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# 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†

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ABSTRACT: Cobalamin-dependent methionine synthase from *Escherichia coli* catalyzes the last step in *de novo* methionine biosynthesis. Conversion of the inactive cob(II)alamin form of the enzyme, formed by the occasional oxidation of cob(I)alamin during turnover, to an active methylcobalamin-containing form requires a reductive methylation of the cofactor in which an electron is supplied by reduced flavodoxin and the methyl group is derived from *S*-adenosyl-L-methionine. *E. coli* flavodoxin acts specifically in this activation reaction, and neither *E. coli* ferredoxin nor flavodoxin from the cyanobacterium *Synechococcus* will substitute, despite their highly similar midpoint potentials for one-electron transfer. As assessed by EPR spectroscopy, the binding of flavodoxin to cob(II)alamin methionine synthase results in a change in the coordination geometry of the cobalt from five-coordinate to four-coordinate. Histidine 759 of methionine synthase, which replaces the normal lower ligand dimethylbenzimidazole on binding of methylcobalamin to methionine synthase, is dissociated from the cobalt of the cobalamin by the binding of flavodoxin. The association of flavodoxin and methionine synthase depends on ionic strength and pH; the pH dependence corresponds to the uptake of one proton on association. The formation of a complex between flavodoxin and methionine synthase perturbs the midpoint potentials of the flavin and cobalamin cofactors only marginally and without any significant thermodynamic advantage for electron transfer to the cobalamin of methionine synthase. No significant binding was seen between oxidized flavodoxin and methylcobalamin methionine synthase. A model for the interaction of methionine synthase with flavodoxin is proposed in which flavodoxin binding leads to changes in the distribution of methionine synthase conformations.

In *Escherichia coli*, flavodoxin provides electrons for the activation of the cobalamin-dependent methionine synthase[1] (Fujii & Huennekens, 1974; Osborne *et al*., 1991), as well as for the activation of three other enzymes: pyruvate formate-lyase (Wong *et al*., 1993), anaerobic ribonucleotide reductase (Bianchi *et al*., 1993a), and biotin synthase (Ifuku *et al*., 1994). Methionine synthase is a 136 kDa methyltransferase that catalyzes the final step in methionine biosynthesis. The enzyme transfers a methyl group to homocysteine from $CH_3$-$H_4$folate to generate methionine and tetrahydrofolate, using methylcobalamin as an intermediate in the reaction (eqs 1 and 2).

$$\text{cob(I)alamin} + \text{CH}_3\text{-H}_4\text{folate} \rightarrow \text{methylcobalamin} + \text{H}_4\text{folate} \quad (1)$$

$$\text{methylcobalamin} + \text{Hcy} \rightarrow \text{cob(I)alamin} + \text{Met} \quad (2)$$

Methionine synthase cycles between methylcobalamin and cob(I)alamin during turnover and under rigorously anaerobic conditions is oxidized to the inactive cob(II)alamin state approximately once in every 2000 turnovers (Drummond *et al*., 1993). During turnover under microaerophilic conditions, cob(II)alamin may be formed once in every 100 turnovers (Fujii *et al*., 1977). To re-enter the catalytic cycle, the cob(II)alamin form of methionine synthase must be reductively methylated using *S*-adenosyl-L-methionine (AdoMet) and flavodoxin semiquinone or hydroquinone (Banerjee & Matthews, 1990). During aerobic growth of *E. coli*, the ultimate source of electrons is NADPH, and the enzyme ferredoxin (flavodoxin):$NADP^+$ oxidoreductase acts to accept hydride ions from NADPH and to donate electrons to flavodoxin (Fujii *et al*., 1977).

While the reactivation reaction is not fully understood, two possible mechanisms may be entertained. Equations 3 and 4 show the mechanism initially proposed, in which flavodoxin reduces cob(II)alamin to cob(I)alamin, which is then methylated by AdoMet. Although the reduction shown in eq 3 is thermodynamically unfavorable, the cob(I)alamin formed in reaction 3 would be trapped by the coupled

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; Met, L-methionine; Hcy, L-homocysteine; $CH_3$-$H_4$folate, $N^5$-methyltetrahydrofolate; $H_4$folate, tetrahydrofolate; methionine synthase, $N^5$-methyltetrahydrofolate−homocysteine methyltransferase (EC 2.1.1.13); Fld, flavodoxin; SHE, standard hydrogen electrode.

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exergonic reaction (eq 4), in which AdoMet transfers a methyl group to the highly nucleophilic cob(I)alamin (Banerjee *et al*., 1990; Fujii *et al*., 1977).

$$\text{cob(II)alamin} + \text{Fld}_{hq}\ (\text{Fld}_{sq}) \rightarrow \text{cob(I)alamin} + \text{Fld}_{sq}\ (\text{Fld}_{ox}) \quad (3)$$

$$\text{cob(I)alamin} + \text{AdoMet} \rightarrow \text{methylcobalamin} + \text{AdoHcy} \quad (4)$$

Although model studies suggest that the mechanism shown in eqs 3 and 4 is plausible (Schrauzer & Deutsch, 1969), it is curious that AdoMet does not compete with methyltetrahydrofolate in the reaction shown in eq 1.

Another mechanism of methylation seemed possible, in which the cob(I)alamin species does not play a role. Equations 5 and 6 show an alternate mechanism of activation in which flavodoxin reduces the positively charged sulfonium group of the AdoMet to the neutral radical, which undergoes an $S_H2$ reaction with cob(II)alamin to generate methylcobalamin and AdoHcy:

$$\text{Ado-S}^{+}\text{-Met} + \text{Fld}_{hq}\ (\text{Fld}_{sq}) \rightarrow \text{Ado-S}^{\bullet}\text{-Met} + \text{Fld}_{sq}\ (\text{Fld}_{ox}) \quad (5)$$

$$\text{Ado-S}^{\bullet}\text{-Met} + \text{cob(II)alamin} \rightarrow \text{AdoHcy} + \text{methylcobalamin} \quad (6)$$

This mechanism is similar to that of other reactions involving AdoMet and reduced flavodoxin, in that the AdoMet is reduced to a radical species. However, in the reactions involving *E. coli* flavodoxin and the activating enzymes of pyruvate formate-lyase (Wong *et al*., 1993) or anaerobic ribonucleotide reductase (Bianchi *et al*., 1993a), reductive cleavage of AdoMet yields the 5′-deoxyadenosyl radical.

The known roles of flavodoxin in *E. coli* are unique compared to roles of flavodoxin in other organisms. In organisms such as *Klebsiella pneumoniae* and *Azotobacter vinelandii*, flavodoxin provides the electrons required for dinitrogen reduction by nitrogenase. In contrast, in *E. coli*, the electrons provided by flavodoxin are required only for activation of methionine synthase, pyruvate formate-lyase, and anaerobic ribonucleotide reductase and not for the overall reactions catalyzed by these enzymes. *E. coli* flavodoxin is also a highly specific electron transfer protein for enzyme activation; the highly homologous flavodoxin from *Synechococcus* (formerly *Anacystis nidulans*) does not efficiently donate electrons to methionine synthase (R. G. Matthews, unpublished data). In many microorganisms that express flavodoxin, ferredoxin can substitute in electron transfer reactions involving flavodoxin (Mayhew & Ludwig, 1975); in *E. coli*, this is not the case with methionine synthase, anaerobic ribonucleotide reductase (Bianchi *et al*., 1993a), or pyruvate formate-lyase (Blaschkowski *et al*., 1982). There is also evidence that only flavodoxin will support the activity of biotin synthase (Ifuku *et al*., 1994).

Structure-based alignment of flavodoxin sequences demonstrates that *E. coli* flavodoxin contains a unique pair of tyrosines (residues 58 and 59) in a loop near the flavin mononucleotide (FMN) cofactor (Hoover *et al*., 1994; Ludwig & Luschinsky, 1992; Osborne *et al*., 1991). These tyrosines are also found in the deduced amino acid sequence for flavodoxin in the bacterium *Haemophilus influenzae* Rd (Fleischmann *et al*., 1995), but they are lacking in flavodoxins from other organisms. The *H. influenzae* genome contains sequences homologous to $B_{12}$-dependent methionine synthase, pyruvate formate-lyase, and anaerobic ribonucleotide reductase. The tyrosines at positions 58 and 59 of the *E. coli* and *H. influenzae* flavodoxins may play a role in the ability of flavodoxin to recognize and activate its physiological partners.

This paper describes the interaction between flavodoxin and methionine synthase. A 1:1 complex of flavodoxin with methionine synthase exhibits changes in both the visible absorbance and electron paramagnetic resonance spectra of the methionine synthase-bound cobalamin cofactor. These spectral changes are indicative of a change in coordination geometry of the cobalt in the bound cobalamin, and they were used to measure the binding of flavodoxin to methionine synthase. We present evidence that *E. coli* flavodoxin not only provides electrons for the activation of methionine synthase but also may cause a global conformation change within methionine synthase when it binds. We propose that this conformation change is required to allow methylation of the cobalamin cofactor by AdoMet rather than by $CH_3$-$H_4$folate and is key to the ability of the enzyme to discriminate between catalytic turnover and reductive methylation.

