Ligand Trans Influence Governs Conformation in Cobalamin-Dependent Methionine Synthase†

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ABSTRACT: Cobalamin-dependent methionine synthase (MetH) of *Escherichia coli* is a large, modular enzyme that uses a cobalamin prosthetic group as a donor or acceptor in three separate methyl transfer reactions. The prosthetic group alternates between methylcobalamin and cob(I)alamin during catalysis as homocysteine is converted to methionine using a methyl group derived from methyltetrahydrofolate. Occasional oxidation of cob(I)alamin to cob(II)alamin inactivates the enzyme. Reductive methylation with flavodoxin and adenosylmethionine returns the enzyme to an active methylcobalamin state. At different points during the reaction cycle, the coordination state of the cobalt of the cobalamin changes. The imidazole side chain of His759 coordinates to cobalamin in a "His-on" state and dissociates to produce a "His-off" state. The His-off state has been associated with a conformation of MetH that is poised for reactivation of cobalamin by reductive methylation rather than catalysis. Our studies on cob(III)alamins bound to MetH, specifically aqua-, methyl-, and *n*-propylcobalamin, show a correlation between the accessibility of the reactivation conformation and the order of the established ligand trans influence. The trans influence also controls the affinity of MetH in the cob(III)alamin form for flavodoxin. Flavodoxin, which acts to shift the conformational equilibrium toward the reactivation conformation, binds less tightly to MetH when the cob(III)alamin has a strong trans ligand and therefore has less positive charge on cobalt. These results are compared to those for cob(II)alamin MetH, illustrating that access to the reactivation conformation is governed by the net charge on the cobalt as well as the trans influence in cob(III)-alamins.

Cobalamin-dependent methionine synthase (MetH)[1] from *Escherichia coli* catalyzes the transfer of a methyl group from methyltetrahydrofolate (CH_3-H_4folate) to homocysteine (Hcy), forming methionine (Met) (Figure 1). During this process, an enzyme-bound cobalamin prosthetic group is used as an intermediate methyl donor and acceptor; i.e., the methyl group of methylcobalamin is transferred to Hcy and the resulting cob(I)alamin is methylated by CH_3-H_4folate to reform methylcobalamin. Cob(I)alamin formed during catalytic turnover is susceptible to occasional oxidation, which leads to the formation of an inactive cob(II)alamin species (*1*). MetH is able to return the prosthetic group to the active methylcobalamin form through reductive methylation with flavodoxin (*2*) and adenosylmethionine (AdoMet) (*3*, *4*).

MetH is a large enzyme composed of four modules that are arranged linearly with single interdomain linkers (*5*). The modules bind Hcy, CH_3-H_4folate, cobalamin, and AdoMet, respectively. The available structures of MetH fragments indicate that only one module at a time may be in contact with the cobalamin-binding module. Therefore, in order to complete catalytic turnover, the enzyme must undergo large conformational changes so that the cobalamin-binding module can alternately access the Hcy- and CH_3-H_4folate-binding modules (*6*). In addition, the AdoMet-binding module, which also contains determinants for flavodoxin binding (*7*, *8*), must occasionally be in contact with the cobalamin-binding module for the reductive methylation of cob(II)alamin to occur; this arrangement of the modules is referred to as the reactivation conformation.

Movement into the reactivation conformation has been associated with a change in the coordination state of the cobalamin. Upon binding to MetH, the dimethylbenzimidazole base that acts as the α-ligand (i.e., the "lower" axial ligand) to the cobalt of cobalamin in solution is replaced by the imidazole side chain of His759 of the cobalamin-binding module (*9*). His759 has been shown to dissociate to yield a His-off cob(II)alamin species upon binding of flavodoxin to cob(II)alamin MetH, which is initially in a His-on conformation as judged by EPR spectroscopy (*10*, *11*). Observations made with the His759Gly mutant of MetH, which cannot exist in a His-on form, suggest that the enzyme is in a different conformation than the His-on form of the wild-type enzyme. His759Gly MetH is not active in catalytic turnover, but is active in reductive methylation (*12*), and the

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[1] Abbreviations: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; CH_3-H_4folate, 5-methyltetrahydrofolate; FPLC, fast protein liquid chromatography; Hcy, homocysteine; MetH, cobalamin-dependent methionine synthase; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy.

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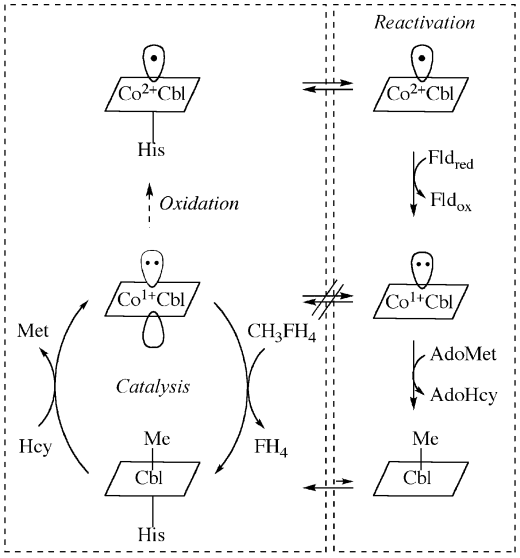


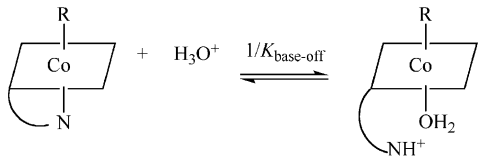
FIGURE 1: Reaction cycle for MetH. During catalysis (lower left), Hcy is converted to Met using a methyl group derived from CH_3-H_4folate (CH_3FH_4). The enzyme remains in one of the catalytic conformations (dashed box on the left) as enzyme-bound cobalamin (Cbl) alternates between methylcobalamin and cob(I)alamin. However, the cob(I)alamin form MetH is susceptible to oxidation, which leads to the formation of a cob(II)alamin species. While this inactive form of the prosthetic group is initially His-on, the enzyme converts to the reactivation conformation (dashed box on the right) and switches to His-off cob(II)alamin in the presence of flavodoxin. Following electron transfer from reduced flavodoxin and methyl transfer from AdoMet, His-off methylcobalamin is initially formed (*10*). The enzyme is subsequently returned to His-on methylcobalamin as MetH converts to the catalytic conformation (*10*). As indicated by the broken arrows, cob(I)alamin enzyme is unable to interconvert between the catalytic and reactivation conformations (*13*).

cob(II)alamin form of His759Gly MetH shows a marked difference in its cleavage pattern from that of wild-type MetH on limited proteolysis of the enzyme with trypsin (*13*). The X-ray crystal structure of a truncated form of His759Gly MetH (residues 649−1227) that only contains the cobalamin- and AdoMet-binding modules further confirmed a link between conformation and the coordination state of the cobalamin. The structure, in which the AdoMet-binding module interacts with the cobalamin-binding module of the truncated protein, revealed that a loop from the AdoMet-binding module is positioned between the corrin ring and the cobalamin-binding module (*14*), forcing the cobalamin into a His-off coordination state when MetH is in the reactivation conformation.

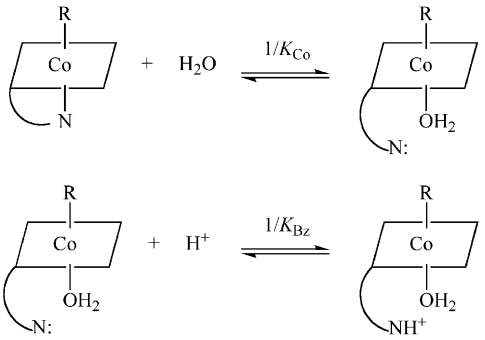
The absorbance properties of cobalamin have been useful in understanding the rules that govern the rearrangements of the modules of MetH and the factors influencing these movements. The His-on and His-off species have distinct spectral properties; for instance, the His-on form of methylcobalamin MetH exhibits an absorbance maximum at 525 nm, whereas the absorbance maximum is shifted to 450 nm for the methylcobalamin form of His759Gly MetH (*12*). With a means to experimentally distinguish between the His-on catalytic and His-off reactivation conformations, previous studies on methylcobalamin MetH revealed that the protein exhibits temperature-dependent changes in its absorbance spectrum, indicating a reversible interconversion between the His-on species, favored at low temperatures, and the His-off species, favored at high temperatures (*15*). Furthermore,

Scheme 1

Overall reaction:



Reaction in two steps:



$$K_{Co} = (K_{base\text{-}off}/K_{Bz}) - 1$$

ligands alter the conformational equilibria of methylcobalamin MetH by selectively stabilizing or destabilizing conformations (*15*). For example, CH_3-H_4folate shifts the equilibrium toward His-off methylcobalamin; because of steric clashes between the methyl groups of CH_3-H_4folate and methylcobalamin, the conformation in which the CH_3-H_4folate- and cobalamin-binding modules are juxtaposed is destabilized. Adenosylhomocysteine (AdoHcy), the product of reductive methylation, shifts the equilibrium toward His-off methylcobalamin by stabilizing the reactivation conformation, whereas AdoMet destabilizes the reactivation conformation due to steric repulsions between its methyl group and the methyl group of methylcobalamin and shifts the equilibrium toward His-on methylcobalamin.

