enzymes have shown that the initial steady-state activity has a nonhyperbolic dependence on the homocysteine concentration, a previously undetected phenomenon. Experiments are in progress to understand this behavior, and a complete analysis of the enzyme-monitored turnover experiments will follow.

Mutant Cob(II)alamin Enzymes Can Be Remethylated with AdoMet and a Reducing System. As discussed in the accompanying paper (Amaratunga et al., 1996), the mutant enzymes are isolated with most of the cobalamin present as cob(II)alamin or cob(III)alamin rather than methylcobalamin. This inability to maintain the methylcobalamin form of the enzyme *in vivo* might be due either to an increased lability of the methylcobalamin enzyme or to a decreased rate of reductive methylation of cob(II)alamin enzyme. We tested the latter possibility by attempting the reductive methylation of each mutant protein by electrochemical reduction and by enzymatic reduction using the physiological reducing system.

Each mutant protein was successfully reductively methylated in an electrochemical cell. In this procedure, the cob(II/III)alamin enzyme is mixed with AdoMet and methyl viologen and poised at −450 mV vs SHE. The wild-type enzyme is typically methylated in 20−30 min, and each mutant was also successfully methylated within 30 min. The assignment of the resulting protein-bound cofactor as methylcobalamin was based on the visible spectrum of the cofactor after denaturing the enzyme in 6 M guanidine hydrochloride, as described in the next section.

Each mutant was also reductively methylated using the physiological reducing system. In this procedure, the cob(II/III)alamin enzyme is incubated under anaerobic conditions with 10 equiv of AdoMet, 0.1 equiv of flavodoxin, 0.05 equiv of ferredoxin (flavodoxin)-$NADP^+$ oxidoreductase, and 50 equiv of NADPH. The wild-type enzyme is methylated at a rate of ~0.01 s^{-1}, and the progress of the reaction can be monitored by visible absorbance spectroscopy. Each mutant was successfully methylated under these conditions, with rates that are comparable to that of wild-type enzyme, ranging between 0.02 and 0.005 s^{-1}. In these experiments, the rates are at least partially limited by the rate of electron flux from NADPH to flavodoxin and thence to methionine synthase and therefore may not directly reflect the rate of methyl transfer. However, the rate of methyl transfer must be least as fast as the overall rate of reductive activation.

To probe the rate of methyl transfer from AdoMet to cob(II)alamin more directly, we turned to stopped-flow spectrophotometry. Under anaerobic conditions, flavodoxin can be photoreduced to yield >90% of the flavin in the hydroquinone oxidation state. Assuming a midpoint potential of −440 mV for the flavodoxin hydroquinone/semiquinone couple (Hoover, Matthews, and Ludwig, unpublished data), this corresponds to an effective reduction potential of −496 mV vs SHE. We reacted excess reduced flavodoxin with cob(II)alamin methionine synthase that had been preincubated with AdoMet. At 410 nm, where flavodoxin hydroquinone and semiquinone have the same absorbance, we were

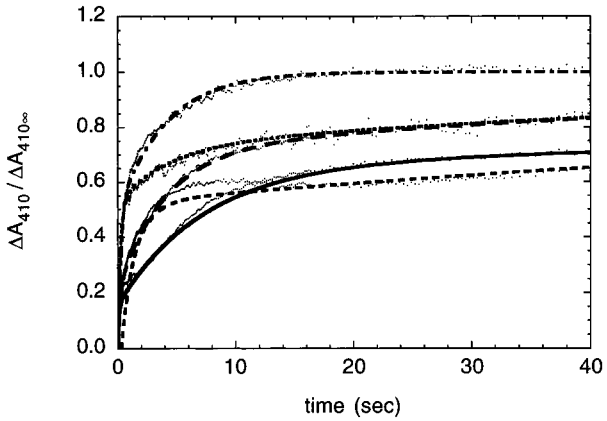


FIGURE 4: Conversion of enzyme preparations in the cob(II)alamin form to methylcobalamin after mixing with AdoMet and flavodoxin hydroquinone under anaerobic conditions in a stopped-flow spectrophotometer at 25 °C. Measurements were made at 410 nm, where the oxidation of flavodoxin hydroquinone to semiquinone does not contribute to the absorbance changes. The rate of reductive methylation is shown for the following forms of methionine synthase: wild-type (—), Ser810Ala (— — —), Asp757Asn (- - -), Asp757Glu (— - —), and His759Gly (···).