## EXPERIMENTAL PROCEDURES

*E. coli* flavodoxin (Bianchi *et al*., 1993a), *Synechococcus* sp. PCC 7942 flavodoxin (Clubb *et al*., 1991), *E. coli* ferredoxin (flavodoxin):$NADP^+$ oxidoreductase (Bianchi *et al*., 1993b), and methionine synthase (Amaratunga *et al*., 1996) were prepared as previously described. Proteolysis and preparation of fragments of methionine synthase followed published methods (Drummond *et al*., 1993) and were carried out by Sha Huang, the University of Michigan. Methyl viologen and safranin O dyes were obtained from Sigma. 5-Deazaflavin-3-sulfonate was a gift of Dr. Vincent Massey, the University of Michigan.

*Aerobic Titrations.* A solution of methionine synthase (∼10−25 $\mu$M) was placed in the sample cuvette of a thermostated spectrophotometer chamber (Perkin-Elmer Lambda 7 model or Varian Cary 3E), and an equal volume of buffer was placed in the reference cuvette. Sample and reference volumes were matched precisely by weight; assuming that the density of aqueous buffered protein solutions does not change significantly within the range of protein concentrations used, matching by weight allowed an accurate balance between the sample and reference cells and maintained matched concentrations of added titrants over a wide range. Portions of a ∼1 mM flavodoxin solution, from either *E. coli* or *Synechococcus*, dissolved in the same buffer, were added to both sample and reference cells and spectra were recorded of the initial solutions and after each addition of flavodoxin. The solutions were gently mixed by stir bars within the cuvettes. All titrations were performed at 20 °C. Difference spectra were calculated by subtracting the absorbance at each wavelength of the initial methionine synthase solution from the dilution-corrected absorbance of the mixed flavodoxin/methionine synthase solution. The same method was also used for ferredoxin:$NADP^+$ oxidoreductase titrations, substituting ferredoxin:$NADP^+$ oxidoreductase for

methionine synthase. The concentration of oxidized *E. coli* flavodoxin was estimated using an $\epsilon_{466}$ of 8250 $M^{-1}$ $cm^{-1}$. The molar absorbance coefficient for oxidized *E. coli* flavodoxin was estimated by titration of free FMN with apoflavodoxin generated by precipitation with trichloroacetic acid (Wassink & Mayhew, 1975); this value agrees with the previously determined extinction coefficient (Vetter & Knappe, 1971). The following molar absorbance coefficients were used to calculate the concentrations of components in the reaction mixture: methionine synthase in the cob(I)alamin form, $\epsilon_{388} = 24\,000$ $M^{-1}$ $cm^{-1}$ (J. Jarrett, unpublished results); methionine synthase in the cob(II)alamin form, $\epsilon_{474} = 9470$ $M^{-1}$ $cm^{-1}$ (Jarrett *et al*., 1996a); methionine synthase in the cob(III)alamin form at pH 7.2, $\epsilon_{357} = 20\,200$ $M^{-1}$ $cm^{-1}$ (Jarrett *et al*., 1996a); oxidized flavodoxin from *Synechococcus*, $\epsilon_{466} = 9200$ $M^{-1}$ $cm^{-1}$ (Entsch & Smillie, 1972); and oxidized ferredoxin:$NADP^+$ oxidoreductase, $\epsilon_{400} = 7400$ $M^{-1}$ $cm^{-1}$ (Jenkins & Waterman, 1994).

*Anaerobic Titrations.* For anaerobic titrations, a modification of the method of Matthews and Williams (1976) was employed and a Perkin-Elmer Lambda 7 model spectrophotometer was used. Methionine synthase was placed in an anaerobic spectrophotometric cuvette containing a glass-coated stir bar, and concentrated oxidized flavodoxin was placed in a side arm of the same cell. The cuvette was sealed with a rubber septum, and an empty gas-tight syringe was passed through the septum and into the side arm. The cuvette contents were made anaerobic with 10 alternate cycles of evacuation and equilibration with oxygen-free argon gas. Oxygen-free argon was produced by passage over a heated copper-containing column and then hydrated by bubbling through water (Matthews *et al*., 1974). The cell was kept on ice during gas exchange to minimize evaporation. The syringe was filled with concentrated flavodoxin from the side arm, and portions of flavodoxin were added to the anaerobic methionine synthase in the sample compartment. A separate syringe outside of the spectrophotometric cuvette was filled with oxidized flavodoxin of an equal concentration, and portions were added to the reference cuvette, which contained only buffer. Spectra were recorded as in aerobic titrations and corrected for dilution, and the blank spectrum was subtracted before analysis.

*Measurement of Binding Competition.* Methods similar to those for aerobic titrations were used, except that the spectra were measured with a Cary 3E spectrophotometer. In this case, the binding of flavodoxin to ferredoxin:$NADP^+$ oxidoreductase was measured in the presence of increasing amounts of methionine synthase. Ferredoxin:$NADP^+$ oxidoreductase was placed in the sample cuvette and methionine synthase in both the sample and reference cuvettes. Spectra were recorded as small portions of oxidized flavodoxin were added to both the sample and reference cuvettes. These spectra were corrected for dilution and for the absorbance of the reference solution. The $K_d$ was estimated by plotting the absorbance at 410 nm versus flavodoxin added, since the spectral changes due to binding to methionine synthase are minimal at this wavelength. Plotting the apparent $K_d$ for flavodoxin binding to ferredoxin:$NADP^+$ oxidoreductase versus the concentration of methionine synthase gave a slope equal to the ratio of the $K_d$ for flavodoxin binding to ferredoxin:$NADP^+$ oxidoreductase to the $K_d$ for flavodoxin binding to methionine synthase.

*Measurement of EPR Spectra.* Because methionine synthase in the cob(II)alamin oxidation state forms a cob(III)alamin/superoxo complex when frozen in the presence of oxygen, and because the binding of oxidized flavodoxin to cob(II)alamin promotes its oxidation to cob(III)alamin, all protein samples used for EPR were first deoxygenated prior to mixing and freezing in liquid $N_2$ (Banerjee *et al*., 1990). The samples were then placed in EPR tubes with an outer diameter of 4 mm and frozen in liquid nitrogen until spectra were measured. Spectra were recorded using an X-band Varian E-line spectrometer with a custom-made gas-phase helium transfer line, as previously described (Frasca *et al*., 1988). Signal averaging was performed on multiple transients using a Tracor-Northern NS-570 signal averager. The digitized data were transferred to a microcomputer for spin quantitation, which was performed by comparison of the second integral of the sample spectra with the integrated spectrum of a cupric perchlorate standard (1.0 mM $CuSO_4$, 10 mM HCl, and 1 M $NaClO_4$).

*Measurement of the Midpoint Potentials of Protein Complexes.* 5-Deazaflavin-3-sulfonate was photoreduced in the presence of EDTA (Massey & Hemmerich, 1977) to provide a source of reducing equivalents. Concentrated stocks of cob(II)alamin methionine synthase and flavodoxin were exchanged into 10 mM potassium phosphate buffer (pH 7.0), containing 15 mM EDTA, by repeated dilution and centrifugal concentration using an appropriate filter (Amicon). For the measurement of the cob(I)alamin/cob(II)alamin midpoint potential in the presence of saturating flavodoxin, the proteins were mixed to give a final volume of 800 $\mu$L of 10 mM potassium phosphate buffer (pH 7.0) containing 15 mM EDTA, 10 $\mu$M methionine synthase, 300 $\mu$M flavodoxin, 1 $\mu$M methyl viologen, and 5 $\mu$M 5-deazaflavin-3-sulfonate. Methyl viologen was added to provide more rapid equilibration between the 5-deazaflavin-3-sulfonate semiquinone and the proteins. The solution was placed in an anaerobic cuvette and exchanged with oxygen-free argon. The cell was immersed in ice-cold water during illumination for brief time periods (5−15 s) with a 500 W tungsten/halogen lamp. To allow equilibration between reduced 5-deazaflavin-3-sulfonate, methyl viologen, flavodoxin, and methionine synthase, the samples were incubated in the spectrophotometer compartments (20 °C) for 10 min after each irradiation before recording the spectra. The samples were illuminated, and spectra were recorded until no further changes occurred (approximately 300 s of total illumination).