Cob(III)alamin, when free in solution, is also known to undergo a base-on to base-off conversion, in which the dimethylbenzimidazole base is displaced by water and the nitrogen of the base is protonated (Scheme 1). The base-on form of cobalamin (a red-colored species) is converted to the base-off form (a yellow-colored species) in sufficiently strong acid. Thus, the large spectral changes, monitored as a function of pH, readily lend themselves to precise measurements of $pK_{base-off}$ (the apparent pK_a for the base-on/base-off reaction). Furthermore, Brown et al. (*16*) noted that the base-on/base-off equilibrium could be viewed as the sum of two equilibria. The model involves, first, the dissociation of the dimethylbenzimidazole and its replacement by water ($1/K_{Co}$) and, second, protonation of dimethylbenzimidazole, described by $1/K_{Bz}$ (Scheme 1). Values of K_{Co}, which should be dependent on the nature of the β-ligand (i.e., the upper axial ligand, R), can therefore be directly calculated from measured values of $pK_{base-off}$ by assuming that the value of pK_{Bz} is the same as that of the detached dimethylbenzimidazole nucleoside (1-α-D-ribofuranosyl-5,6-dimethylbenzimidazole) (*16*). The change in free energy required for the dissociation of the dimethylbenzimidazole base and its replacement by water (ΔG_{Co}), calculated from K_{Co}, reveals the influence of the β-ligand on the base-on/base-off reaction.

The values of $pK_{base-off}$ and ΔG_{Co} measured for cob(III)alamins while varying the β-ligand illustrate that the properties of this ligand affect the affinity of the dimethylbenzimidazole for the cobalt of the cob(III)alamin cofactor. In fact, the values of ΔG_{Co} vary from $+10.4$ kcal·mol^{-1} for a weak, uncharged β-ligand such as aqua (aquacobalamin; $pK_{base-off} = -2.13$) to $+1.9$ kcal·mol^{-1} for a strongly electron-donating ligand such as *n*-propyl (*n*-propylcobalamin; $pK_{base-off} = 4.10$) (*17*). Hence, as the β-ligand becomes more electron-donating, the affinity for the dimethylbenzimidazole base lessens, resulting in a shift in the equilibrium toward base-off cob(III)alamin. This phenomenon has been termed the ligand trans influence[2] and states that the equilibrium between the base-off and base-on forms of cob(III)alamin shifts toward the base-off form as the donor strength of the β-ligand increases in the order $H_2O < CN^- < CH_3 < CH_2CH_2CH_3$ (*18*).

Here, we asked whether the well-characterized ligand trans influence described for free cob(III)alamins applies to cob(III)alamins bound to MetH. The aquacobalamin, methylcobalamin, and *n*-propylcobalamin forms of MetH were subjected to conditions that were known to influence the distribution of conformers, including changes in temperature and the addition of ligands to MetH, including flavodoxin. The access of these different cob(III)alamin forms of MetH to the His-off reactivation conformation varied with the electron-donating properties of the β-ligand in the order of the established ligand trans influence. We also found that the affinity of flavodoxin for cob(III)alamin forms of MetH varied inversely with the ligand trans influence. Furthermore, because cob(II)alamin MetH is the form of the enzyme that requires reactivation, the cob(II)alamin form of MetH was characterized and found to lie between the methylcobalamin and *n*-propylcobalamin forms of MetH in terms of accessibility to the reactivation conformation.

MATERIALS AND METHODS

Materials. L-Homocysteine thiolactone, titanium(III) chloride, *n*-propyl iodide, 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), AdoHcy, and AdoMet were purchased from Sigma-Aldrich. (6*R*,*S*)-CH_3-H_4folate (calcium salt) was purchased from Schirks Laboratories. L-Hcy was prepared by the hydrolysis of L-homocysteine thiolactone and quantified as described previously (*19*). Titanium(III) citrate was prepared from titanium(III) chloride as described previously (*19*, *20*). *E. coli* flavodoxin (*7*, *8*) and MetH (*19*) were prepared as previously described. MetH was reductively methylated with AdoMet in an electrochemical cell prior to use, as described (*19*). A Varian Cary Bio 300 UV−vis thermostatted spectrophotometer fitted with both sample- and reference-cell holders was used for all spectroscopic experiments, and KaleidaGraph 3.6 (Synergy Software) was used to obtain all fits.

Formation of the Aquacobalamin, n-Propylcobalamin, and Cob(II)alamin Forms of MetH. The methylcobalamin form of MetH was converted to the aquacobalamin, *n*-propylcobalamin, and cob(II)alamin forms for use in this study. Methylcobalamin MetH was converted to the aquacobalamin form through aerobic demethylation with Hcy, as described previously (*19*). Briefly, Hcy (0.5 mM) was added to methylcobalamin MetH (10−100 μM in 1 mL of 0.1 M potassium phosphate buffer, pH 7.2) at 25 °C. The conversion was monitored spectrophotometrically at 350 nm for 5−15 min. The enzyme was then concentrated and exchanged into 10 mM potassium phosphate buffer, pH 7.2, for further use. The formation of *n*-propylcobalamin MetH began with the enzyme in the aquacobalamin form. Aquacobalamin MetH (10−100 μM in 1 mL of 0.1 M potassium phosphate buffer, pH 7.2) was placed in an anaerobic glass cuvette fitted with a septum and screw cap and equilibrated with Ar(g). Titanium(III) citrate (0.5 mM final concentration; added to reduce the enzyme to the cob(I)alamin form) and *n*-propyl iodide (0.5−1 mM final concentration) were added via a syringe at 37 °C. The conversion was monitored spectrophotometrically at 430 nm for 1−2 h. The enzyme was then concentrated and exchanged into 10 mM potassium phosphate buffer, pH 7.2, for further use. The cob(II)alamin MetH was formed by the photolysis of methylcobalamin MetH as described previously (*19*). Briefly, methylcobalamin MetH (10−100 μM in 1 mL of 0.1 M potassium phosphate buffer, pH 7.2) and 0.4 mM TEMPO were placed in an anaerobic glass cuvette and equilibrated with Ar(g). The cuvette was immersed in a beaker of ice water and irradiated with a tungsten/halogen lamp for 10 s. An absorbance spectrum was recorded after each irradiation until no further spectral changes were observed. The enzyme was concentrated and exchanged into 10 mM potassium phosphate buffer, pH 7.2, which also removed the TEMPO, for further use.

Temperature-Dependent Spectroscopic Experiments. In these experiments, MetH (5−10 μM in 50 mM potassium phosphate buffer, pH 7.2) was placed in the sample cuvette of a thermostatted spectrophotometer, and an equal amount of buffer was placed in the reference cuvette. The sample was allowed to equilibrate for 2 min after the desired temperature was reached before a spectrum was recorded. Spectra were recorded at intervals as the temperature was lowered from 25 to 10 °C, then raised from 10 to 40 °C, and finally lowered from 40 to 25 °C to ensure that the spectral changes were reversible.

Spectral deconvolution was used to determine the contributions of the His-on and His-off species to the observed spectra, as described previously for MetH (*21*). Briefly, the appropriate reference spectra were identified for His-on and His-off MetH. Varying amounts of the reference spectra were combined, and the resulting spectra were visually compared to the experimentally obtained spectra. The spectrum that most closely reproduced the experimental spectrum was identified and used as the fraction of the enzyme in the His-on and His-off forms. The His-off reference for *n*-propylcobalamin MetH was the spectrum of the propylated enzyme at 40 °C. The His-on reference was the spectrum of the *n*-propylcobalamin form of the cobalamin-binding module (residues 643−896), which was isolated by digestion of the native enzyme with trypsin followed by purification on MonoQ FPLC (*1*). The His759Gly variant of MetH, in which the His is not available for coordination, was used as a His-off reference. The spectrum of reductively methylated His759Gly MetH was used as the His-off reference for methylcobalamin MetH, and the His-off reference for cob(II)alamin MetH was the spectrum of five-coordinate cob-

[2] Early papers referred to a ligand trans effect. At present, thermodynamic influences are referred to as trans influences while the term trans effect is reserved for kinetic influences.