able to isolate changes in the cobalamin spectrum and found that the reaction is very complex, involving at least four kinetic phases. However, an overlay of the normalized traces of the wild-type enzyme and ligand triad mutants shows that the mutants generally are methylated faster than the wild-type enzyme (Figure 4). We fit these kinetic curves to four sequential exponential phases, with rates ranging from 200 to 0.01 s^{-1}; only the final two phases are visible in Figure 4. We calculated a half-time for remethylation of the enzyme from the absorbance changes measured at 410 nm to allow a simple comparison of the mutants. The His759Gly and Asp757Glu mutants are methylated 14-fold faster than the wild-type enzyme (Table 1, column 4). Thus, although the His759Gly mutation results in a decrease of five orders of magnitude in the rate of reaction of enzyme-bound methylcobalamin with homocysteine and loss of all activity in catalytic turnover, the rate of methylation of this mutant enzyme in the cob(II)alamin form by AdoMet is unimpaired. Our results suggest that the presence of a lower axial ligand may actually impede AdoMet-dependent cob(II)alamin methylation, a result completely opposite that obtained for the other substrates. Indeed, when flavodoxin binds to wild-type enzyme in the cob(II)alamin form, the histidine ligand dissociates (Hoover et al., 1995). These observations may signal a difference in the mechanisms of methyl transfer to and from homocysteine and CH_3-H_4folate as compared to the mechanism of methyl transfer from AdoMet.

Mutations Affect the Visible Spectrum of the Enzyme-Bound Cobalamin. The UV/visible absorbance spectra of wild-type enzyme and each mutant protein in the methylcobalamin and cob(II)alamin forms were recorded in phosphate buffer, pH 7.2 (Table 2 and Figure 5). The enzyme was methylated using the electrochemical procedure described above and purified by gel filtration to remove excess

[3] The term "base-on" refers to cobalamin derivatives in which a nitrogen ligand, usually dimethylbenzimidazole, is coordinated to the lower axial position of the cobalt. "Base-off" refers to cobalamin derivatives in which the nitrogen ligand has been displaced from the cobalt, usually by protonation in acidic solution. In most cases, a water molecule or other oxygen ligand (e.g., aspartate or glutamate) then serves as the ligand to cobalt, although in methionine synthase the base-off cofactor may lack a ligand in the lower axial position.

Table 2: Wavelengths and Extinction Coefficients for Absorption Maxima of Mutant Enzymes in Selected Oxidation States

mutant protein	methylcobalamin[a]		cob(II)alamin[b]		cob(III)alamin[c]	
	λ_{max}	ϵ (M^{-1} cm^{-1})	λ_{max}	ϵ (M^{-1} cm^{-1})	λ_{max}	ϵ (M^{-1} cm^{-1})
wild type	525	8910	474	9470	352	20200
Ser810Ala	527	8950	475	9760	352	24700
Asp757Asn	527	9130	473	10260	356	24600
Asp757Glu	533	8620	465	11600	353	24800
His759Gly	450	11660	466	12770	ND[d]	ND[d]
cobalamin	520	9100[e]	475	9250	350	26200[c]
cobinamide	463	8740[e]	469	12300[b]	348	28000[c]

[a] The absorbance of free methylcobalamin is used as the basis for all protein-bound cobalamin extinction coefficient calculations (Hill et al., 1964). [b] The free cob(II)alamin extinction coefficient was determined experimentally, while the cob(II)inamide extinction coefficient is from Lexa et al. (1980). [c] The extinction coefficient of cob(III)alamin is reported for aquocob(III)alamin and that reported for cob(III)inamide is for diaquocob(III)inamide (Hill et al., 1965). The spectrum of aquocob(III)alamin is pH dependent, and the water ligand ionizes to form hydroxocobalamin with a pK of 7.6. The hydroxocob(III)alamin absorbance maximum is shifted to 357 nm, and a value of ϵ_{357} = 21 000 was determined experimentally. His759Gly could not be oxidized to the cob(III)alamin oxidation state. [d] ND, not determined. [e] The methylcobinamide extinction coefficient reported is actually an experimental determination of the extinction coefficient of base-off methylcobalamin in 0.1 M H_3PO_4.

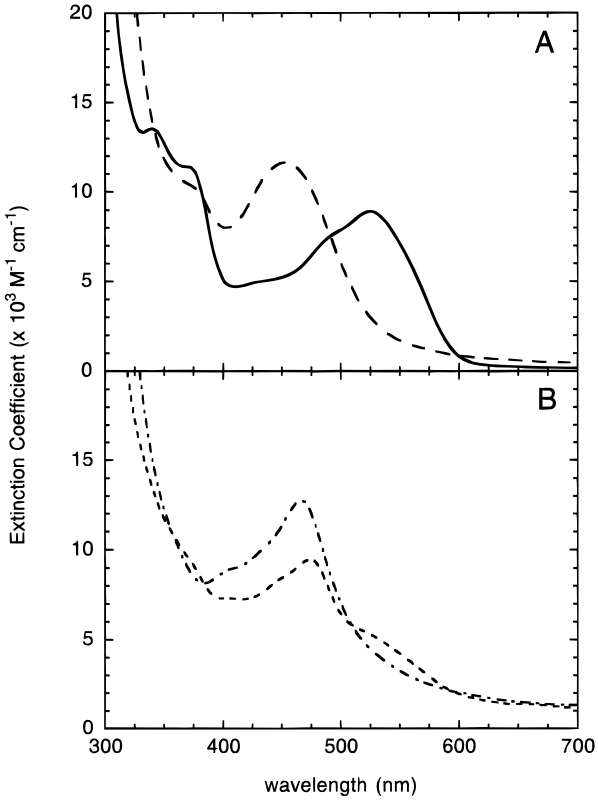


FIGURE 5: UV/visible absorbance spectra of methylcobalamin and cob(II)alamin proteins in phosphate buffer, pH 7.2. (A) Spectra of enzymes in the methylcobalamin form: wild-type (—) and His759Gly (— — —). (B) Spectra of enzymes in the cob(II)alamin form: wild-type (- - -) and His759Gly (— - —).