For the measurement of the flavodoxin oxidized/semiquinone and semiquinone/hydroquinone midpoint potentials in the presence of saturating methionine synthase, the method of Drummond (Drummond & Matthews, 1994) was used. Eight hundred microliters of 10 mM potassium phosphate buffer (pH 7.0) containing 100 $\mu$M methionine synthase, 10 $\mu$M flavodoxin, 1 $\mu$M methyl viologen, 5 $\mu$M 5-deazaflavin-3-sulfonate, 10 mM potassium phosphate buffer, and 15 mM EDTA were placed in an anaerobic spectrophotometric cuvette and exchanged with oxygen-free argon. The cuvette, immersed in an ice bath, was illuminated for 90 s. This duration of photoreduction typically produced ∼70−75% cob(I)alamin, based on the molar absorbance of cob(I)alamin at 388 nm (24 000 $M^{-1}$ $cm^{-1}$; J. Jarrett, unpublished data). The system was allowed to equilibrate for 5 min, and spectra were recorded every 5−10 min thereafter. Under these conditions, the contents of the cuvette slowly oxidize, perhaps

because cob(I)alamin can reduce the protons of solvent to hydrogen gas (Tackett, 1963). At pH 7.0, oxidation of cob(I)alamin occurs sufficiently slowly to permit equilibration of methyl viologen, flavodoxin, and methionine synthase.

*Analysis of Spectra.* To obtain the concentration of reduced and oxidized species in complex mixtures (three or more chromophores), each spectrum was deconvoluted using absorbance properties of each of the individual components. Absorbances for the spectra of each component [cob(I)alamin [$A_{\mathrm{MS\ Co(I)}}$]; cob(II)alamin, both bound to flavodoxin [$A_{\mathrm{MS\ Co(II)b}}$] and free [$A_{\mathrm{MS\ Co(II)f}}$]; the hydroquinone, semiquinone, and oxidized states of flavodoxin ($A_{\mathrm{F\ hq}}$, $A_{\mathrm{F\ sq}}$, and $A_{\mathrm{F\ ox}}$, respectively); methyl viologen radical cation ($A_{\mathrm{MV}}$); and oxidized and semiquinone 5-deazaflavin-3-sulfonate [$A_{\mathrm{dF\ ox}}$ and $A_{\mathrm{dF\ rd}}$)] were measured individually beforehand and then scaled to the concentration of that component in the titration. Absorbances were summed to give a model spectrum. Total concentrations for flavodoxin, methionine synthase, methyl viologen, and 5-deazaflavin-3-sulfonate were known, so only the fraction oxidized, α, was varied for each component. Equations 7−11 were used to calculate the concentrations of each component.

$$\text{simulated spectrum} = A_{\mathrm{MS}} + A_{\mathrm{F}} + A_{\mathrm{MV}} + A_{\mathrm{dF}} \quad (7)$$

$$A_{\mathrm{MS}} = [A_{\mathrm{MS\ Co(I)}}(1 - \alpha_{\mathrm{MS}}) + [A_{\mathrm{MS\ Co(II)b}} \text{ or } A_{\mathrm{MS\ Co(II)f}}]\alpha_{\mathrm{MS}}] \quad (8)$$

$$A_{\mathrm{F}} = A_{\mathrm{F\ hq}}(1 - \alpha_{\mathrm{F}}) + A_{\mathrm{F\ sq}}\alpha_{\mathrm{F}} \quad E_{\mathrm{h}} < -370 \text{ mV}$$
$$= A_{\mathrm{F\ sq}}(1 - \alpha_{\mathrm{F}}) + A_{\mathrm{F\ ox}}\alpha_{\mathrm{F}} \; E_{\mathrm{h}} > -320 \text{ mV} \quad (9)$$

$$A_{\mathrm{MV}} = A_{\mathrm{MV}}(1 - \alpha_{\mathrm{MV}}) \quad (10)$$

$$A_{\mathrm{dF}} = A_{\mathrm{dF\ rd}}(1 - \alpha_{\mathrm{dF}}) + A_{\mathrm{dF\ ox}}\alpha_{\mathrm{dF}} \quad (11)$$

The oxidized form of methyl viologen is colorless, and it does not contribute to the simulated spectrum. For potentials below −370 mV versus SHE, the concentration of oxidized flavodoxin is negligible. Above −370 mV, methionine synthase was present entirely as cob(II)alamin, and the absorbance of methyl viologen was negligible, due both to its low concentration (1 μM) relative to other components and to its midpoint potential of −440 mV. In the presence of saturating flavodoxin, the bound cob(II)alamin spectrum [$A_{\mathrm{MS\ Co(II)b}}$] was used, while in the presence of saturating methionine synthase, the data were best fit with a mixture of 91% free cob(II)alamin and 9% flavodoxin-bound cob(II)alamin. Between −370 and −320 mV, all three flavodoxin oxidation states were included in the simulation, while above −320 mV, only oxidized and semiquinone states were included. Thus, each simulated spectrum was empirically derived using four or fewer variables: $\alpha_{\mathrm{MS}}$, $\alpha_{\mathrm{F}}$, $\alpha_{\mathrm{MV}}$, and $\alpha_{\mathrm{dF}}$. The α values were varied manually between 0 and 1 to give the best fit of the simulated spectrum to the observed spectrum at all wavelengths (300−700 nm).

Initial estimates of the α values could be obtained by inspection of absorbance changes at wavelengths where these changes are dominated by a single component. The initial α values were refined by iterative cycling, and refinement was stopped when the difference between observed and simulated spectra was less than ±0.002 absorbance unit at each wavelength. This analysis is similar to previous methods of spectral analysis (Batie & Kamin, 1981; Lambeth *et al.*, 1976), except that all wavelengths were used in the fitting, rather than a few discrete wavelengths.

*Calculation of Midpoint Potentials.* Once the concentrations of oxidized and reduced species have been determined, the system potential can be determined from the ratio of oxidized to reduced species of known potentials. Either methyl viologen or the protein in excess could be used as redox indicators. However, because of the low concentration and inherent problems of dimerization and photochemical damage of the dye (Drummond & Matthews, 1994), it was more reliable to use the protein in excess, rather than methyl viologen, as the redox indicator. Midpoint potentials for free flavodoxin [$E_{\mathrm{m7}} = -260$ mV versus SHE (oxidized/semiquinone) and $E_{\mathrm{m7}} = -440$ mV (semiquinone/hydroquinone)] were measured by equilibrating a redox dye [safranin O ($E_{\mathrm{m7}} = -289$ mV) for the oxidized/semiquinone couple and methyl viologen ($E_{\mathrm{m7}} = -440$ mV) for the semiquinone/reduced couple] with flavodoxin and then slowly photoreducing the system using 5-deazaflavin-3-sulfonate and EDTA. Spectra were measured at intervals, and midpoint potentials were calculated for the free flavodoxin using the dyes as redox indicators (Clark, 1960; Dutton, 1978). The values obtained differ slightly from those previously reported [$E_{\mathrm{m7}} = -244$ mV versus SHE (oxidized/semiquinone) and $E_{\mathrm{m7}} = -455$ mV (semiquinone/hydroquinone) (Vetter & Knappe, 1971)]. The midpoint potential for the cob(II)alamin/cob(I)alamin couple of methionine synthase used for these analyses was −484 mV versus SHE (C. Y. Choi and R. G. Matthews, unpublished results).

*Measurement of Flavodoxin Binding to Methionine Synthase in Cob(III)alamin Forms with Different Axial Ligands.* Methods similar to those used for aerobic titrations (described above) were used to measure the binding of oxidized flavodoxin to cob(III)alamin in the presence of different anions. Titrations were performed in 50 mM potassium phosphate buffer at pH 7.4.

The ligand concentrations in reference and sample cells were 2 mM for azide, thiocyanate, and cyanide and 200 mM for chloride and bromide. After addition of the sodium or potassium salt of the anion, spectra were recorded until no further changes in the cobalamin spectrum were seen. Concentrated oxidized *E. coli* flavodoxin was then added to both the sample and reference cells in small portions, and spectra were recorded. $K_{\mathrm{d}}$ values for flavodoxin binding were calculated from the maxima in the difference spectra.

## RESULTS

*Complex Formation between Oxidized Flavodoxin and Methionine Synthase Can Be Demonstrated by Visible Absorbance Spectroscopy.* Methionine synthase reactivation by flavodoxin has been shown to be specific for *E. coli* flavodoxin (R. G. Matthews, unpublished results), and the rate of methionine formation is half-maximal at a flavodoxin concentration of 5 nM (Fujii *et al.*, 1977). However, direct evidence for complex formation between flavodoxin and methionine synthase has not been reported in the literature.