(II)alamin His759Gly MetH, in which one axial position is occupied by a water ligand (*22*). The His-on reference for methylcobalamin MetH was the spectrum of the methylated enzyme at 37 °C. The spectrum of cob(II)alamin MetH at 25 °C was corrected to account for 15% of the cob(II)alamin enzyme in the His-off form (*12*) to create its His-on reference spectrum.

The fraction of the enzyme in the His-on and His-off forms, as determined by spectral deconvolution, was used to calculate the thermodynamics of the interconversion of the His-on and His-off forms of MetH. The equilibrium constant [K = (% His-off)/(% His-on)] was calculated at each temperature and used in eq 1, where T is temperature in Kelvin and R is the gas constant.

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (1)$$

The values for the enthalpy (ΔH) and entropy (ΔS) changes were calculated from the slope and intercept, respectively, of a linear fit from a van't Hoff plot of ln K versus $1/T$. The free energy differences were calculated using $\Delta G = \Delta H - T\Delta S$.

Addition of Ligands to MetH. To monitor spectral changes due to the addition of the ligands CH_3-H_4folate and/or AdoHcy to MetH, equal volumes of the ligands (1 mM final concentration each) were added at the indicated temperature to both the sample cuvette (containing 10 μM aqua-, methyl-, or *n*-propylcobalamin MetH in 50 mM potassium phosphate buffer, pH 7.2) and the reference cuvette (containing buffer). After mixing the contents of each of the cuvettes with stir bars, the contents were allowed to equilibrate for 2 min prior to recording spectra. Spectra presented in the figures were corrected for dilution.

AdoMet was added to the cob(II)alamin form of MetH under anaerobic conditions to prevent oxidation to the cob(III)alamin form. Cob(II)alamin MetH (10 μM in 50 mM potassium phosphate buffer, pH 7.2) was contained in an anaerobic glass cuvette with a side arm, in which AdoMet was placed. After equilibration with Ar(g), AdoMet (1 mM final concentration) was mixed with the enzyme. The cuvette was placed in the sample compartment of the spectrophotometer, and a cuvette with an equal volume of AdoMet in buffer was placed in the reference compartment. A spectrum was recorded after the contents were allowed to equilibrate for 2 min. Spectra presented in the figures were corrected for dilution.

Difference Titrations with Oxidized Flavodoxin. Titrations of MetH with oxidized flavodoxin were performed as described previously (*11*) with minor modifications. For titrations with the aqua-, methyl-, and *n*-propylcobalamin forms of MetH, the enzyme was added to the sample cuvette (~10 μM MetH in 50 mM potassium phosphate buffer, pH 7.2), and an equal volume of buffer was added to the reference cuvette. The volumes in the sample and the reference cuvettes were matched precisely by weight to ensure that the concentrations of flavodoxin in each cuvette remained matched after each addition, as discussed previously (*11*). After an initial spectrum was recorded, aliquots of a concentrated oxidized flavodoxin solution, dissolved in the same buffer, were added to both cuvettes. The contents were gently mixed with a stir bar and allowed to equilibrate for 2 min before a spectrum was recorded. Spectra were recorded after each flavodoxin addition and were corrected for dilution before analysis.

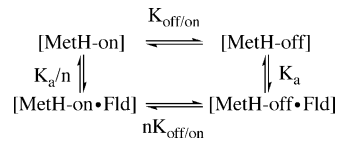


FIGURE 2: Thermodynamic box defining flavodoxin binding to MetH. Flavodoxin binding to His-on and His-off MetH is defined in this thermodynamic box, where MetH-on represents the average of all of the His-on states and MetH-off represents the His-off state. The change in the equilibrium constant ($K_{off/on}$) and in the affinity of flavodoxin for MetH (K_a) at saturating flavodoxin concentrations is given by the value n.

Titrations of cob(II)alamin MetH with oxidized flavodoxin were performed similarly to those above, but were done under anaerobic conditions to prevent oxidation to the cob(III)alamin form, as noted previously (*11*). MetH (10 μM MetH in 50 mM potassium phosphate buffer, pH 7.2) was added to a gastight glass cuvette and then equilibrated with Ar(g). A concentrated oxidized flavodoxin solution in a separate container was also equilibrated with Ar(g) and then added to the enzyme in the anaerobic cuvette. Portions of the same flavodoxin solution were also added to the reference cuvette, which contained only buffer. The contents in each cuvette were gently mixed with a stir bar and allowed to equilibrate for 2 min before a spectrum was recorded. Spectra were recorded after each flavodoxin addition and were corrected for dilution before analysis.

Equations Used to Determine Flavodoxin Binding to MetH. For the *n*-propylcobalamin, methylcobalamin, and cob(II)alamin forms of MetH, the following equations were derived to describe flavodoxin binding. These forms of MetH contain both His-on and His-off forms in the absence of flavodoxin, and the His-on and His-off forms were assumed to have different affinities for flavodoxin. The thermodynamic box shown in Figure 2 is used in order to define flavodoxin binding to His-on and His-off forms of MetH, where MetH-on represents the average of all of the His-on states and MetH-off represents the His-off state. The equilibrium between the two forms in the absence of ligands was defined as $K_{off/on}$. The change in this equilibrium was given by the value n at saturating flavodoxin so that the equilibrium between MetH-on·Fld and MetH-off·Fld, the flavodoxin-bound His-on and His-off forms, is $nK_{off/on}$. If the affinity of flavodoxin for MetH-off is given as K_a, then the thermodynamic box dictates that the affinity for MetH-on is K_a/n.

The observable spectral signal was the conversion of the His-on to the His-off forms induced by flavodoxin binding. However, the flavodoxin-bound and unbound forms of His-off MetH could not be distinguished from each other spectrally. Therefore, the term Y_t was defined by eq 2, where $[E]_t$ is the total MetH concentration.

$$Y_t = \frac{[\text{MetH-off}] + [\text{MetH-off·Fld}]}{[E]_t} \quad (2)$$

The information provided in the thermodynamic box was then combined with eq 2 to produce an equation for Y_t in terms of flavodoxin concentration, as given in eq 3. The

complete derivation of eq 3 is included in the Supporting Information.

$$Y_t = \frac{1 + K_a[\mathrm{Fld}]}{(1 + K_a[\mathrm{Fld}]) + (1/K_{off/on})(1 + (K_a/n)[\mathrm{Fld}])} \quad (3)$$

Y_t was calculated at each flavodoxin concentration used in the titrations by spectral deconvolution as described above, and a plot of Y_t versus flavodoxin was created. The values for n and K_a were extracted from the data by fitting the resulting plot to eq 3. Below, values are reported as K_d ($1/K_a$) and nK_d.

The experimental data from the titration of aquacobalamin MetH with flavodoxin could not be strictly analyzed in the same manner as described above. Increasing the concentration of flavodoxin resulted in an absorption change at 350 nm, which was fit to eq 4 for tight binding to obtain a value for K_d (*23*).

$$\Delta A_{350} = \frac{\Delta A_{350,max}}{2[\mathrm{E}]_t}\{([\mathrm{E}]_t + [\mathrm{Fld}] + K_d) - \sqrt{([\mathrm{E}]_t + [\mathrm{Fld}] + K_d)^2 - 4[\mathrm{E}]_t[\mathrm{Fld}]}\} \quad (4)$$

RESULTS

n-Propylcobalamin MetH. The *n*-propylcobalamin form of MetH showed a large, reversible temperature dependence. This form of MetH changed with temperature from mostly His-on at 10 °C to completely His-off at 40 °C, as shown in Figure 3A. The temperature-dependent changes were used to calculate an equilibrium constant [K = (% His-off)/(% His-on)]. From a plot of ln K versus $1/T$ (Figure 3B), ΔH was calculated to be 30 ± 1 kcal·mol^{-1} and ΔS was calculated to be 105 ± 5 cal·K^{-1}·mol^{-1}, yielding a value for ΔG of −2.6 ± 1.8 kcal·mol^{-1} at 37 °C (see Table 1). The negative value of ΔG reflects the preference of *n*-propylcobalamin MetH to remain in the His-off conformation.

In order to relate the temperature-dependent changes to conformational changes, CH_3−H_4folate and AdoHcy were added to *n*-propylcobalamin MetH at 10 °C. As this form of MetH was nearly completely His-off at higher temperatures, these experiments were performed at lower temperatures where there is a significant amount of enzyme in the His-on conformations. The addition of CH_3−H_4folate shifted the equilibrium toward the His-off conformation (Figure 4). As predicted, steric conflicts between the methyl group of CH_3−H_4folate and the propyl group of *n*-propylcobalamin MetH disfavored the conformation in which the CH_3−H_4folate-binding module is positioned above the cobalamin, shifting the equilibrium toward the His-off conformation. However, the addition of AdoHcy shifted the equilibrium toward His-on, in contrast to its effect on methylcobalamin MetH, where AdoHcy stabilizes the His-off form (*15*). A propyl group is much larger than a methyl group, and thus the reactivation conformation may not be able to accommodate both AdoHcy and *n*-propylcobalamin. In this case, the His-off reactivation conformation would be disfavored, shifting the equilibrium toward the His-on conformations. The effect of ligands on the absorbance properties of *n*-propylcobalamin MetH are consistent with the observed shifts between His-on and His-off forms being correlated with conformational changes of the protein.