AdoMet. The spectrum of this methylcobalamin enzyme was recorded, and the extinction coefficient was determined by denaturing the protein in guanidine hydrochloride and recording a spectrum of the liberated cofactor. The wild-type enzyme gave a spectrum consistent with base-on[3] methylcobalamin (Figure 5A), with absorbance maxima at 525, 375, and 340 nm. The extinction coefficient at 525nm of 8910 M^{-1} cm^{-1} (Table 2) was significantly lower than

had previously been reported (Fujii & Huennekens, 1974) but is comparable to that of free cofactor which has an ϵ_{520} of 9100 M^{-1} cm^{-1} (Hill et al., 1964). The spectra of each of the other mutant proteins except His759Gly were similar to that of wild-type enzyme. The His759Gly methylcobalamin spectrum differed from those of the other proteins, with a λ_{max} at 450 nm and an extinction coefficient of 11 660 M^{-1} cm^{-1} (Figure 5A). This spectrum is similar to, but not identical with, that of free base-off[3] methylcobalamin (methylcobalamin at pH 0), which has a λ_{max} at 462 nm and an extinction coefficient of 8740 M^{-1} cm^{-1}. The observed differences suggest that, while free base-off cofactor has water as a lower ligand, the His759Gly mutant may have no lower ligand and the methylcobalamin form of His759Gly protein may be truly five-coordinate.

The cob(II)alamin enzyme was produced quantitatively by photolysis under anaerobic conditions. The addition of TEMPO as a radical trap accelerated this reaction and prevented damage to the protein due to random insertion of the methyl radical. The extinction coefficient of the cob(II)alamin enzyme was determined relative to the previously determined methylcobalamin extinction coefficient. The spectrum of wild-type enzyme in the cob(II)alamin form (Figure 5B, $\epsilon_{474} = 9740$ M^{-1} cm^{-1}) is similar to that of free cob(II)alamin ($\epsilon_{475} = 9250$ M^{-1} cm^{-1}). The His759Gly cob(II)alamin enzyme ($\epsilon_{466} = 12\,770$ M^{-1} cm^{-1}) exhibited a spectrum similar to cob(II)inamide which has an ϵ_{469} of 12 300 M^{-1} cm^{-1} (Hill et al., 1965; Lexa et al., 1980), consistent with the cobalamin being "base-off" in this mutant protein (Figure 5B). The other mutations in residues of the ligand triad resulted in a gradual increase in the extinction coefficient of the cob(II)alamin enzyme, in the order Ser810Ala, Asp757Asn, and Asp757Glu. The measured extinction coefficients suggest that the wild-type enzyme and the Ser810Ala mutant may be primarily base-on while the Asp757Glu protein is primarily base-off in the cob(II)alamin oxidation state. Note that this trend parallels the loss of steady-state activity and the decrease in the pre-steady-state demethylation rate.

Ligand Triad Mutations Affect the EPR Spectra of Cob(II)alamin Enzymes. The visible spectra of mutant proteins in the cob(II)alamin form suggested that there is a gradual shift from base-on cob(II)alamin in the wild-type enzyme to base-off cob(II)alamin in the His759Gly mutant. Cob(II)alamin contains an unpaired electron in the d_z^2 orbital, and the EPR spectrum is sensitive to the presence of an axial nitrogen ligand (Banerjee et al., 1990b; Bayston et al., 1970). The spectrum of the base-off cob(II)alamin shows a complex low-field signal (at $g = 2.5$) and eight high-field singlets (centered at $g = 2$) due to hyperfine coupling with the cobalt nucleus. In the spectrum of base-on cob(II)alamin, the low-field signal merges into a single broad unresolved signal ($g = 2.3$), while the eight high-field singlets are each split into triplets due to superhyperfine coupling with the nucleus of the nitrogen ligand. The presence of a nitrogen ligand also results in a change in the coupling constant for cobalt hyperfine splitting that alters the spacing of the peaks, allowing simultaneous observation of both singlets and triplets in mixed samples. Spectra which contain both base-off singlets and base-on triplets probably contain a distribution of protein molecules frozen in either the base-on or base-off conformation. Wild-type enzyme contains an equilibrium mixture of base-on and base-off cob(II)alamin in the native state that is detectable by EPR spectroscopy and is sensitive