Previous studies have shown distinct absorbance changes on binding of flavodoxins to other redox partners (Foust *et*

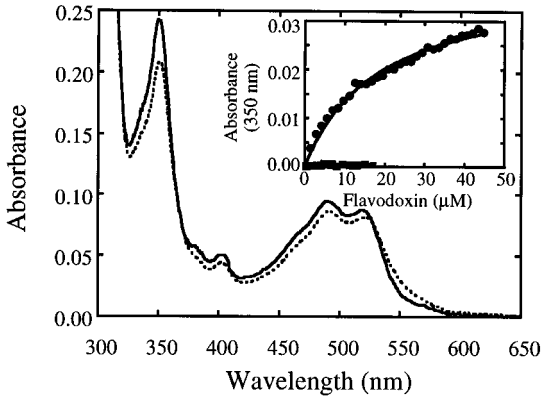


FIGURE 1: Absorbance changes associated with complex formation between the hydroxocob(III)alamin form of methionine synthase and oxidized flavodoxin from *E. coli*. The spectrum of methionine synthase was measured in the absence (dashed line) or presence (solid line) of 45 $\mu$M flavodoxin. Eight hundred microliters of 10 $\mu$M methionine synthase in 100 mM potassium phosphate buffer at pH 7.4 and 20 °C was titrated with 1 $\mu$L aliquots of concentrated flavodoxin (1.09 mM). (Inset) Plot of the change in absorbance at 350 nm versus the concentration of flavodoxin added. A $K_d$ of 18.5 $\mu$M and a $\Delta\epsilon_{350}$ of 3.86 $mM^{-1}$ $cm^{-1}$ can be estimated from these data. Circles show the changes associated with addition of flavodoxin from *E. coli*, and squares show the changes associated with addition of flavodoxin from *Synechococcus* sp. PCC 7942.

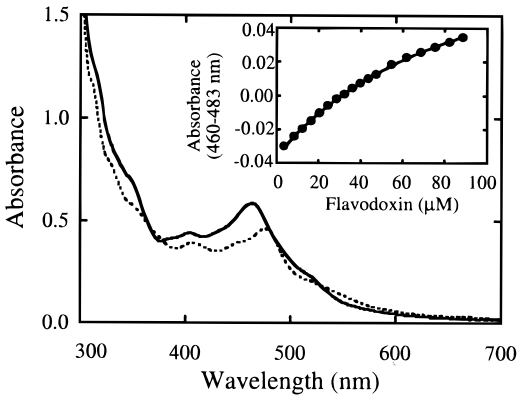


FIGURE 2: Absorbance changes associated with complex formation between the cob(II)alamin form of methionine synthase and oxidized flavodoxin. Spectra of methionine synthase in the absence (dashed line) or presence (solid line) of 88 $\mu$M flavodoxin are shown. Eight hundred microliters of 42.3 $\mu$M cob(II)alamin methionine synthase in 50 mM potassium phosphate buffer at pH 7.0 and 20 °C was titrated with 5 $\mu$L portions of oxidized flavodoxin (680 $\mu$M) in an anaerobic cuvette. (Inset) Replot of the absorbance difference ($A_{460\ nm} - A_{483\ nm}$) as a function of added flavodoxin concentration. Analysis of these data gives a $K_d$ of 46.5 $\mu$M and a $\Delta\epsilon_{460-483}$ of 3.6 $mM^{-1}$ $cm^{-1}$ for the interaction of oxidized flavodoxin with the cob(II)alamin form of methionine synthase.

*al*., 1969), so we looked for absorbance changes associated with the interaction of methionine synthase and flavodoxin. At low ionic strengths, addition of oxidized flavodoxin was found to induce changes in the visible spectrum of methionine synthase in the hydroxocob(III)alamin form (Figure 1). The hydroxocob(III)alamin form of methionine synthase was initially investigated because mixing oxidized flavodoxin with the cob(II)alamin form of methionine synthase resulted in the oxidation of the cobalt (data not shown). Oxidation of methionine synthase was only seen in aerobic solutions, and later titrations with the cob(II)alamin form of the enzyme were performed anaerobically to eliminate oxidation (see below). The absorbance changes are similar to those accompanying weakening or substitution of the upper ligand−Co bond (Pratt, 1972). Flavodoxin binds methionine synthase with a 1:1 stoichiometry and a $K_d$ of 18.5 $\mu$M in 100 mM potassium phosphate buffer at pH 7.4 (Figure 1, inset). When flavodoxin from *Synechococcus* is added under identical conditions, no absorbance changes in methionine synthase are seen (Figure 1, inset). This observation is rather striking, since the two flavodoxins are extremely similar in sequence (49% identity) and structure (0.8 Å root mean-squared deviation for backbone atoms) (D. M. Hoover, unpublished data) and is consistent with the specificity of the *E. coli* flavodoxin previously noted for the reductive activation of methionine synthase.

The hydroxocob(III)alamin form of methionine synthase is, however, not a physiological state of the protein; methionine synthase cycles between methylcob(III)alamin and cob(I)alamin forms, and the cofactor occasionally oxidizes to cob(II)alamin. The hydroxocob(III)alamin form of methionine synthase can easily be reduced, e.g. by thiols, and presumably would not be present in the bacterium. *In vitro*, flavodoxin is capable of reducing enzyme-bound cob(III)alamin to the cob(II)alamin form (data not shown). Because the interaction of flavodoxin with the cob(II)alamin form of methionine synthase is more relevant to the reactivation reaction, interaction with cob(II)alamin methionine synthase was investigated. Spectral changes were observed when oxidized flavodoxin and cob(II)alamin methionine synthase were mixed anaerobically, indicating complex formation (Figure 2). The absorbance changes in the cob(II)alamin form are characteristic of those seen when the lower axial ligand is weakened or dissociated from the cobalamin (Jarrett *et al*., 1996a). Titration of the cob(II)alamin form of methionine synthase with flavodoxin is associated with a $K_d$ of 46.5 $\mu$M in 50 mM potassium phosphate buffer at pH 7.0.

*The Methylcobalamin Form of Methionine Synthase Does Not Bind Flavodoxin.* When oxidized flavodoxin was added to methylated methionine synthase and to buffer in a reference cuvette at low ionic strengths, no change in absorbance was seen (data not shown). This observation does not fully answer the question of whether flavodoxin can form a complex with the methylated form of methionine synthase, since such a complex might not perturb the methionine synthase spectrum. The ability of the methylcobalamin form of methionine synthase to compete with ferredoxin:$NADP^+$ oxidoreductase for binding to flavodoxin was therefore examined.

Ferredoxin:$NADP^+$ oxidoreductase and ferredoxin have long been known to play important roles in exchange of electrons between NADPH-dependent enzymes and electron transfer chains, and in some species, flavodoxin can substitute for ferredoxin in this role (Bianchi *et al*., 1993b). Both ferredoxin and flavodoxin bind to ferredoxin:$NADP^+$ oxidoreductase very tightly at low ionic strengths (Foust *et al*., 1969). If flavodoxin binds to both methionine synthase and ferredoxin:$NADP^+$ oxidoreductase, and binding is mutually exclusive, methionine synthase will compete with ferredoxin:$NADP^+$ oxidoreductase for the binding to flavodoxin and thus allow an independent measurement of the affinity of methionine synthase for flavodoxin. Such experiments are similar to studies of the binding of cytochrome $b_5$ to $P_{450cam}$ (Stayton *et al*., 1989). In this case, cytochrome $b_5$ was shown to compete with putidaredoxin, the natural electron donor, for binding to $P_{450cam}$.

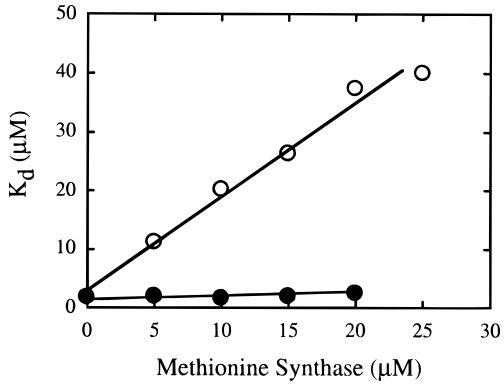


FIGURE 3: Competition of different forms of methionine synthase with ferredoxin:$NADP^+$ oxidoreductase for binding to flavodoxin. The proteins were titrated in 10 mM potassium phosphate buffer at pH 7.0 as described in Experimental Procedures. The slope of each line is equal to the ratio of the $K_d$ for flavodoxin binding to ferredoxin:$NADP^+$ oxidoreductase to the $K_d$ for flavodoxin binding to methionine synthase. Under the conditions of these experiments, the $K_d$ for flavodoxin binding to ferredoxin:$NADP^+$ oxidoreductase is 1.0 $\mu$M; the $K_d$ for flavodoxin binding to the hydroxocob(III)-alamin form of methionine synthase (open circles) is calculated to be 0.3 $\mu$M, while that for flavodoxin binding to the methylcobalamin form of methionine synthase (solid circles) is calculated to be >70 $\mu$M.