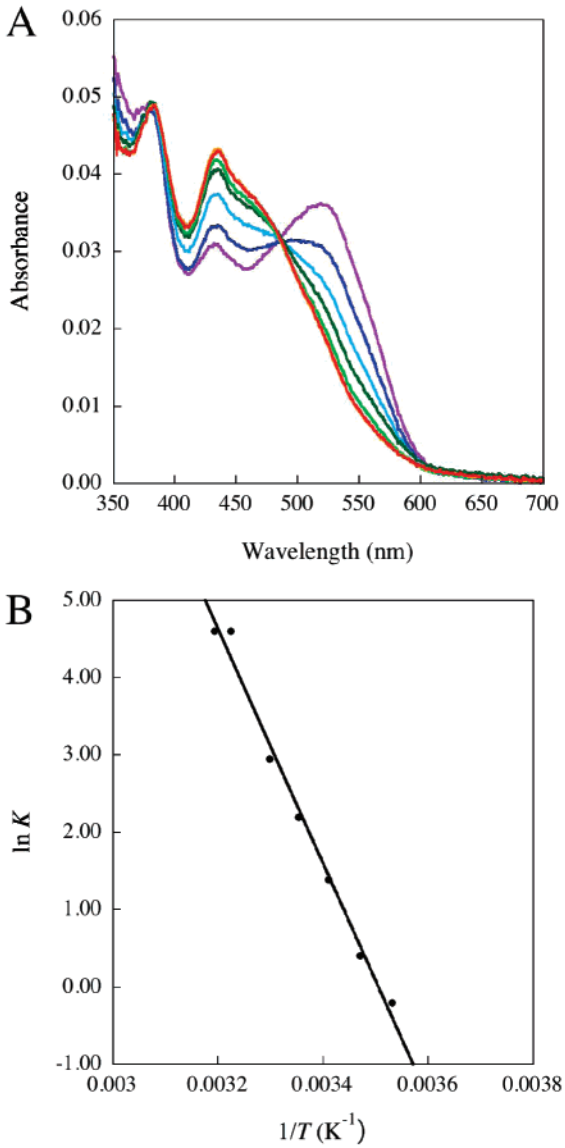


FIGURE 3: Effect of temperature on the absorbance of *n*-propylcobalamin MetH. The spectra of MetH (~5 μM) in 50 mM KPi buffer, pH 7.2, were recorded after equilibration for 2 min at 10 (purple), 15 (dark blue), 20 (light blue), 25 (dark green), 30 (light green), 37 (orange), and 40 °C (red) (A). The equilibrium constant [K = (% His-off)/(% His-on)] was calculated at each temperature and used to create a van't Hoff plot (B). Values for ΔH and ΔS were calculated from the slope and intercept, respectively, of a linear fit.

The titration of *n*-propylcobalamin MetH with flavodoxin at 5 °C is shown in Figure 5. With increasing concentrations of flavodoxin, the equilibrium was shifted further toward the His-off conformation. The spectra were deconvoluted using appropriate reference spectra (see Materials and Methods) to determine Y_t at each flavodoxin concentration, and the resulting data was fit to eq 3. For *n*-propylcobalamin MetH, the value of n was 23 ± 14, K_d was 12 ± 3 μM, and nK_d was 280 ± 180 μM (see Table 2).

Methylcobalamin MetH. The methylcobalamin form of MetH remained fully His-on between 10 and 40 °C, and the addition of either CH_3−H_4folate or AdoHcy singly did not result in any changes in the spectrum of MetH (data not shown and ref *15*). However, it had been previously observed that the addition of both CH_3−H_4folate and AdoHcy to methylcobalamin MetH at 37 °C results in a detectable shift

Table 1: Thermodynamic Effects of Temperature on MetH

form of MetH	ΔS (cal·K^{-1}·mol^{-1})	ΔH (kcal·mol^{-1})	ΔG at 37 °C (kcal·mol^{-1})
n-propylcobalamin	105 ± 5	30 ± 1	−2.6 ± 1.8
cob(II)alamin	44.1 ± 0.9	14.2 ± 0.3	0.5 ± 0.4
methylcobalamin + ligands	44.7 ± 1.4	14.9 ± 0.4	1.0 ± 0.6
methylcobalamin − ligands[a]	N.D.[b]	N.D.[b]	≈2.5

[a] The value of ΔG in the absence of ligands was calculated from the value obtained in the presence of ligands by assuming that CH_3−H_4folate and AdoHcy destabilize the His-on conformation of methylcobalamin MetH by 0.6 and 0.9 kcal·mol^{-1}, respectively, as determined in ref *15*. [b] Values could not be determined due to a lack of spectral changes.

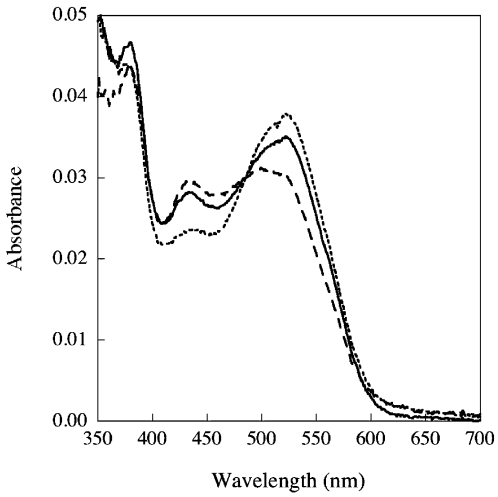


FIGURE 4: Effects of ligands on the absorbance of *n*-propylcobalamin MetH. The spectra of MetH (∼5 μM) in 50 mM KPi buffer, pH 7.2, were recorded after equilibration for 2 min at 10 °C in the absence of ligands (solid line), in the presence of 1 mM CH_3−H_4folate (dashed line), or in the presence of 1 mM CH_3−H_4folate and 1 mM AdoHcy (dotted line).

in the equilibrium toward the His-off conformation (*15*). Therefore, the spectrum of the methylcobalamin form of MetH was recorded at various temperatures in the presence of CH_3−H_4folate and AdoHcy. As shown in Figure 6, the spectra reveal that although there was little to none of the His-off species present at low temperature, there was a significant amount of His-off present at higher temperatures.

The analysis of the temperature dependence of the absorbance spectra illustrated the extent to which the His-on conformation is preferred by methylcobalamin MetH in comparison to the *n*-propylcobalamin form. The values of ΔH and ΔS were calculated to be 14.9 ± 0.4 kcal·mol^{-1} and 44.7 ± 1.4 cal·K^{-1}·mol^{-1}, respectively. These values, much smaller than those seen for the *n*-propylcobalamin form, yielded a ΔG of 1.0 ± 0.6 kcal·mol^{-1} at 37 °C (see Table 1). As previously reported, CH_3−H_4folate and AdoHcy destabilize the His-on conformation of methylcobalamin MetH by 0.6 and 0.9 kcal·mol^{-1} at 37 °C, respectively (*15*). The ΔG for methylcobalamin MetH in the absence of ligands is then approximated to be ≈2.5 kcal·mol^{-1} at 37 °C, indicating more than a 5 kcal·mol^{-1} difference between the *n*-propylcobalamin and methylcobalamin forms of MetH in the absence of added ligands.

Upon the addition of flavodoxin to the methylcobalamin form of MetH there were no spectral changes (data not shown and ref *11*). However, if CH_3−H_4folate and AdoHcy were present as described above for the temperature dependence experiments, the addition of flavodoxin at 37 °C resulted in a further shift toward the His-off form (Figure 7). In the presence of these two ligands, the value of *n* was 16.3 ± 1.0, K_d was 6.4 ± 0.6 μM, and nK_d was 104 ± 11 μM for methylcobalamin MetH (see Table 2). This form of MetH therefore exhibits tighter flavodoxin binding than the *n*-propylcobalamin form in both His-on and His-off conformations.