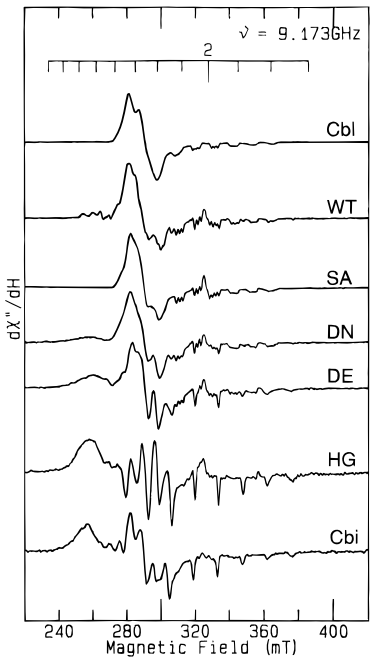


FIGURE 6: EPR spectra of methionine synthase mutant and wild-type proteins in the cob(II)alamin form. Spectra shown: free cob(II)alamin (Cbl), wild-type (WT), Ser810Ala (SA), Asp757Asn (DN), Asp757Glu (DE), His759Glu (HG), and free cob(II)inamide (Cbi). All samples were in 50 mM potassium phosphate buffer, pH 7.2, except cobalamin and cobinamide which contain 50% methanol as a cosolvent. The small signal at $g = 2$ in the protein spectra is probably due to protein radicals introduced by the photolysis process.

to the solvent (Banerjee et al., 1990b), and we thought that we might be able to detect increasing amounts of base-off cobalamin in the mutant proteins by EPR.

The EPR spectra of wild-type enzyme and mutant proteins Ser810Ala, Asp757Asn, Asp757Glu, and His759Gly are shown in Figure 6, along with spectra of cob(II)alamin and cob(II)inamide. The His759Gly mutant is completely base-off as shown by the absence of high-field triplets and the presence of high-field singlets, similar to the spectrum of cob(II)inamide. For the other proteins, the approximate fraction (±10%) of base-off cofactor was estimated by subtracting the scaled spectrum for His759Gly enzyme from each of the other spectra until a spectrum resembling free cob(II)alamin was obtained. Wild-type and Ser810Ala enzymes appear to be 15% and 5% base-off, respectively, while the Asp757Asn protein is 25% base-off and the Asp757Glu protein is 65% base-off according to the EPR analysis. Taken together, the EPR and UV/visible absorbance spectra provide evidence for weakening of the cobalt−nitrogen bond in the lower axial position of enzyme-bound cob(II)alamin when aspartate or serine residues of the ligand triad are mutated.

Mutations Affect the Stability of Methylcobalamin Enzyme toward Photolysis. Alkylcobalamins are unstable toward light in the 350−470 nm region (Schrauzer et al., 1970). Binding of the methylcobalamin cofactor to methionine synthase results in a significant increase in the photostability of the C−Co bond (Taylor & Weissbach, 1968). We had observed that while the wild-type enzyme is isolated as 80% methylcobalamin enzyme, the mutants were isolated as varying mixtures of cob(II)alamin and cob(III)alamin enzyme (Amaratunga et al., 1996). The reactivation experiments described above showed that this was not due to defective *in vivo* AdoMet-dependent reductive methylation and sug-

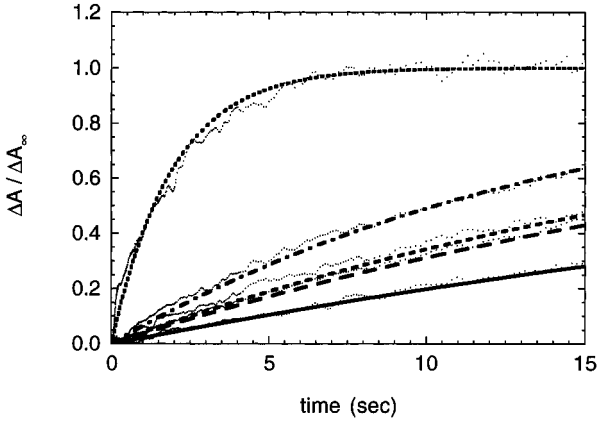


FIGURE 7: Rates of photolysis of methylcobalamin enzyme by a xenon lamp in air-saturated phosphate buffer, pH 7.2, at 25 °C. Spectra were followed at the wavelengths of maximal absorbance changes (see Table 2), and the kinetic traces were normalized. Photolysis traces are shown for the following forms of methionine synthase: wild-type (—), Ser810Ala (— — —), Asp757Asn (- - -), Asp757Glu (— - —), and His759Gly (···).

gested that the mutations had resulted in a decrease in the ability to protect methylcobalamin enzyme against loss of the methyl group.