Ferredoxin:$NADP^+$ oxidoreductase from *E. coli* was titrated with oxidized flavodoxin. A complex could easily be detected in 10 mM potassium phosphate buffer by difference spectrophotometry. In Figure 3, the apparent $K_d$ of flavodoxin for ferredoxin:$NADP^+$ oxidoreductase is plotted as a function of the concentration of methionine synthase in the hydroxocob(III)alamin form (open circles) or in the methylcobalamin form (closed circles). The calculated $K_d$ for flavodoxin binding to methionine synthase in the hydroxocob(III)alamin form is 0.3 $\mu$M, in good agreement with the value of 0.5 $\mu$M measured under the same conditions in the absence of ferredoxin:$NADP^+$ oxidoreductase (data not shown). In contrast, binding of flavodoxin to methylcobalamin is associated with a $K_d$ of >70 $\mu$M. Thus, methionine synthase can partially exclude flavodoxin during turnover by disfavoring its association with enzyme in the methylcobalamin state.

*Binding of Oxidized Flavodoxin to Methionine Synthase in the Cob(II)alamin Form Results in the Dissociation of the Histidine Ligand to the Cobalt.* Binding of cobalamin to methionine synthase replaces the dimethylbenzimidazole ligand to cobalt with a histidine residue from the protein (Drennan, 1994). Histidine 759 is linked by a hydrogen bond to Asp757, which in turn is hydrogen bonded to the hydroxyl of Ser810; these residues constitute a ligand triad (Jarrett *et al*., 1996a). When methylcobalamin is demethylated to form cob(I)alamin, the ligand presumably dissociates (Lexa & Saveant, 1976), giving rise to four-coordinate "base-off" cob-(I)alamin. A series of mutants of the ligand triad residues has been constructed and characterized (Amaratunga *et al*., 1996; Jarrett *et al*., 1996a), one of which is His759Gly. The His759Gly protein, which lacks the histidine ligand, has absorbance characteristic of four-coordinate cob(II)alamin (Pratt, 1972). The spectrum of the cob(II)alamin methionine synthase/flavodoxin complex (Figure 2) is similiar to the spectrum of the His759Gly mutant protein (Jarrett *et al*., 1996a), providing initial evidence that flavodoxin binding leads to dissociation of His759 from the cob(II)alamin cofactor.

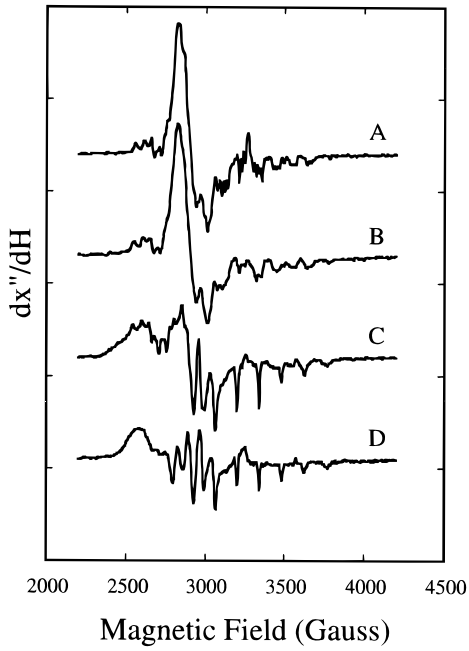


FIGURE 4: EPR spectra of methionine synthase with and without flavodoxin: (A) 200 $\mu$M methionine synthase in 50 mM potassium phosphate buffer at pH 7.2, (B) 200 $\mu$M methionine synthase and 380 $\mu$M oxidized flavodoxin from *Synechococcus* sp. PCC 7942 in 50 mM potassium phosphate buffer at pH 7.2, (C) 200 $\mu$M methionine synthase and 550 $\mu$M oxidized *E. coli* flavodoxin in 50 mM potassium phosphate buffer at pH 7.2, (D) 120 $\mu$M His759Gly mutant methionine synthase in 50 mM potassium phosphate buffer at pH 7.2. On the basis of the spectrum of the His759Gly mutant protein, methionine synthase in the presence of *E. coli* flavodoxin (4C) was calculated to be greater than 95% base off.

Since cob(II)alamin contains a single unpaired electron centered on the cobalt, EPR spectroscopy provides a second technique for monitoring the cobalamin environment and the consequences of flavodoxin binding. Because cobalt has a spin of $^7/_2$, the interaction of this unpaired electron with the cobalt nucleus produces a hyperfine splitting that is clearly evident in the $g_z$ signal, giving an EPR spectrum containing eight high-field peaks centered at $g = 2.0004$ (Bayston *et al*., 1970; Pilbrow, 1982). In methionine synthase, the cobalt of cob(II)alamin is ligated to the $\epsilon$-nitrogen (spin = 1) of His759. Superhyperfine couplings caused by this interaction result in each of the eight singlets being split into triplets (Banerjee *et al*., 1990). The cob(II)alamin form of methionine synthase thus has a spectrum that is diagnostic for the presence of an axial nitrogen ligand (Figure 4A). Addition of oxidized flavodoxin from *Synechococcus* does not affect the EPR spectrum of methionine synthase (Figure 4B), but addition of oxidized flavodoxin from *E. coli* to methionine synthase leads to a loss of the superhyperfine splittings, indicative of dissociation of the nitrogenous lower ligand from the cobalt (Figure 4C). A similar EPR spectrum is also seen in the His759Gly mutant (Figure 4D). This indicates that the cob(II)alamin coordination induced by the binding of *E. coli* flavodoxin is similar to that in the His759Gly mutant, namely four-coordinate with the base off.

*pH Dependence of Complex Formation between Flavodoxin and Methionine Synthase.* Dissociation of the histidine ligand upon reduction of cob(II)alamin to cob(I)-alamin is known to be associated with proton uptake (Drummond & Matthews, 1994). If the ligand dissociation observed upon flavodoxin binding is also associated with

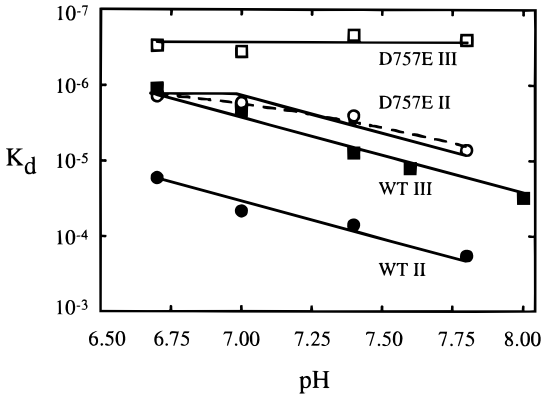


FIGURE 5: Variation of the dissociation constants for binding of oxidized flavodoxin to wild-type and mutant methionine synthase enzymes with pH. Methionine synthase in the hydroxocob(III)alamin form (2−3 $\mu$M) was mixed with aerobic potassium phosphate buffers of the indicated pH that had been adjusted to an ionic strength of 0.12. Difference spectra were recorded, and $K_d$ values were calculated as described in Experimental Procedures. Experiments with cob(II)alamin forms of methionine synthase were conducted under anaerobic conditions. The following plots are shown: wild-type enzyme in the cob(II)alamin form (solid circles), wild-type enzyme in the hydroxocob(III)alamin form (solid squares), Asp757Glu enzyme in the cob(II)alamin form (open circles), and Asp757Glu enzyme in the aquocob(III)alamin form (open squares). The solid lines represent simple fits of the $K_d$ values assuming either no pH dependence or uptake of a single proton on complex formation. The dashed line was calculated assuming that a residue with a p$K$ of 6.9 was being protonated in the titration of Asp757Glu mutant methionine synthase in the cob(II)alamin form.

proton uptake, then complex formation should be pH-dependent. The binding of oxidized flavodoxin to both hydroxocob(III)alamin and cob(II)alamin forms of methionine synthase is pH-dependent between pH 6.7 and pH 7.8 (Figure 5). The slopes of these plots indicate that one proton is indeed taken up on formation of the complex within this pH range as shown in eq 12.

$$\text{E-hydroxocob(III)alamin} + \text{flavodoxin} + \text{H}^+ \rightarrow \text{E-aquocob(III)alamin/flavodoxin} \quad (12)$$

However, we have not yet proved that the proton is used to convert hydroxo- to aquocob(III)alamin, and in fact, the proton may be taken up elsewhere in the protein due to conformational changes associated with flavodoxin binding. With the cob(II)alamin form of methionine synthase, the pH dependence of flavodoxin binding is consistent with the ligand triad being protonated as shown in eq 13.

$$\text{E-cob(II)alamin} + \text{flavodoxin} + \text{H}^+ \rightarrow \text{EH}^+\text{-cob(II)alamin/flavodoxin} \quad (13)$$

*Effects of Ligand Triad Mutations of Methionine Synthase on Complex Formation between Cob(II)alamin Methionine Synthase and Flavodoxin.* Because the binding of *E. coli* flavodoxin to methionine synthase in the cob(II)alamin form causes the histidine ligand to dissociate from the cobalt, mutations that disrupt or weaken the histidine ligation should affect the binding of flavodoxin. Two site-directed mutants previously described (Amaratunga *et al*., 1996; Jarrett *et al*., 1996a) were employed to test this hypothesis: His759Gly, in which the lower histidine ligand was removed by conversion to a glycine, and Asp757Glu, in which the aspartate residue of the ligand triad was changed to a glutamate.