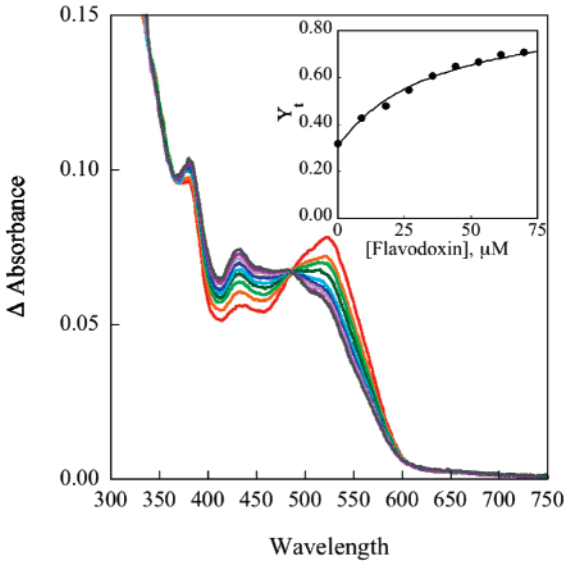


FIGURE 5: Titration of *n*-propylcobalamin MetH with flavodoxin. A solution of MetH (∼10 μM) in 50 mM KPi buffer, pH 7.2, was added to the sample cuvette of a double-beam spectrophotometer, and an equal volume of buffer was added to the reference cuvette at 5 °C. After the initial spectrum was recorded (red), equal amounts of concentrated flavodoxin (1.8 mM) were added to both cuvettes. The contents were gently mixed and then allowed to equilibrate for 2 min before the spectrum was recorded. Flavodoxin was added in increments up to 70 μM (orange). Inset: Plot of Y_t vs the concentration of flavodoxin added. The spectra from the titration were adjusted for dilution and then fit using reference spectra to determine Y_t at each flavodoxin concentration. The data were fit using eq 3 to determine nK_d.

Aquacobalamin MetH. The aquacobalamin form of MetH showed no temperature dependence, and the addition of AdoHcy had no effect on conformational equilibrium (data not shown). Furthermore, the addition of flavodoxin to this form of the enzyme at 25 °C did not result in any spectral changes consistent with formation of a His-off species (Figure 8). With increasing flavodoxin concentrations, there was a small increase in absorbance at 350 nm. The change in absorbance at 350 nm was fit to eq 4, and the K_d for binding to the His-on form (comparable to nK_d for the other forms of MetH) was calculated to be approximately 0.7 ± 0.4 μM. Although the data for aquacobalamin MetH was not analyzed in the same manner as the other forms of MetH, the results indicate that this form has a much higher affinity for flavodoxin than the other forms analyzed in this study.

Cob(II)alamin MetH. The cob(II)alamin form of MetH, as prepared, has been shown by EPR spectroscopy to have approximately 15% of the enzyme in a His-off conformation (*12*). When the temperature was varied (Figure 9A), lower temperatures favored the His-on conformations and higher temperatures favored the His-off conformation. The values of ΔH and ΔS were calculated from the plot of ln *K* versus

Table 2: Affinities of the His-On and His-Off Forms of MetH for Flavodoxin

form of MetH	K_d (μM)	$K_{off/on}$	n	nK_d (μM)
n-propylcobalamin	12 ± 3	0.44 ± 0.03	23 ± 14	280 ± 180
cob(II)alamin	2.2 ± 0.4	0.17 ± 0.02	30 ± 6	66 ± 18
methylcobalamin + ligands	6.4 ± 0.6	0.148 ± 0.007	16.3 ± 1.0	104 ± 11
aquacobalamin	N.D.[a]	N.D.[a]	N.D.[a]	0.7 ± 0.4

[a] Values could not be determined.

$1/T$ to be 14.2 ± 0.3 kcal·mol^{-1} and 44.1 ± 0.9 cal·K^{-1}·mol^{-1}, respectively. These values yielded a ΔG of 0.5 ± 0.4 kcal·mol^{-1} at 37 °C (See Table 1). Thus, access to the His-off conformation is more favorable for cob(II)alamin MetH than for the methylcobalamin form.

The addition of AdoMet shifted the equilibrium toward the His-off conformation at 37 °C (Figure 9B). This observation is in contrast to the effect of AdoMet on the methylcobalamin form of MetH. As discussed previously, AdoMet shifts the equilibrium of methylcobalamin MetH toward His-on due to a predicted steric clash between methyl groups. However, due to the demethylated state of the cob(II)alamin form of MetH, steric clashes are not as likely as for the methylcobalamin form discussed above. Thus, ligands might be expected to shift the conformational equilibria differently for cob(II)alamin than methylcobalamin MetH, as observed here.

The anaerobic titration of cob(II)alamin MetH with flavodoxin at 25 °C resulted in the formation of His-off cob(II)alamin (Figure 10). As with the *n*-propylcobalamin and methylcobalamin forms of MetH, Y_t was determined by spectral deconvolution at each flavodoxin concentration. The spectral changes associated with a conversion from His-on to His-off cob(II)alamin MetH are much more subtle than for the methylcobalamin form (*12*), making analysis of this form of the protein more difficult. Analysis of these data gives values of n as 30 ± 6, K_d as 2.2 ± 0.4 μM, and nK_d as 66 ± 11 μM (see Table 2).

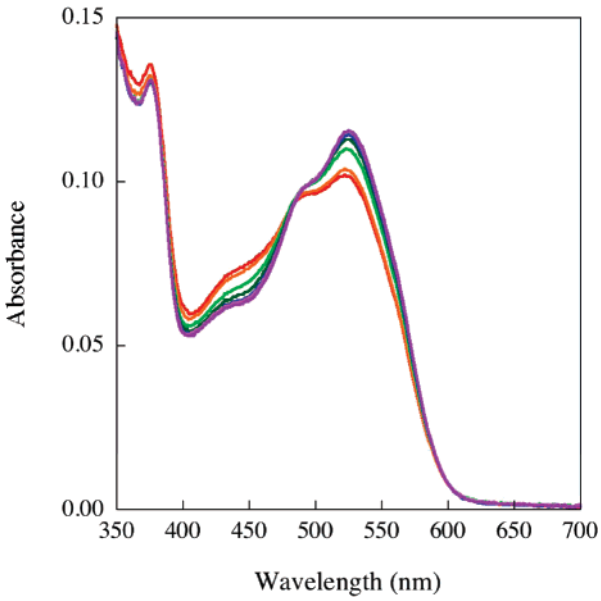


FIGURE 6: Effect of temperature on the absorbance of methylcobalamin MetH in the presence of ligands. A solution of MetH (~10 μM) in 50 mM KPi buffer, pH 7.2, with 1 mM CH_3-H_4folate and 1 mM AdoHcy was added to the sample cuvette of a double-beam spectrophotometer, and an equal volume of buffer with 1 mM CH_3-H_4folate and 1 mM AdoHcy was added to the reference cuvette. The spectra of MetH were recorded after equilibration for 2 min at 10 (purple), 15 (dark blue), 20 (light blue), 25 (dark green), 30 (light green), 37 (orange), and 40 °C (red).

DISCUSSION

Temperature Effects on Cobalamin Conformation in the Free and the MetH-Bound Forms. Despite the large size and conformational complexity of MetH, the principle of the ligand trans influence, initially described for cob(III)alamins in solution, applies to MetH. The order $H_2O < CH_3 < CH_2CH_2CH_3$, which is the same order previously observed with these cob(III)alamin derivatives in solution, was also observed with MetH. Our thermodynamic measurements, summarized in Table 1, indicate that the conversion to the His-off reactivation conformation is favorable for *n*-propylcobalamin MetH, which has a very electron-donating ligand. The same conversion is difficult for methylcobalamin MetH but becomes more facile in the presence of CH_3-H_4folate and AdoHcy. However, a value for ΔG could not even be measured in the present study for aquacobalamin MetH.

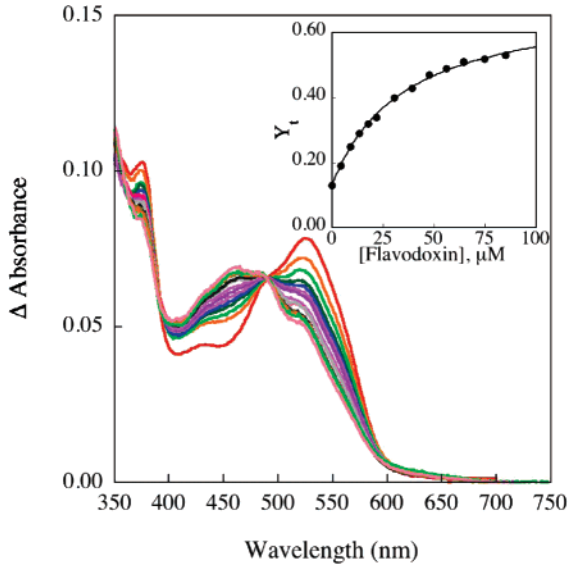


FIGURE 7: Titration of methylcobalamin MetH with flavodoxin in the presence of ligands. A solution of MetH (~10 μM) in 50 mM KPi buffer, pH 7.2, with 1 mM CH_3-H_4folate and 1 mM AdoHcy was added to the sample cuvette of a double-beam spectrophotometer, and an equal volume of buffer with 1 mM CH_3-H_4folate and 1 mM AdoHcy was added to the reference cuvette at 37 °C. After the initial spectrum was recorded (orange), equal amounts of concentrated flavodoxin (2.21 mM) were added to both cuvettes. The contents were gently mixed and then allowed to equilibrate for 2 min before the spectrum was recorded. Flavodoxin was added in increments up to 85 μM (pink). The spectrum of the enzyme in the absence of ligands is shown in red. Inset: Plot of Y_t vs the concentration of flavodoxin added. The spectra from the titration were adjusted for dilution and then fit using reference spectra to determine Y_t at each flavodoxin concentration. The data were fit using eq 3.

