Mechanism of Reductive Activation of Cobalamin-Dependent Methionine Synthase: An Electron Paramagnetic Resonance Spectroelectrochemical Study†

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ABSTRACT: The mechanism of reductive methylation of cobalamin-dependent methionine synthase (5-methyltetrahydrofolate:homocysteine methyltransferase, EC 2.1.1.13) has been investigated by electron paramagnetic resonance (EPR) spectroelectrochemistry. The enzyme as isolated is inactive, and its UV/visible absorbance and EPR spectra are characteristic of cob(II)alamin. There is an absolute requirement for catalytic amounts of AdoMet and a reducing system for the formation and maintenance of active enzyme during in vitro turnover. The midpoint potentials of the enzyme-bound cob(II)alamin/cob(I)alamin and cob(III)alamin/cob(II)alamin couples have been determined to be -526 ± 5 and $+273 \pm 4$ mV (versus the standard hydrogen electrode), respectively. The presence of either CH_3-H_4folate or AdoMet shifts the equilibrium distribution of cobalamin species observed during reduction by converting cob(I)alamin to methylcobalamin. The magnitude of these shifts is however vastly different, with AdoMet lowering the concentration of cob(II)alamin at equilibrium by a factor of at least 3×10^7, while CH_3-H_4folate lowers it by a factor of 19. These studies of coupled reduction/methylation reactions elucidate the absolute requirement for AdoMet in the in vitro assay system, in which the ambient potential is approximately -350 mV versus the standard hydrogen electrode. At this potential, the equilibrium distribution of cobalamin in the presence of CH_3-H_4folate would be greatly in favor of the cob(II)alamin species, whereas in the presence of AdoMet the equilibrium favors methylated enzyme. In these studies, a base-on form of cob(II)alamin in which the dimethylbenzimidazole substituent of the corrin ring is the lower axial ligand for the cobalt has been observed for the first time on methionine synthase. Glycerol was found to influence the base-on/base-off equilibrium on the enzyme. The midpoint potential of the base-off cob(II)alamin/cob(I)alamin couple was determined to be -572 ± 24 mV.

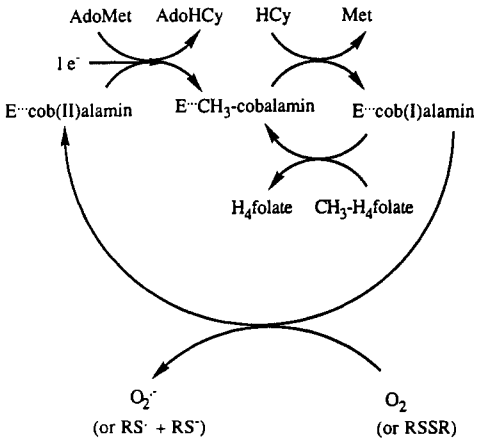
Methionine synthase catalyzes the terminal step in the de novo biosynthesis of methionine. The transmethylation reaction involves CH_3-H_4folate[1] as the one-carbon donor and homocysteine as the acceptor (eq 1). Two forms of the en-

$$CH_3\text{-}H_4\text{folate} + HCy \rightarrow H_4\text{folate} + Met \quad (1)$$

zyme exist in nature: one form requires cobalamin as a prosthetic group (the *metH* gene product) and the other does not (the *metE* gene product). The gene for the cobalamin-dependent methionine synthase from *Escherichia coli* K-12 has been cloned and expressed recently in this laboratory, and its nucleotide sequence has been determined (Banerjee et al., 1988, 1989).

As isolated from *E. coli*, the enzyme is inactive, has the visible absorbance properties of cob(II)alamin (Taylor & Weissbach, 1967; Fujii & Huennekens, 1974), and exhibits an EPR spectrum that is consistent with the +2 oxidation state of cobalt (Frasca et al., 1988). There is an absolute requirement for catalytic amounts of AdoMet (Mangum & Scrimgeour, 1962; Foster et al., 1964) and a reducing system (Guest et al., 1962) for the formation and maintenance of active enyzme during in vitro turnover. Fujii and Huennekens (1974) have shown that two flavoproteins isolated from cell extracts of *E. coli* can activate methionine synthase in the presence of AdoMet and NADPH. These flavoproteins, termed the R and F proteins, show properties analogous to ferredoxin:$NADP^+$ oxidoreductase and flavodoxin, respectively. Anaerobic incubation of methionine synthase with these two proteins and an excess of NADPH and AdoMet results in absorbance changes that are consistent with the conversion of the enzyme-bound cobalamin prosthetic group from cob(II)alamin to methylcobalamin (Fujii & Huennekens, 1979).

Scheme I: Interconversion of Forms of Methionine Synthase



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[1] Abbreviations: EPR, electron paramagnetic resonance; AdoMet, *S*-adenosylmethionine; CH_3-H_4folate, 5-methyltetrahydrofolate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; AdoHCy, *S*-adenosylhomocysteine; HCy, homocysteine; KP_i, potassium phosphate; bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; MES, 2-(*N*-morpholino)-ethanesulfonate; 5-deazaflavin, 7,8-didemethyl-N^3-methyl-5-deazalumiflavin.