Cob(II)alamin in the His759Gly mutant protein is four-coordinate and would be expected to bind flavodoxin tightly. However, when flavodoxin was mixed with the His759Gly protein, no spectral changes could be detected. We therefore employed the competitive binding assay using ferredoxin:$NADP^+$ oxidoreductase in order to measure the dissociation constant for the complex between oxidized flavodoxin and the cob(II)alamin form of the His759Gly protein. The mutant methionine synthase was seen to compete with ferredoxin:$NADP^+$ oxidoreductase for the binding of flavodoxin (data not shown). The $K_d$ for oxidized flavodoxin binding to His759Gly methionine synthase in the cob(II)alamin form in 50 mM potassium phosphate buffer at pH 7.0 was 2.5 $\mu$M. This value is approximately 20-fold smaller than the $K_d$ for wild-type cob(II)alamin methionine synthase measured under identical conditions, as expected from the observation that the binding of flavodoxin to wild-type methionine synthase causes the dissociation of the histidine ligand to cobalamin.

Previous studies indicated that the Asp757Glu mutation weakens the bond between the histidine ligand and cobalamin; the cob(II)alamin form is present as a mixture of four-coordinate and five-coordinate states (Jarrett *et al*., 1996a). While the midpoint potential of the cob(II)alamin/cob(I)alamin couple for the wild-type enzyme is pH-dependent (Drummond & Matthews, 1994), the $E_{m7}$ of the Asp757Glu mutant is higher than that of the wild-type enzyme and shows a diminished pH dependence within the pH range from 6.7 to 8.0 (C. Y. Choi and R. G. Matthews, unpublished results). The pH dependence of flavodoxin binding to the Asp757Glu protein in the cob(II)alamin state was investigated (Figure 5, open circles). Compared with that of the wild-type protein, the affinity of oxidized flavodoxin for the Asp757Glu mutant protein shows diminished pH dependence over the pH range from 6.7 to 7.8, and the dissociation constants are approximately 10-fold smaller than for the wild-type enzyme under the same conditions. The spectral changes associated with complex formation involving the mutant enzyme are smaller than those for formation of the wild-type complex, probably because at least half of the mutant enzyme adopts the base-off conformation even in the absence of flavodoxin (Jarrett *et al*., 1996a). The pH dependence can be fit to a p$K_a$ of ~6.9 in the cob(II)alamin form of the Asp757Glu protein. Referring back to eq 13, the p$K$ of E-cob(II)alamin (the ligand triad) in the mutant enzyme is now 6.9, and at low pH, the dissociation constant for the flavodoxin complex becomes pH-independent. The effects of the Asp757Glu mutation indicate that the pH dependence of the dissociation constant for interaction of flavodoxin with the cob(II)alamin form of methionine synthase arises from the linked dissociation of histidine and protonation of His-Asp. This mutation presumably disrupts the hydrogen-bonding network and alters the observed pH dependence of flavodoxin binding.

*Effect of Ligand Triad Mutations on Complex Formation between Flavodoxin and the Cob(III)alamin Form of Methionine Synthase.* The dissociation constant for binding of oxidized flavodoxin to the Asp757Glu mutant protein in the cob(III)alamin state was measured over the pH range from 6.7 to 7.8 (Figure 5, open squares). The binding of flavodoxin to the mutant is at least 10 times tighter than the binding to the wild-type enzyme and is no longer pH-dependent within this pH range. In the cob(III)alamin form,

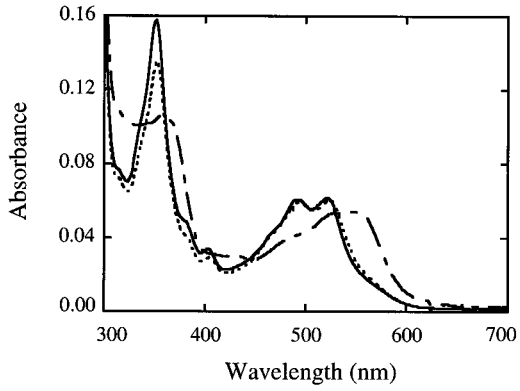


FIGURE 6: Displacement of the thiocyanate ligand to the cob(III)alamin form of methionine synthase by the binding of oxidized flavodoxin. KSCN (final concentration of 2 mM) was added to a cuvette containing 6.7 $\mu$M methionine synthase in the cob(III)alamin form in 10 mM potassium phosphate buffer at pH 7.0. After absorbance changes associated with ligand exchange were complete, portions of oxidized flavodoxin were added to a final concentration of 250 $\mu$M. Spectra are shown for the initial hydroxocob(III)alamin methionine synthase (- - -), cob(III)alamin methionine synthase in the presence of 2.0 mM KSCN (− - −), and cob(III)alamin methionine synthase in the presence of 2.0 mM KSCN and 250 $\mu$M oxidized flavodoxin (—).

the mutant protein exists predominantly in the aquocob(III)alamin form, as judged by its absorbance properties (Jarrett *et al*., 1996a). We may conclude then that binding of flavodoxin to the hydroxocobalamin form of wild-type methionine synthase is accompanied by protonation of the hydroxide ligand, forming aquocob(III)alamin.

*Effects of Ligand Substitution on Complex Formation between Flavodoxin and the Cob(III)alamin Form of Methionine Synthase*. In the preceding sections, we have shown that oxidized flavodoxin binding to methionine synthase in the cob(III)alamin form leads to the protonation of a coordinated hydroxide ligand and that oxidized flavodoxin does not bind to enzyme in the methylcobalamin form. These experiments suggested that the upper axial ligand might be expelled when flavodoxin binds. Accordingly, we decided to measure the binding of flavodoxin to cob(III)alamin enzyme that had alternate anionic ligands in the upper axial position. Since ligand substitution of cob(III)alamin is associated with spectral changes, we could monitor the fate of the ligand when oxidized flavodoxin binds.

Figure 6 shows the spectral changes associated with binding of flavodoxin to thiocyanate-substituted cob(III)alamin methionine synthase. It is clear from this experiment that binding of flavodoxin to thiocyanatocob(III)alamin results in expulsion of the thiocyanate ligand, since the final absorbance spectrum of the flavodoxin/methionine synthase complex is identical to the spectrum of the complex formed when flavodoxin binds methionine synthase in the hydroxocob(III)alamin form. The apparent $K_d$ values for flavodoxin binding measured in the presence of 2 mM potassium thiocyanate were pH-independent, consistent with release of the thiocyanate ligand as an anion on flavodoxin binding (data not shown). Flavodoxin also binds to enzyme substituted with azide, chloride, or bromide ligands with expulsion of the ligand. A similar experiment was performed to measure binding of flavodoxin to enzyme partially substituted with cyanide, and no flavodoxin binding was detected. Since the affinity of free cob(III)alamin for cyanide (log $K_a > 12$) is much greater than its affinity for azide (log $K_a = 4.9$), chloride (log $K_a = 0.1$), bromide (log $K_a = 0.3$), thiocyanate (log $K_a = 3.1$), or hydroxide (log $K_a = 7.1$) (Pratt, 1972), these experiments suggest that expulsion of the upper axial ligand is required for flavodoxin binding to the cob(III)alamin form of methionine synthase and that strongly basic and tightly bound ligands such as cyanide or the methyl carbanion block flavodoxin binding.

*What Regions of Methionine Synthase Are Required for Flavodoxin Binding?* Methionine synthase from *E. coli* has been shown to contain several distinct functional regions (Banerjee *et al*., 1989; Drummond *et al*., 1993). Tryptic cleavage of the native enzyme yields an N-terminal 98 kDa domain that binds methylcobalamin, $CH_3$-$H_4$folate, and homocysteine and that catalyzes the methyltransferase reaction and a C-terminal 38 kDa domain that binds AdoMet and functions to reactivate the protein in the cob(II)alamin form. The cleaved enzyme can no longer be reductively methylated with AdoMet, even if an artificial reducing system is substituted for flavodoxin (Drummond *et al*., 1993). We examined whether proteolytic cleavage of methionine synthase would affect flavodoxin binding and/or the conformational change induced on binding that results in dissociation of the histidine ligand to cobalamin.

The 98 kDa N-terminal fragment of methionine synthase generated by tryptic proteolysis of the enzyme in the methylcobalamin form was converted to the hydroxocob(III)alamin form by incubation with homocysteine and ferricyanide and mixed with oxidized flavodoxin after removal of excess reagents; no difference spectrum was evident on addition of a 4-fold excess of oxidized flavodoxin. Therefore, in the absence of the C-terminal domain, productive complex formation is impaired, although nonspecific binding of flavodoxin cannot be excluded. Addition of the purified C-terminal 38 kDa domain of methionine synthase to flavodoxin did not lead to complex formation as seen by the ferredoxin:$NADP^+$ oxidoreductase competition assay (data not shown). Thus, separating the AdoMet-binding C-terminal domain from the catalytic N-terminal fragment disfavors productive interactions between flavodoxin and methionine synthase. Both regions of the protein are apparently required for flavodoxin-induced effects on cobalamin coordination.