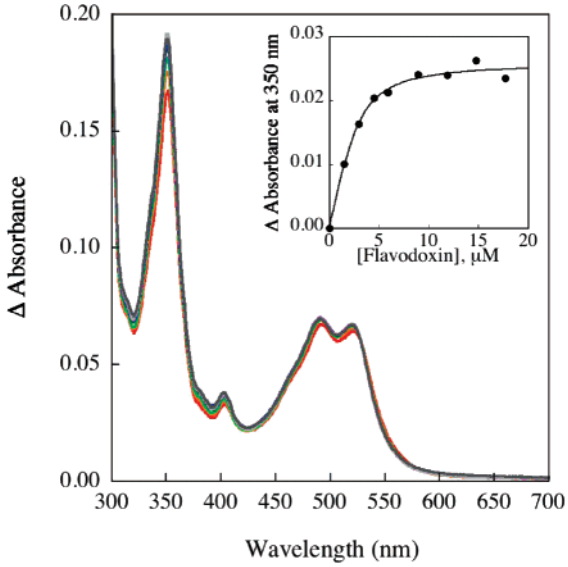


FIGURE 8: Titration of aquacobalamin MetH with flavodoxin. A solution of MetH (~10 μM) in 50 mM KPi buffer, pH 7.2, was added to the sample cuvette of a double-beam spectrophotometer, and an equal volume of buffer was added to the reference cuvette at 25 °C. After the initial spectrum was recorded (red), equal amounts of concentrated flavodoxin (2.97 mM) were added to both cuvettes. The contents were gently mixed and then allowed to equilibrate for 2 min before the spectrum was recorded. Flavodoxin was added in increments up to 18 μM (black). Inset: Plot of the change in absorbance at 350 nm vs the concentration of flavodoxin added. The data were fit using eq 4, yielding a K_d of 0.7 μM.

Access to the His-off reactivation conformation is extremely unfavorable for this form of MetH, which has a poor electron-donating group as the β-ligand to the cobalt of cobalamin.

The reader may wonder why we have not examined the conformation of MetH with other cob(III)alamin derivatives such as cyanocobalamin, azidocobalamin, or thiocyanatocobalamin. In no case could we observe temperature- or ligand-dependent changes indicating formation of His-off MetH with these exchangeable ligands. Furthermore, as demonstrated previously (*11*), addition of flavodoxin to MetH in the presence of subsaturating concentrations of these ligands results in exchange to form aquacobalamin MetH, complicating attempts to determine a binding affinity for flavodoxin.

The thermodynamic measurements also illustrate that cob(II)alamin MetH requires less free energy to convert to the reactivation conformation than the methylcobalamin form but requires more free energy than that necessary for the *n*-propylcobalamin form (Table 1). Thus, enzyme in the cob(II)alamin form more easily accesses the reactivation conformation than enzyme in the methylcobalamin form, as it should in order to ensure the reactivation of this inactive form of the prosthetic group. Furthermore, although the ligand trans series was first described for the behavior of cob(III)alamins, an indication of the position of free cob(II)alamin relative to the series of cob(III)alamins is given by $pK_{base-off}$, the apparent pK_a of the base-on/base-off reaction (Scheme 1). For cob(II)alamin, $pK_{base-off}$ was reported to be 3.10 (*24*), a value that lies between that of methylcobalamin ($pK_{base-off}$ = 2.90) and that of *n*-propylcobalamin ($pK_{base-off}$ = 4.10) (*17*). Therefore, the ligand series (H_2O < CH_3 < cob(II)alamin < $CH_2CH_2CH_3$) observed here for MetH is again the same order as previously observed for cobalamin derivatives in solution.

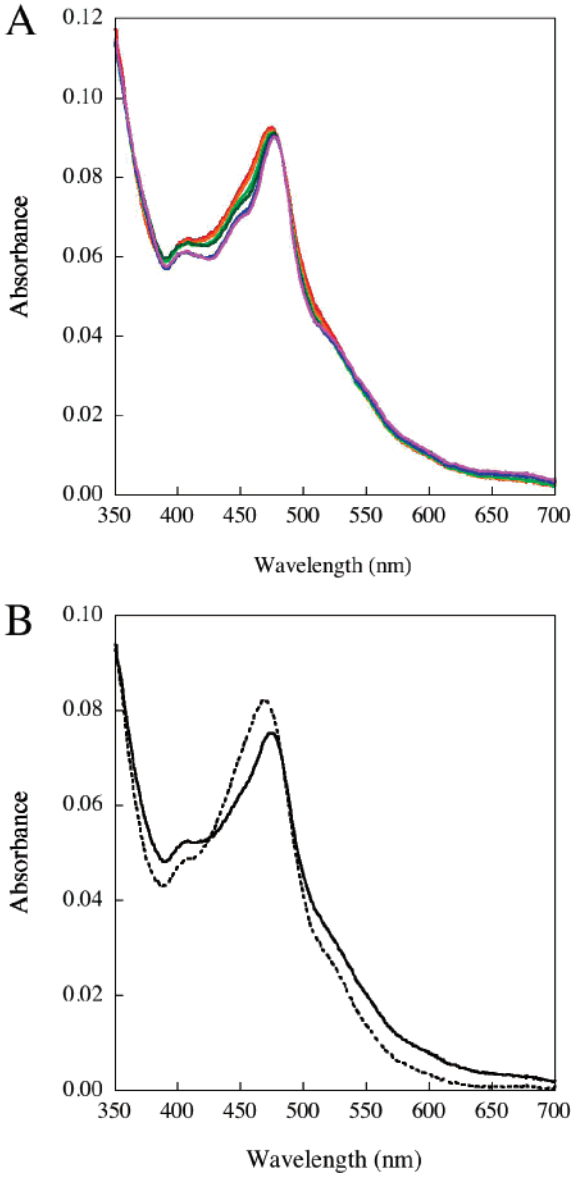


FIGURE 9: Effect of temperature and ligands on the absorbance of cob(II)alamin MetH. (A) Effect of temperature on the absorbance of cob(II)alamin MetH. The spectra of MetH (~10 μM) in 50 mM KPi buffer, pH 7.2, were recorded after equilibration for 2 min at 15 (purple), 20 (blue), 25 (dark green), 30 (light green), 37 (orange), and 40 °C (red). (B) Effect of AdoMet on the absorbance of cob(II)alamin MetH at 37 °C. A solution of MetH (~10 μM) in 50 mM KPi buffer, pH 7.2, was placed in an anaerobic cuvette, and a concentrated solution of AdoMet (38 mM) was added in the side arm. The spectrum of MetH was recorded after equilibration for 2 min (solid line). AdoMet (1 mM, final concentration) was then added from the side arm under anaerobic conditions, and the spectrum was recorded after equilibration for 2 min (dotted line).

The order of the ligand trans influence series is also reflected in the change in free energy required for the dissociation of the dimethylbenzimidazole base and its replacement by water (ΔG_{Co}) for cobalamin derivatives in solution (Scheme 1). The values of ΔG_{Co} for free cobalamin report specifically on the replacement of the base by water and are therefore corrected for the protonation of the base once it has dissociated (see the introductory remarks). The values of ΔG reported here for MetH-bound cobalamin are not corrected for any possible protonation of His759.