We measured the rate of photolysis of the mutant enzymes and free cofactor under continuous-wave photolysis conditions. We chose to use a xenon lamp because its high intensity and even spectral distribution were less likely to result in preferential cleavage of certain mutants due to differences in spectral overlap between the sample and lamp, unlike previous experiments with tungsten lamps (Schrauzer et al., 1970). We measured the photolysis rates under aerobic conditions; under these conditions the methyl radical is rapidly trapped by oxygen to give $CH_3OO^{\cdot}$, which in turn is converted to a mixture of products with the predominant species being formaldehyde (Hogenkamp, 1966). In air-saturated solution, the free cob(II)alamin cofactor is oxidized by oxygen-derived radicals to cob(III)alamin at a rate that is similar to the photolysis rate (Hogenkamp, 1966), while the enzyme-bound cofactor remains as cob(II)alamin. The addition of a spin trap (TEMPO, 500 μM) to the experiment had no effect on the photolysis rate measured under aerobic conditions, suggesting that the methyl radical is effectively scavenged by oxygen.

The wild-type enzyme is photolyzed ~40-fold slower than free methylcobalamin (Table 1). This is less stabilization than has previously been detected and may have resulted from a better overlap of the axial bond molecular orbital transitions with the xenon lamp as compared to the tungsten lamp used in previous experiments (Schrauzer et al., 1970; Taylor & Weissbach, 1968). In the protein series wild-type, Ser810Ala, Asp757Asn, Asp757Glu, and His759Gly, the rate of photolysis is increased 23-fold (Table 1 and Figure 7), even though all of these residues lie beneath the cobalamin cofactor and would not be expected to alter the solvent exposure of the methyl group on the upper face of the cobalamin. Note that this order of photolysis parallels the loss of activity in steady-state turnover and in approach to steady state and also parallels the changes in the spectra of cob(II)alamin enzymes described above.

DISCUSSION

Methionine synthase catalyzes successive transfers of a methyl group from CH_3-H_4folate to cob(I)alamin and from methylcobalamin to homocysteine. Such difficult reactions do not proceed at an appreciable rate in the absence of enzyme (Hilhorst, 1993; Hogenkamp et al., 1985). The enzyme-catalyzed reaction proceeds with net retention of stereochemistry in the transferred methyl group, consistent with transfer of the methyl group from CH_3-H_4folate to homocysteine via two successive heterolytic S_N2 reactions (Zydowsky et al., 1986). In order to catalyze these reactions, methionine synthase must modify the reactivity of both substrates and the cofactor to promote facile heterolytic carbon−cobalt bond formation and cleavage. In particular, the cobalamin cofactor must alternate between six-coordinate methylcobalamin, the resting state of the enzyme, and the highly reactive four-coordinate cob(I)alamin intermediate. This transition requires that the lower axial ligand be alternately coordinated to the cobalt and then moved away from the cobalt. In addition, the enzyme must promote heterolytic cleavage of the carbon−cobalt bond by a factor of $>10^5$ when homocysteine is bound, relative to the uncatalyzed rate of demethylation seen with 2-mercaptoethanol (Hogenkamp et al., 1985). Finally, the enzyme must prevent inadvertent loss of the methyl group as a methyl radical and inadvertant oxidation of cob(I)alamin enzyme to cob(II)alamin, processes which are both metabolically wasteful and potentially damaging to the cell.

The crystal structure of the 27 kD cobalamin-binding region of *E. coli* methionine synthase suggested several residues that were likely to be involved in modifying the properties of the cofactor (Drennan et al., 1994a). The most impressive feature of the structure was the coordination of a histidine residue of the protein to the lower axial position of the cobalt in methylcobalamin. His759, Asp757, and Ser810 form an extended hydrogen-bonded network which leads from the cobalt to the solvent (Figure 1). We suggested that this network might promote catalysis by shuttling a proton from solvent into the ligand triad to promote formation of the four-coordinate cob(I)alamin intermediate (Drennan et al., 1994a,b). In this way, one could envision alternate stabilization of methylcobalamin by a strongly coordinating histidyl ligand as well as stabilization of cob(I)alamin by protonation and dissociation of the histidyl residue.

Evidence that Asp757 and Ser810 Contribute to the Ligation Strength of His759 to Cobalt and Affect Carbon−Cobalt Bond Strength. The spectroscopic properties of Asp757 and Ser810 mutant proteins suggest that these residues are important in promoting strong coordination of the histidine ϵ-nitrogen to the cobalt. The UV/visible spectra of cob(II)alamin from enzymes with mutations in the ligand triad show an increase in the extinction coefficient of the absorbance band at ~470 nm (Table 2 and Figure 5). Such changes in absorbance are also seen when the spectra of cobinamide and cobalamin are compared. We believe that the absorbance changes in the mutant proteins reflect a decrease in the base-on/base-off ratio of the cobalamin cofactor, a postulate which is supported by the EPR spectra of the cob(II)alamin proteins (Figure 6). If the extinction coefficient of free cofactor is used as a value for 100% base-on cobalamin and the extinction coefficient of His759Gly protein is used as the value for 100% base-off cobalamin, then the wild-type, Ser810Ala, Asp757Asn, and Asp757Glu proteins are estimated to be 6.3, 14, 29, and 67% base-off, respectively. The maximal observed change in the base-on/base-off ratio of enzyme in the cob(II)alamin form corresponds to an increase in the coordination strength of

the His759 ϵ-nitrogen of ~2.0 kcal/mol due to the hydrogen-bonding network involving Asp757 and Ser810. Model studies have shown that the coordination strength of the axial ligand increases with increasing basicity of the free ligand (Brown et al., 1972). Thus, an important role of Asp757 and Ser810 may be to increase the basicity of His759, thereby increasing the strength of the bond between His759 and the cobalt.