*Redox Potentials of Complexes: Binding of Flavodoxin Hydroquinone to the Cob(II)alamin Form of Methionine Synthase Does Not Favor Electron Transfer to Cob(II)alamin.* The electron transfer step of reductive reactivation, according to the model described in eqs 3 and 4 in which electrons are transferred to cob(II)alamin, is dependent on the difference in midpoint potentials between the FMN of flavodoxin and the cobalamin of methionine synthase. However, the midpoint potentials disfavor electron transfer from flavodoxin hydroquinone to the cob(II)alamin form of methionine synthase. Because the experiments already described had shown that *E. coli* flavodoxin binds to methionine synthase and induces a conformation change at the cobalamin, it seemed possible that the binding of flavodoxin to methionine synthase might perturb the midpoint potentials of the two proteins to make electron transfer more favorable. Protonation and dissociation of the dimethylbenzimidazole lower ligand raise the cob(II)alamin/cob(I)alamin midpoint potential for free cobalamin by approximately 100 mV (Lexa & Saveant, 1983). Thus, the binding of *E. coli*

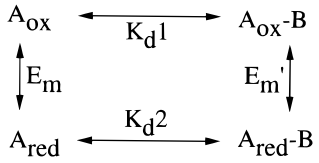


FIGURE 7: Thermodynamic cycle describing the relationship between binding constants and midpoint potentials for the interaction of a protein ligand, B, with a redox-active protein, A.

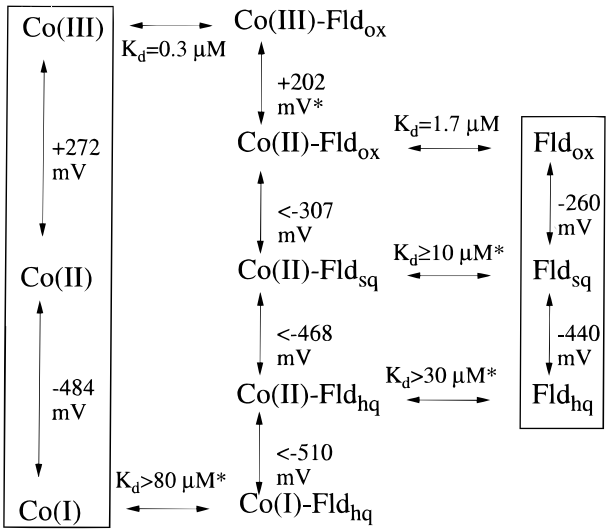


FIGURE 8: Thermodynamic cycles relating binding constants and midpoint potentials for the various oxidiation states of the cofactors of flavodoxin and methionine synthase. Midpoint values in shaded boxes are for the free proteins. Values marked by an asterisk were calculated on the basis of the other measured values using eq 14. All $K_d$ values were determined from measurements in 10 mM potassium phosphate buffer at pH 7.0 and 20 °C; the $K_d$ values determined in Figures 1 and 2 are different than those reported here because they were determined in 50 mM buffer.

flavodoxin to cob(II)alamin methionine synthase might make electron transfer more favorable. To test this hypothesis, we measured the midpoint potentials of the two proteins in complex with one another.

In a system in which a redox active protein A can bind another protein B, a simple thermodynamic cycle can be constructed that describes the effect of binding on the redox potentials of protein A (Figure 7). It can be shown that, under saturating concentrations of component B, the midpoint potential of A in the bound complex is given by eq 14 (Batie & Kamin, 1981). Extending this concept to the interactions

$$E_m'(A_{ox}\text{-}B/A_{red}\text{-}B) = E_m(A_{ox}/A_{red}) + (RT/nF)\log(K_{d1}/K_{d2}) \quad (14)$$

of methionine synthase and flavodoxin is more complicated because both proteins are redox-active. However, because the one-electron midpoint potentials of the three oxidation states of flavodoxin and of the cobalamin cofactor of methionine synthase are widely separated, a single thermodynamic diagram representing all relevant complexes can be drawn (Figure 8). Measurement of the midpoint potential of protein A complexed to protein B requires the liganding protein (protein B) to be in saturating concentrations relative to protein A. However, if both proteins are redox-active, as in the case of flavodoxin and methionine synthase, the protein present in excess can be used as both a ligand and an internal redox indicator, since most of the liganding protein is free.

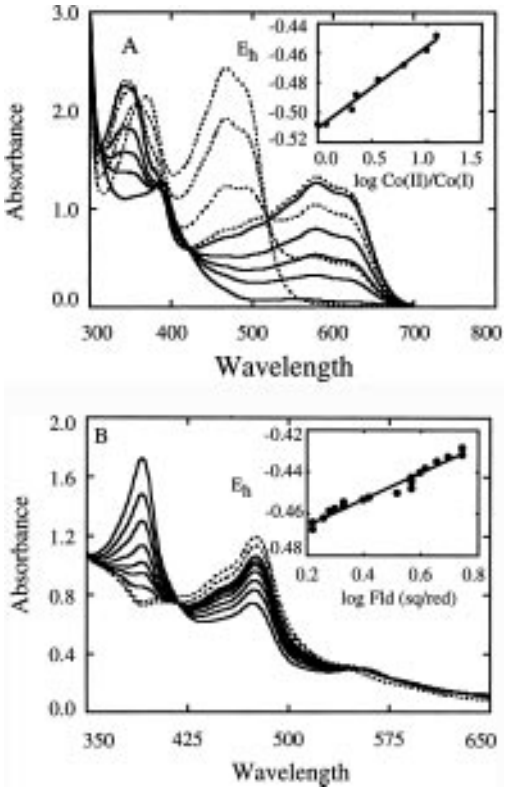


FIGURE 9: Midpoint potential determinations in flavodoxin/methionine sythase complexes. (A) Flavodoxin is present in excess over methionine synthase and serves as a redox indicator as well as a ligand. The system, containing 5-deazaflavin-3-sulfonate, EDTA, and methyl viologen, was gradually photoreduced, and spectra were recorded at intervals. Dashed lines show spectral changes associated with the reduction of oxidized to semiquinone forms of flavodoxin, and solid lines show reduction of flavodoxin from the semiquinone to hydroquinone forms concomitant with reduction of the cob(II)alamin form of methionine synthase to cob(I)alamin. The inset shows a Nernst plot of the system potential versus the logarithm of the cob(II)alamin/cob(I)alamin couple. (B) Methionine synthase is present in excess over flavodoxin and serves as redox indicator and ligand. The system, containing 5-deazaflavin-3-sulfonate, EDTA, and methyl viologen, is completely photoreduced and then allowed to oxidize slowly. Spectra shown as solid lines show the oxidation of the cob(I)alamin form of methionine synthase to cob(II)alamin concomitant with oxidation of flavodoxin from the hydroquinone to the semiquinone state. Dashed lines show the conversion of flavodoxin from the semiquinone to the oxidized form. The inset shows a Nernst plot of the system potential versus the logarithm of the semiquinone/hydroquinone couple of flavodoxin.

Such a method was used by Batie and Kamin (1981) to measure the effect of complexation on the midpoint potentials of ferredoxin and ferredoxin:$NADP^+$ oxidoreductase.

In the experiment summarized in Figure 9A, flavodoxin was present at a 25-fold higher concentration than methionine synthase, along with a trace of methyl viologen to equilibrate the two proteins, and the entire system was slowly photoreduced. Concentrations of the oxidized and reduced species were determined from absorbance spectra, and the system potential was determined by the ratio of either oxidized to semiquinone forms of flavodoxin or semiquinone to hydroquinone forms of flavodoxin, using the respective free midpoint potentials previously determined (−260 and −440 mV at pH 7.0). A Nernst plot gives a midpoint potential for the cob(II)alamin/cob(I)alamin couple of methionine synthase bound to hydroquinone flavodoxin of −510 mV, 26 mV lower than that for free methionine synthase (inset).