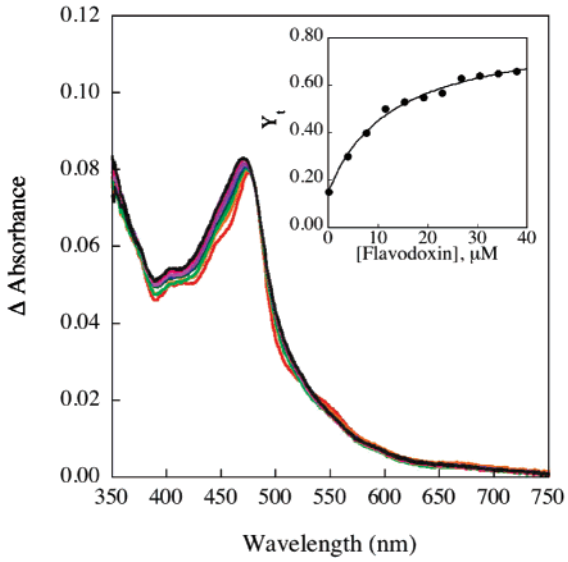


FIGURE 10: Titration of cob(II)alamin MetH with flavodoxin. A solution of MetH (~10 μM) in 50 mM KPi buffer, pH 7.2, was placed in an anaerobic cuvette and equilibrated with argon. An equal volume of buffer was added to the reference cuvette. After the initial spectrum was recorded (red), equal amounts of concentrated flavodoxin (1.94 mM) were added to both cuvettes at 25 °C. The contents were gently mixed and then allowed to equilibrate for 2 min before the spectrum was recorded. Flavodoxin was added in increments up to 38 μM (black). Inset: Plot of Y_t vs the concentration of flavodoxin added. The spectra from the titration were adjusted for dilution and then fit using reference spectra to determine Y_t at each flavodoxin concentration. The data were fit using eq 3.

Table 3: Comparison of ΔG Values for Cobalamin Derivatives in Solution and Bound to MetH[a]

R	ΔG_{Co}, cobalamin (kcal·mol^{-1})[b]	ΔG, MetH-bound cobalamin (kcal·mol^{-1})
H_2O	+10.4	N.D.[c]
CH_3	+3.6	+2.5
Co^{2+}	+3.4	+0.5
$CH_2CH_2CH_3$	+1.9	−2.6

[a] The values for cobalamin derivatives in solution are at 25 °C, but the MetH values are at 37 °C. [b] Refs *17* and *24*. [c] Values could not be determined due to a lack of spectral changes consistent with formation of His-off aquacobalamin.

However, at pH 7.2, protonation of this ligand, with a pK_a of ~5.6, is expected to be only a minor contributor to the value of ΔG. Therefore, the values of ΔG_{Co} for free cobalamin reported by Brown and co-workers (*17*, *24*) are compared to the values of ΔG reported here for MetH-bound cobalamins in Table 3. The trend in values of ΔG and ΔG_{Co} is the same for the free and the MetH-bound forms of cobalamin. However, it must be noted that there are differences in the changes in entropy and enthalpy associated with a change in cobalamin conformation in the free and the MetH-bound forms. In solution, the values of ΔH_{Co} for alkylcobalamins are virtually independent of the nature of the β-ligand, with average values of ~8 kcal·mol^{-1} for the conversion of the base-on species to the base-off species (*17*). The values of ΔS_{Co} for alkylcobalamins in solution, however, are dependent on the β-ligand and range from ~5 to 25 cal·K^{-1}·mol^{-1} (*17*). Presumably the much larger values of ΔS and ΔH for the MetH-bound cobalamins reflect the conformational changes associated with the base-on to base-off transition. Nonetheless, the trend in the values of ΔG reflects the trend in values of ΔG_{Co} for cobalamin derivatives in solution. Indeed, the value of ΔG_{Co} for free cobalamin suggests a very unfavorable value of ΔG for aquacobalamin MetH, which helps to explain why His-off aquacobalamin MetH could not be formed within the temperature range used for the experiments discussed here and why ligands were unable to affect the conformational equilibria.

Effect of Ligands on the Conformation of MetH. Previously, the effect of the ligands AdoHcy and CH_3-H_4folate on the conformation of methylcobalamin MetH helped to confirm a link between the coordination of His759 and the conformation of MetH (*15*). Bound ligands form an important part of the contact surface between domains of MetH, as illustrated for AdoMet in the structure of the C-terminal fragment of MetH in the reactivation conformation (*14*). Here, the observed shifts between the His-on and His-off forms of *n*-propylcobalamin MetH with the addition of ligands are also consistent with the correlation of coordination and conformation. As with the methylcobalamin form of MetH, steric interactions between the β-ligand of the cobalamin and the methyl group of added ligands act to disfavor select conformations. The ability to relate the coordination state of the His759 ligand to the conformation of MetH for the *n*-propylcobalamin form helps to explain the results of Taylor and Weissbach (*25*). They showed that upon propylation of MetH, the enzyme was inactivated and exhibited an absorbance maximum at 430 nm. The propylated form of cobalamin is expected to react ~75 times slower than the methylated form with the dealkylating agent (i.e., Hcy) based on the organic precedent (*26*), but this cannot fully account for the almost complete inhibition of MetH by propylation. From the current study, the observation that the propylated MetH is in a His-off reactivation conformation can additionally account for the inhibition of MetH upon propylation. Furthermore, we can now relate the observed spectral properties of the propylated MetH to the fact that it is in a His-off reactivation conformation.

Binding of Oxidized Flavodoxin Generally Varied Inversely with the Ligand trans Influence. Our analysis of the flavodoxin titration data allows for comparison across the cobalamin series by specifically reporting on the affinity of oxidized flavodoxin for His-on MetH. It was observed that the affinity of flavodoxin for His-on MetH generally varies inversely with the ligand trans influence (Table 2). However, the values obtained from the flavodoxin titration experiments contain some inherent inaccuracies, which is especially apparent in the error associated with affinity of flavodoxin for the *n*-propylcobalamin form of (His-on) MetH (nK_d). The largest contribution to the error in the values of nK_d is from the error associated with the values of *n* (Table 2). The values of *n* are most sensitive to the endpoint of the titration, where the high absorbance of added flavodoxin (the spectrum of which obscures the spectrum of MetH, hence the need to do difference titrations) increases the error in the final data points of the titration and dictates when the titration must be stopped. Furthermore, in order to maximize the spectral changes of MetH, the titrations of the various forms of MetH with flavodoxin could not be executed at the same temperature and some forms required the addition of ligands. Therefore, despite the inherent inaccuracies in the measurements, it can be noted that the affinity of flavodoxin for His-

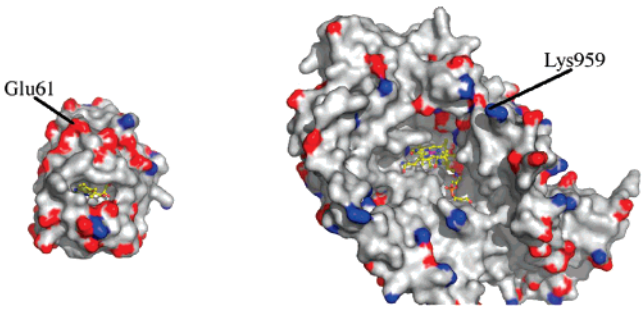


FIGURE 11: Flavodoxin and MetH docking surfaces. The surfaces of flavodoxin (left) and the C-terminal half of MetH (right) that are proposed to interact are shown here, with negatively charged residues in red and positively charged residues in blue. Cross-linking experiments linked Glu61 of flavodoxin and Lys959 of MetH (*8*). Note that both surfaces contain a ring of ionic residues around a hydrophobic interior, with FMN and cobalamin shown in yellow as stick figures, which places importance on the cobalt of cobalamin in the electrostatic interactions between flavodoxin and MetH.

on MetH generally varies inversely with the ligand trans influence.

The data presented here demonstrates that flavodoxin has a very strong affinity for aquacobalamin MetH, which has the most net positive charge on the cobalt of the cobalamin of the species investigated here. Alternatively, flavodoxin has a weak affinity for MetH when the β-position to the cobalt is occupied by a strongly electron-donating group such as *n*-propyl, which results in less net positive charge on the cobalt than in the aquacobalamin form of MetH. As the net positive charge on the cobalt is varied by changing the β-ligand of the enzyme-bound cob(III)alamin, the affinity of flavodoxin appears to vary to reflect this change.

As noted previously, when flavodoxin binds to cob(III)-alamin forms of MetH with exchangeable β-ligands such as azidocobalamin or thiocyanatocobalamin, it induces ligand exchange (*11*). In each case, the absorbance changes are consistent with conversion to aquacobalamin. Since the binding of oxidized flavodoxin to enzyme in the aquacobalamin form should be much tighter than the binding of flavodoxin to enzyme-bound cobalamin with a more electron-donating β-ligand, we may rationalize the exchange as due to preferential binding of flavodoxin to the aquacobalamin form of the enzyme in the presence of subsaturating concentrations of the exchangeable ligand.

Also consistent with the hypothesis that oxidized flavodoxin has an affinity that varies with the net positive charge on the cobalt is our observation that it has a greater affinity for aquacobalamin MetH (+3 oxidation state) than for cob(II)alamin MetH (+2 oxidation state). The latter of course has a reduced net positive charge on the cobalt.