In both the methylcobalamin and cob(III)alamin forms of wild-type enzyme, the histidine is more strongly coordinated to cobalt than in the cob(II)alamin enzyme, with no significant base-off species detected in the physiological pH range. Mutations of Asp757 and Ser810 do not lead to formation of significant base-off methylcobalamin, so that the coordination strength of the histidine in mutant forms of methylcob(III)alamin enzyme cannot be measured directly and correlated with variations in the strength of the carbon−cobalt bond. However, the spectra of mutant forms of aquocob(III)alamin enzymes show an increase in the extinction coefficient at 350 nm (Table 2), suggesting a shift from hydroxocobalamin in the wild-type enzyme to aquocobalamin in the Asp757 and Ser810 mutant proteins. The pK_a for protonation of the hydroxide ligand in wild-type hydroxocob(III)alamin enzyme is below 6.0 and cannot be measured due to the instability of the enzyme at low pH, while the pK_a for protonation of free hydroxocob(III)alamin cofactor is 7.6. The extinction coefficients of the Ser810 and Asp757 mutant cob(III)alamin proteins suggest that, at pH 7.2, these proteins are mixtures of aquo- and hydroxocob(III)alamin and that the pK_a for protonation of the hydroxide ligand has increased as a result of these mutations. These observations suggest that mutation of Asp757 and Ser810 results in a decrease in the coordination strength of the upper oxygen ligand or, more generally, that the coordination strength of the lower axial ligand affects the strength with which the upper ligand bonds to cobalt in the cob(III)alamin state. Further, these effects suggest that mutation of Asp757 and Ser810 does affect the coordination behavior of the histidyl ligand in cob(III)alamin enzyme. For purposes of our analysis, we suggest that the strength of the Co−N bond in methylcob(III)alamin enzyme is directly related to the strength of the Co−N bond in the cob(II)alamin enzyme, which is detected as a change in the base-on/base-off ratio. Thus we have plotted the base-on/base-off ratio derived from visible spectra of the cob(II)alamin proteins in Figure 8 as a measure of the relative strength of the Co−N bond in the methylcobalamin proteins.

The coordination strength of the lower axial ligand is likely to affect the strength of a carbon−cobalt bond in the upper axial position (Ng et al., 1982). Direct measurement of the bond strength by thermal dissociation of the C−Co bond is not possible, since the temperatures required for measurable thermolysis (Martin & Finke, 1992) are well above the temperature range where the protein is stable. The rate of photolysis of the methylcobalamin enzyme provided an indirect way to measure the strength of this bond. When the methylcobalamin cofactor absorbs light in the 350−470 nm region of the spectrum, an electron is excited into the σ^* antibonding orbital of the carbon−cobalt bond (Schrauzer et al., 1970). The observed rate of photolysis is affected by several factors, including the strength of the resulting excited state C−Co bond, possible thermal relaxation of the excited state, and radical pair recombination due to protein cage effects. Mutations in the ligand triad below the cobalamin

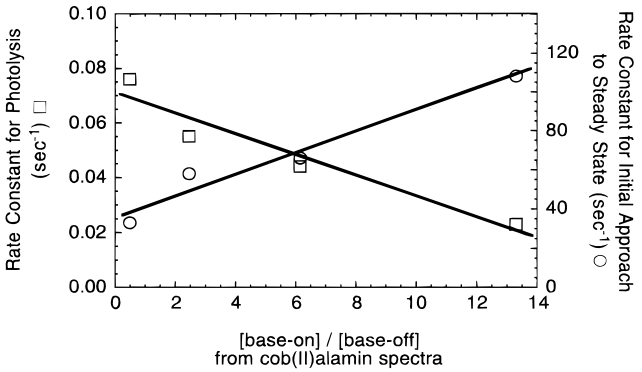


FIGURE 8: Inverse correlation between the effects of mutations of Asp757 and Ser810 on the rate constants for heterolytic demethylation (derived from the initial rate of approach to steady-state) and for homolytic demethylation (photolysis). The base-on/base-off ratio in the cob(II)alamin oxidation state is used to indicate the relative strength of the Co−N bond in the methylcobalamin state, and is calculated from the extinction coefficients for cob(II)alamin forms of the enzymes given in Table 2, assuming that free cob(II)alamin is 100% base-on and the His759Gly protein is 100% base-off. The rate constants for pre-steady-state demethylation (○) and photolysis (□) are given in Table 1.