Similarly, in the experiment shown in Figure 9B, methionine synthase was present at a 12-fold higher concentration than flavodoxin. After photoreduction, the system was allowed to oxidize slowly, and concentrations of the oxidized and reduced species were again determined from absorbance spectra. The system potential was calculated using the ratio of cob(I)alamin/cob(II)alamin and the midpoint potiential for free methionine synthase [$E_{m7} = -484$ mV (C. Y. Choi and R. G. Matthews, unpublished results)]. The midpoint potential for the flavodoxin semiquinone/hydroquinone couple bound to methionine synthase was determined to be −468 mV, 28 mV lower than that for the equivalent potential for free flavodoxin. The midpoint potential of the oxidized/semiquinone couple of flavodoxin could be determined from the percentage of semiquinone formed during the reduction (92%) using eqs 15 and 16 (Clark, 1960).

$$E_m(\text{ox/sq}) = (RT/nF) \log K_{sq} + E_m(\text{sq/red}) \quad (15)$$

$$\text{fraction semiquinone formed} = \sqrt{K_{sq}}/(2 + \sqrt{K_{sq}}) \quad (16)$$

Once these midpoint potentials are determined, the $K_d$ values indicated with asterisks in Figure 8 can be calculated. Note that the concentration of phosphate buffer, and therefore the ionic strength, is lower in these experiments than in Figures 1 and 2; this lower concentration of phosphate was chosen to increase the affinity between flavodoxin and methionine synthase and to maximize the amount of complex formation. After $K_d$ values for the reduced complexes were calculated, the fraction of protein bound in the complexes could be estimated. In the cases of the complexes between the cob(I)alamin form of methionine synthase and flavodoxin hydroquinone and between cob(II)alamin and flavodoxin hydroquinone, it was estimated that ∼50% of the limiting protein was bound in the complex. This indicates that the true $E_m$ values would be somewhat lower than the calculated $E_m$ values; thus, the values stated for the midpoint potentials of these complexes should be regarded as upper limits, while the values given for the dissociation constants are lower limits.

As the flavodoxin and methionine synthase cofactors become more reduced, the affinity of these proteins for one another diminishes. In addition, methionine synthase in the cob(II)alamin form is more difficult to reduce when it is complexed with flavodoxin hydroquinone. Since free cob(II)alamin becomes easier to reduce when it is in the base-off form (Lexa & Saveant, 1983), this observation is surprising, but consistent with other observations. Addition of glycerol to the cob(II)alamin form of methionine synthase, which promotes dissociation of the histidine from the cobalt, similarly leads to a lowered midpoint potential (Banerjee *et al*., 1990). Furthermore, the His759Gly mutant protein has a significantly lower midpoint potential than the wild-type enzyme (C. Y. Choi and R. G. Matthews, unpublished data).

## DISCUSSION

Studies of complex formation between flavodoxin and methionine synthase, described in this paper, have established that flavodoxin binding is accompanied by axial ligand dissociation from the cobalt of the cobalamin cofactor. When flavodoxin binds to the cob(II)alamin form of methionine synthase, the His759 ligand is displaced from the lower axial position and a proton is taken up, presumably by the His-Asp pair. Binding of flavodoxin to the hydroxocob(III)alamin form of methionine synthase results in protonation of the upper ligand and, on the basis of experiments with other anionic ligands, expulsion of the water. Upper ligands such as thiocyanate, which exist as anions at pH 7, are expelled without protonation when flavodoxin binds. Analogous changes in the coordination geometry of a metal center have been seen when substrates bind other metalloproteins. For example, in the absence of camphor, cytochrome P-450$_{cam}$ contains a single low-spin ferric heme, coordinated to a cysteine thiolate in the lower axial position and to water in the upper axial position. Binding of camphor leads to the formation of a five-coordinate high-spin ferric heme with the cysteinate sulfur as the sole axial ligand and is accompanied by expulsion of the solvent ligand (Raag & Poulos, 1989).

It is not clear whether flavodoxin binding leads to axial ligand dissociation from the cobalt of cobalamin because of electrostatic perturbations associated with the binding of a highly negatively charged molecule to methionine synthase or because binding induces a conformational change in methionine synthase that results in altered coordination geometry. However, preliminary experiments (S. Huang and R. G. Matthews, unpublished data) show that the sites of tryptic proteolysis of native methionine synthase are altered in the presence of flavodoxin, suggesting that the conformation of methionine synthase is changed. We cannot exclude the possibility that flavodoxin binding to methionine synthase results in ligand substitution at the upper axial position of the cobalamin, introducing an oxygen or sulfur ligand from the protein that is not detected by EPR or absorbance changes.

The cob(II)alamin form of methionine synthase is five-coordinate in the absence of flavodoxin, and the present studies demonstrate that flavodoxin binding results in dissociation of the histidine from cobalt. Mutations of the ligand triad that weaken (Asp757Glu) and finally abolish (His759Gly) interactions between His759 and the cobalt of cob(II)alamin have been shown to decrease the rate of methyl transfer from methyltetrahydrofolate to homocysteine and to increase the rate of reductive methylation of the cob(II)alamin form of methionine synthase in the presence of AdoMet and flavodoxin (Jarrett *et al*., 1996a). The present finding that flavodoxin binding is accompanied by dissociation of the histidine provides a structural rationale for these observations. If the coordination change induced by flavodoxin binding is partially rate-limiting in reductive activation, then mutant cob(II)alamin proteins that already have the His-Asp pair dissociated will undergo more rapid reductive methylation.

Unexpectedly, our results have shown that binding of flavodoxin hydroquinone to the cob(II)alamin form of methionine synthase, accompanied by proton uptake and by dissociation of the lower axial ligand, does not favor reduction of cob(II)alamin to cob(I)alamin within the error of measurement. While protonation and displacement of the dimethylbenzimidazole ligand of free cob(II)alamin raises the midpoint potential for the cob(II)alamin/cob(I)alamin couple by 100 mV, binding of flavodoxin hydroquinone to the cob(II)alamin form of methionine synthase lowers the midpoint potential of the cob(II)alamin/cob(I)alamin couple by at least 26 ± 10 mV.

It is possible that the relevant complex for electron transfer during aerobic growth is actually the complex between flavodoxin semiquinone and the cob(II)alamin form of methionine synthase. When flavodoxin is incubated with an excess of NADPH and flavodoxin:$NADP^+$ oxidoreductase, only the flavodoxin semiquinone is formed (Fujii *et al*., 1977). Furthermore, in stopped-flow measurements of the rate of reductive methylation, either flavodoxin semiquinone or hydroquinone can serve as the electron donor to methionine synthase, although the semiquinone reacts more slowly (data not shown). While the flavodoxin hydroquinone may be the major electron donor to methionine synthase during anaerobic growth, it seems likely that the semiquinone is the major electron donor during aerobic growth.

Why, then, does flavodoxin binding induce a change in coordination geometry at the cobalt? Although our present knowledge does not permit an unambiguous answer to this question, we will present a hypothesis that can be tested. The Rossmann domain that interacts with the lower face of the cobalamin cofactor and contains the His759 ligand to the cobalt may play an important role in regulating both access to and reactivity of the cobalamin cofactor. In the X-ray structure of the proteolytic fragment that binds methylcobalamin, the upper face of the corrin ring is covered by an α-helical or "cap" domain that shields the methyl group from solvent and would be expected to block methyl transfer from methylcobalamin to homocysteine. Mutations of residues from the α-helical domain that contact the cobalamin establish that the cap also shields the cobalamin in the resting methylcobalamin state of the intact enzyme (Jarrett *et al*., 1996b). In some as yet unknown manner, the binding of substrates and activators must lead to conformational changes in the protein that expose the cobalamin for reaction at its upper face and position the appropriate region of the protein vis-à-vis the cobalt. We propose that the changes in the coordination geometry of the cobalamin seen on flavodoxin binding to the cob(II)alamin form of methionine synthase reflect just such conformational changes, in this case rearrangements that position the C-terminal domain of methionine synthase with its bound AdoMet to transfer a methyl group to the cobalt of the cobalamin cofactor.

The ability of flavodoxin to bind methionine synthase in the cob(II)alamin form or the hydroxocob(III)alamin form, but not in the methylcobalamin form, may be important for excluding AdoMet from participation in the catalytic cycle of the enzyme. Methylation of homocysteine by AdoMet is a futile and wasteful reaction, since a major metabolic fate of methionine is conversion to AdoMet. However, the conformation of methionine synthase in the absence of flavodoxin may sterically disfavor interactions of AdoMet with cob(I)alamin, and the conformation required for reaction of AdoMet with the cofactor may only be significantly populated when flavodoxin is bound.

Dissociation of the lower ligand of an enzyme-bound cob(II)alamin during reductive activation is apparently not unique to methionine synthase. Studies on the reductive methylation of the cob(II)amide form of methanol:5-hydroxybenzimidazolylcobamide methyltransferase from the stricly anaerobic methanogen *Methanosarcina barkeri* have also demonstrated ligand dissociation (Daas *et al*., 1996). This enzyme also cycles between methylcobamide and cob(I)amide forms during turnover, and the cob(II)amide species is inactive. Activation requires both an electron, supplied by reduced ferredoxin, and a methyl donor, in this case the substrate methanol. Furthermore, a phosphorylated activator protein, methyltransferase activation protein, is required. Vogels and his colleagues have shown that addition of the methyltransferase activation protein to methyltransferase in the cob(II)amide form results in dissociation of the nitrogen ligand to the enzyme-bound cob(II)amide.

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