Finally, the binding of flavodoxin to the *n*-propylcobalamin, cob(II)alamin, and methylcobalamin forms of (His-on) MetH leads to the formation of His-off species. In these derivatives lacking exchangeable ligands, flavodoxin binds more strongly to the His-off than to the His-on conformation of the enzyme, again shifting the equilibrium to favor the form with the greatest net positive charge on the cobalt.

Why is flavodoxin so sensitive to the charge on the cobalt of the cobalamin prosthetic group of this large polyionic protein? Figure 11 shows the face of flavodoxin that presents the dimethylbenzene ring of bound flavin mononucleotide (FMN) to partner proteins juxtaposed to the surface of the C-terminal half of MetH that is postulated to bind to flavodoxin. These surfaces were inferred from cross-linking experiments that linked Lys959 on MetH to Glu61 of flavodoxin (*8*) and from saturation transfer NMR experiments mapping out the flavodoxin surface that is presented to the AdoMet-binding domain of MetH (*7*). It can be seen that both surfaces contain a hydrophobic interior surrounded by a ring of ionic residues. The ionic ring of flavodoxin is predominantly negatively charged, whereas that on MetH is largely positively charged. The cobalt of cobalamin would be expected to make an important contribution to the electrostatic attraction experienced by flavodoxin as it binds MetH in the reactivation conformation.

Flavodoxin binds a His-on conformation of MetH in the aquacobalamin form, and here we have no structures to guide our understanding. Current efforts center on attempting to crystallize aquacobalamin MetH in the presence of oxidized flavodoxin.

In summary, our results provide support for an exquisitely balanced ensemble of conformations of MetH. The equilibria between these conformations are sensitive to temperature, the presence or absence of ligands including flavodoxin, and the β-ligand of the cobalamin prosthetic group. We have shown that inactivation of the prosthetic group by oxidation to form cob(II)alamin leads to an enzyme form that more readily accesses the reactivation conformation and that the addition of flavodoxin tips the balance to favor the His-off form of the enzyme. In the presence of reduced flavodoxin, electron transfer followed by methylation with the methyl group of AdoMet will return the prosthetic group to its methylcobalamin form, which will then lead to dissociation of flavodoxin and return of the enzyme to one of the His-on catalytic conformations.

SUPPORTING INFORMATION AVAILABLE

The derivation of eq 3. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Drummond, J. T., Huang, S., Blumenthal, R. M., and Matthews, R. G. (1993) Assignment of enzymatic function to specific protein regions of cobalamin-dependent methionine synthase from *Escherichia coli*, *Biochemistry 32*, 9290−9295.
2. Fujii, K., and Huennekens, F. M. (1974) Activation of methionine synthetase by a reduced triphosphopyridine nucleotide-dependent flavoprotein system, *J. Biol. Chem. 249*, 6745−6753.
3. Mangum, J. H., and Scrimgeour, K. G. F. (1962) Cofactor requirements and intermediates in methionine biosynthesis, *Fed. Proc. 21*, 242.
4. Taylor, R. T., and Weissbach, H. (1967) N^5-Methyltetrahydrofolate-homocysteine transmethylase: role of *S*-adenosylmethionine in vitamin B_{12}-dependent methionine synthesis, *J. Biol. Chem. 242*, 1517−1521.
5. Goulding, C. W., Postigo, D., and Matthews, R. G. (1997) Cobalamin-dependent methionine synthase is a modular protein with distinct regions for binding homocysteine, methyltetrahydrofolate, cobalamin, and adenosylmethionine, *Biochemistry 36*, 8082−8091.
6. Evans, J. C., Huddler, D. P., Hilgers, M. T., Romanchuk, G., Matthews, R. G., and Ludwig, M. L. (2004) Structures of the N-terminal modules imply large domain motions during catalysis by methionine synthase, *Proc. Natl. Acad. Sci. U.S.A. 101*, 3729−3736.
7. Hall, D. A., Vander Kooi, C. W., Stasik, C. N., Stevens, S. Y., Zuiderweg, E. R. P., and Matthews, R. G. (2001) Mapping the interactions between flavodoxin and its physiological partners

flavodoxin reductase and cobalamin-dependent methionine synthase, *Proc. Natl. Acad. Sci. U.S.A. 98*, 9521−9526.
8. Hall, D. A., Jordan-Starck, T. C., Loo, R. O., Ludwig, M. L., and Matthews, R. G. (2000) Interaction of flavodoxin with cobalamin-dependent methionine synthase, *Biochemistry 39*, 10711−10719.
9. Drennan, C. L., Huang, S., Drummond, J. T., Matthews, R. G., and Ludwig, M. L. (1994) How a protein binds B_{12}: a 3.0 Å X-ray structure of B_{12}-binding domains of methionine synthase, *Science 266*, 1669−1674.
10. Jarrett, J. T., Hoover, D. M., Ludwig, M. L., and Matthews, R. G. (1998) The mechanism of adenosylmethionine-dependent activation of methionine synthase: a rapid kinetic analysis of intermediates in reductive methylation of cob(II)alamin enzyme, *Biochemistry 37*, 12649−12658.
11. Hoover, D. M., Jarrett, J. T., Sands, R. H., Dunham, W. R., Ludwig, M. L., and Matthews, R. G. (1997) Interaction of *Escherichia coli* cobalamin-dependent methionine synthase and its physiological partner flavodoxin: binding of flavodoxin leads to axial ligand dissociation from the cobalamin cofactor, *Biochemistry 36*, 127−138.
12. Jarrett, J. T., Amaratunga, M., Drennan, C. L., Scholten, J. D., Sands, R. H., Ludwig, M. L., and Matthews, R. G. (1996) Mutations in the B_{12}-binding region of methionine synthase: how the protein controls methylcobalamin reactivity, *Biochemistry 35*, 2464−2475.
13. Jarrett, J. T., Huang, S., and Matthews, R. G. (1998) Methionine synthase exists in two distinct conformations that differ in reactivity toward methyltetrahydrofolate, adenosylmethionine, and flavodoxin, *Biochemistry 37*, 5372−5382.
14. Bandarian, V., Pattridge, K. A., Lennon, B. W., Huddler, D. P., Matthews, R. G., and Ludwig, M. L. (2002) Domain alternation switches B_{12}-dependent methionine synthase to the activation conformation, *Nat. Struct. Biol. 9*, 53−56.
15. Bandarian, V., Ludwig, M. L., and Matthews, R. G. (2003) Factors modulating conformational equilibria in large modular proteins: a case study with cobalamin-dependent methionine synthase, *Proc. Natl. Acad. Sci. U.S.A. 100*, 8156−8163.
16. Brown, K. L., Hakimi, J. M., Nuss, D. M., Montejano, Y. D., and Jacobsen, D. W. (1984) Acid−base properties of α-ribazole and the thermodynamics of dimethylbenzimidazole association in alkylcobalamins, *Inorg. Chem. 23*, 1463−1471.
17. Brown, K. L., and Peck-Siler, S. (1988) Heteronuclear NMR studies of cobalamins. 9. Temperature-dependent NMR of organocobalt corrins enriched in ^{13}C in the organic ligand and the thermodynamics of the base-on/base-off reaction, *Inorg. Chem. 27*, 3548−3555.
18. Pratt, J. M. (1972) *Inorganic Chemistry of Vitamin B_{12}*, Academic Press, London.
19. Jarrett, J. T., Goulding, C. W., Fluhr, K., Huang, S., and Matthews, R. G. (1997) Purification and assay of cobalamin-dependent methionine synthase from *Escherichia coli*, *Methods Enzymol. 281*, 196−213.
20. Zehnder, A. J. B., and Wuhrmann, K. (1976) Titanium(III) citrate as a nontoxic oxidation−reduction buffering system for culture of obligate anaerobes, *Science 194*, 1165−1166.
21. Bandarian, V., and Matthews, R. G. (2004) Measurement of energetics of conformational change in cobalamin-dependent methionine synthase, *Methods Enzymol. 380*, 152−169.
22. Liptak, M. D., Fleischhacker, A. S., Matthews, R. G., and Brunold, T. C. (2007) Probing the role of the histidine 759 ligand in cobalamin-dependent methionine synthase, *Biochemistry 46*, 8024−8035.
23. Segal, I. H. (1993) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, Wiley-Interscience, New York.
24. Brown, K. L., and Zou, X. (1991) Facile α/β diastereomerism in organocobalt corrins: evidence for thermodynamic control in the synthesis of alkylcobamides, *Inorg. Chem. 30*, 4185−4191.
25. Taylor, R. T., and Weissbach, H. (1967) N^5-Methyltetrahydrofolate-homocysteine transmethylase: propylation characteristics with use of a chemical reducing system and purified enzyme, *J. Biol. Chem. 242*, 1509−1516.
26. March, J. (1992) *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, 4th ed., Wiley-Interscience, New York.

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