probably have little effect on the protein structure above the cobalamin, and therefore we assume that the cage effects are similar for the wild-type and ligand triad mutant proteins. Our experiment cannot distinguish between changes in the C−Co bond strength and effects on thermal relaxation mechanisms. However, it is clear that the rate of photolysis depends on the coordination strength of the lower axial ligand to the cobalt, and this effect may be due to changes in the intrinsic C−Co bond strength. The rate of photolysis is plotted with squares in Figure 8 as a function of the base-on/base off ratio of cob(II)alamin seen in the wild-type (far-right) and mutant enzymes. The ratio of base-on to base-off cob(II)alamin is approximately related to the strength of the cobalt−nitrogen bond in the cob(II)alamin enzyme by eq 3, where C is an arbitrary constant. The rate of photolysis

$$[\text{base-on Cbl}]/[\text{base-off Cbl}] = \exp(-C\Delta G_{\text{Co-N}}/RT) \quad (3)$$

depends on the activation energy for cleavage of the excited state carbon−cobalt bond as described by the Ahrrenius equation (eq 4). The linear relationship between the photolysis rate and the base-on/base-off ratio suggests that the activation energy for photolysis is directly related to the strength of the cobalt−nitrogen bond (eqs 5 and 6). These

$$k_{\text{photo}} = k_{\text{o}} \exp(-E_{\text{aC-Co*}}/RT) \quad (4)$$

$$k_{\text{photo}} = C'[\text{base-on Cbl}]/[\text{base-off Cbl}] \quad (5)$$

$$\Delta E_{\text{aC-Co*}} = C''\Delta\Delta G_{\text{Co-N}} \quad (6)$$

photolysis experiments suggest that a strong cobalt−nitrogen bond results in a more stable excited-state carbon−cobalt bond. A comparison of the data for wild-type and Asp757Glu enzymes indicates a 29-fold decrease in the base-on/base-off ratio in the cob(II)alamin form of the mutant protein, implying a weakening of the cobalt−nitrogen bond by ~2.0 kcal/mol. In the methylcobalamin form of the mutant protein, weakened ligation of the histidine results in a weakening of the excited-state carbon−cobalt bond toward photolysis of ~0.7 kcal/mol. A similar argument was made by Halpern and co-workers for model compounds (Ng et al., 1982). In this study, the authors found a linear

relationship between the activation enthalpy for carbon−cobalt bond thermolysis and the pK_a of the lower ligand. A more basic ligand coordinates more strongly to the cobalt and increases the activation energy for homolytic cleavage of the carbon−cobalt bond and therefore increases the bond dissociation energy of the carbon−cobalt bond.

Correlation between the Strength of the Cobalt−Nitrogen Bond and the Rates of Heterolytic Methyl Transfer and Catalytic Turnover. Although it is important to stabilize the carbon−cobalt bond against homolytic cleavage, the primary catalytic role of methionine synthase is to promote heterolytic carbon−cobalt bond cleavage. Since the methyl transfer reactions in binary enzyme−substrate complexes do not occur at catalytically competent rates (Banerjee et al., 1990a), it is difficult to measure the rate of any isolated methyl transfer reaction. The rate of pre-steady-state demethylation observed in the presence of both substrates depends on methyl transfer reactions from methylcobalamin to homocysteine and between CH_3-H_4folate and cob(I)alamin (Banerjee et al., 1990a). The initial rate of this reaction depends primarily on the rate of methyl transfer from methylcobalamin to homocysteine and provides an estimate of the relative rates of this heterolytic methyl transfer. The ligand triad mutants exhibit increasingly impaired pre-steady-state demethylation rates at 25 °C (Figure 3A); the corresponding rate constants (Table 1) are plotted with circles in Figure 8 as a function of the base-on/base off ratio of cob(II)alamin seen in the wild-type (far-right) and mutant enzymes. The relationship between the rate of approach to steady-state and the base-on/base-off ratio suggests that there is a direct relationship between the activation energy for heterolytic bond cleavage and the strength of the cobalt−nitrogen bond. In this case, a strengthening of the cobalt−nitrogen bond by ∼2 kcal/mol decreases the activation energy for heterolytic bond cleavage by ∼0.7 kcal/mol and therefore increases the rate of heterolytic methyl transfer. Thus we believe that the His759 is bonded to the cobalt in the transition state for methyl transfer between substrates and cobalamin and that the Asp757 and Ser810 residues lower the energy of this transition state by hydrogen bonding to His759. Complete removal of the lower axial nitrogen ligand in the His759Gly mutant results in the loss of catalytic activity and in a decrease of five orders of magnitude in the rate of demethylation of methylcobalamin enzyme by homocysteine.

The small magnitude of the effects on the rate of approach to steady state of proteins with mutations of Asp757 and Ser810 suggests that these residues play lesser roles in catalysis of methyl transfer to and from cobalamin and that their effects are due primarily to perturbation of the strength of ligation of His759 to the cobalt. However, mutation of Asp757 results in a 15−30-fold decrease in steady-state activity (Amaratunga et al., 1996), which is also observed under anaerobic turnover conditions (Table 1, column 2), a change much larger than would be predicted by changes in the pre-steady-state demethylation rates. Previous experiments have shown that reduction of cob(II)alamin enzyme to cob(I)alamin is associated with proton uptake from solvent (Drummond & Matthews, 1994), possibly by the ligand triad residues, and we have proposed that this proton uptake also occurs during catalytic turnover (Drennan et al., 1994a). Preliminary studies on the Asp757Glu mutant enzyme indicate that the reduction of cob(II)alamin enzyme to cob(I)alamin is no longer associated with proton uptake in the physiological pH range (Choi and Matthews, unpublished data), suggesting that proton uptake would also be perturbed during catalytic turnover. The stopped-flow kinetic studies of turnover with the Asp757 mutants suggest that the postulated protonation and deprotonation of the ligand triad has only a minor role in stabilizing the transition state for methyl transfers to and from cobalamin but may play a larger role in effecting product release, which is largely rate-limiting in turnover of wild-type enzyme (Banerjee et al., 1990a). A possibility to be subjected to further testing is that protonation and deprotonation of the ligand triad provides electrostatic signals that can be propagated to other regions of the enzyme, triggering conformational changes that bring substrates into position for reaction with cobalamin and that allow release of products.

Effects of Mutations of the Ligand Triad on the Rate of AdoMet-Dependent Reductive Activation. Reductive activation of methionine synthase in the cob(II)alamin form can be driven by *E. coli* flavodoxin in either the hydroquinone or semiquinone oxidation states as well as by the chemical reducing system (dithiothreitol/B_{12}) employed in steady-state assays. Reactivation is fastest when flavodoxin hydroquinone is employed as the reducing system and is slower when flavodoxin semiquinone or dithiothreitol/B_{12} are used for reduction. To study the effect of ligand triad mutations on reactivation, we used the flavodoxin hydroquinone to minimize the dependence of the reaction on the rate of electron transfer. As the data in Table 1 show, the rate of AdoMet-dependent reactivation increases in ligand triad mutants, and the overall rate of reductive methylation is more than 14-fold faster in the His759Gly mutant than in the wild-type enzyme when flavodoxin hydroquinone is the reductant. These results are in direct contrast to the effect of the His759Gly mutation on methyl transfer reactions involving homocysteine and CH_3-H_4folate. These observations provide strong evidence that the mechanism for methyl transfer from AdoMet is not the same as for methyl transfer involving homocysteine and CH_3-H_4folate. For methyl transfers involving CH_3-H_4folate and homocysteine, the data presented in the previous section suggest a heterolytic mechanism with partial bonding of the nitrogen ligand to the cobalt in the transition state. Cob(I)alamin is a kinetically competent intermediate in these transfers (Banerjee et al., 1990a), but not for methylation by AdoMet. Incubation of enzyme in the cob(I)alamin form with AdoMet under anaerobic conditions does not lead to methylation of the cobalamin cofactor (Drummond, Jarrett, and Matthews, unpublished data). The effects of mutations in the ligand triad on AdoMet-dependent methyl transfer parallel the effects of the same mutations on photolysis and suggest that these two reactions may have similar transition states with no requirement for bonding of the lower axial ligand to cobalt. Due to this similarity, a radical mechanism for AdoMet-dependent reductive methylation is an attractive mechanistic hypothesis. Interestingly, the other enzymes that are known to interact with *E. coli* flavodoxin: pyruvate-formate lyase (Wong et al., 1993), AdoMet-dependent ribonucleotide reductase (Bianchi et al., 1993a), and biotin synthase (Ifuku et al., 1994), all apparently use reduced flavodoxin and AdoMet to form an adenosyl radical, which is then involved in generating a protein-based radical. As a hypothesis to guide further experiments, we propose that the AdoMet-dependent reactivation of methionine synthase may involve a direct transfer of the methyl group from AdoMet to cob(II)alamin, in a reaction that could be

described as a homolytic recombination of the unpaired electron in cob(II)alamin with a transient methyl radical. Such a mechanism requires that the electron needed for reductive activation be transferred to AdoMet rather than cobalamin. Efforts are underway in our laboratories to elucidate the mechanism of this reaction.

In summary, we have begun to dissect the mechanism by which methionine synthase controls the reactivity of enzyme-bound cobalamin. Cofactor binding is accompanied by replacement of the dimethylbenzimidazole ligand to the cobalt with a histidine residue (His759) from the protein. We have shown that a nitrogen ligand in the lower axial position is extremely important for heterolytic methyl transfers. The coordination of the lower axial ligand is strengthened through an extended hydrogen-bond network involving Asp757 and Ser810. The hydrogen-bonded network promotes heterolytic methyl transfer to and from substrates and inhibits homolytic loss of the methyl group by photolysis.

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