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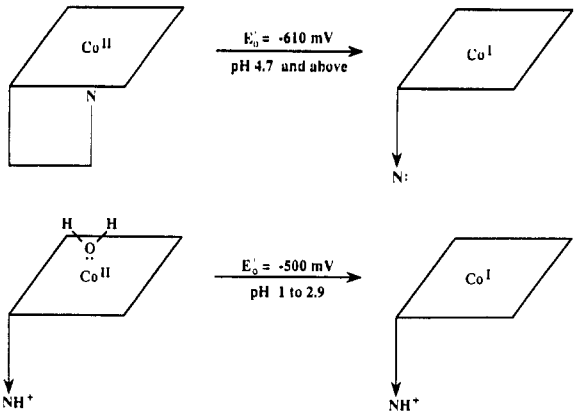
Scheme II: Ligation State and Redox Midpoint Potentials of Cob(II)alamin



These and other studies have led to the formulation of the catalytic mechanism shown in Scheme I. Activation of the enzyme has generally been postulated to proceed via reduction of the enzyme-bound cobalamin to the cob(I)alamin oxidation state with concomitant trapping of this species by methylation with AdoMet. However, it has not been clear why CH_3-H_4folate, which serves as the methyl donor during turnover, can not serve the same role in reductive methylation.

The pH dependence of the midpoint potentials for the cob(II)alamin/cob(I)alamin couple of free cobalamin has been described by Lexa and Saveant (1983). There are two pH-independent regions, where reduction is not associated with changes in protonation. At pH values above 4.7, cob(II)alamin assumes the 5-coordinate base-on conformation as shown in Scheme II, and the midpoint potential in this region is −610 mV versus the standard hydrogen electrode. At pH values between 1 and 2.9, cob(II)alamin is present in the 5-coordinate base-off conformation, with a protonated dimethylbenzimidazole substituent and a presumed water ligand as shown in Scheme II. In this region the midpoint potential is −500 mV.

Harder et al. (1989a) have recently measured the midpoint potentials of two corrinoid proteins from *Clostridium thermoaceticum*. The first, a corrinoid/Fe-S protein, exhibits the UV/visible absorbance and EPR properties of a base-off cob(II)amide and has a midpoint potential of −504 mV, in good agreement with the value measured for free base-off cobalamin. The other, a 27-kDa corrinoid protein, exhibits the properties of a base-on cob(II)amide and has a midpoint potential of −630 mV. Hence, the protein environment in these two proteins does not appear to perturb the midpoint potentials relative to the cofactor free in solution.

The cob(II)alamin/cob(I)alamin potential thus appears to lie somewhat beyond the range of biological reductants, and it remains to be explained how systems such as R/F/NADPH with redox potentials in the range of −350 mV are able to accomplish these difficult reductions. It has been postulated that an endergonic reduction is driven by the exergonic demethylation of the methylsulfonium of AdoMet by cob(I)alamin, a powerful nucleophile (Fujii et al., 1977). Alternatively, the possibility that the cob(II)alamin may react with AdoMet in a homolytic methyl transfer reaction and that the reducing system is required for the subsequent reduction of the AdoHCy radical cation has been proposed by Matthews et al. (1986). Either mechanism would obviate the need for delivery of electrons at very low potentials that are seemingly beyond the realm of biological reductants.

In this study, spectroelectrochemical titrations were employed to determine the potential at which electrons are delivered during reduction of cobalamin bound to methionine synthase. The midpoint potentials of both the cob(II)alamin/cob(I)alamin and cob(III)alamin/cob(II)alamin couples have been determined. Both AdoMet and CH_3-H_4folate have been shown to shift the apparent midpoint potential to more positive values, albeit with vastly different free energy changes accompanying methyl transfer. Our studies support the conclusion that the reductive activation of methionine synthase occurs as a coupled reaction, where a difficult reduction is coupled to a methylation reaction with a large free energy decrease. This mechanism obviates the need for delivering electrons at very low potentials.

Additionally, the current investigation has revealed a solvent effect on the base-on/base-off equilibrium of cobalamin bound to methionine synthase and has led to the discovery of a base-on cob(II)alamin that has a considerably higher midpoint potential for the cob(II)alamin/cob(I)alamin couple than the cofactor free in solution or that bound to the 27-kDa corrinoid protein in *C. thermoaceticum*. Previous EPR studies on the inactive enzyme (Frasca et al., 1988) had shown a base-off cob(II)alamin species. The spectroelectrochemical titrations and their mechanistic significance are discussed.

Experimental Procedures

Materials. The following materials were purchased from Sigma: AdoMet (iodide salt), AdoHCy, and L-homocysteine thiolactone. The following dyes were employed for the titrations: 5,5′-dimethyl-1,1′-trimethylene-2,2′-dipyridinium bromide (TRIQUAT, $E^{\circ\prime}$ = −540 mV), methylviologen ($E^{\circ\prime}$ = −440 mV), benzylviologen ($E^{\circ\prime}$ = −359 mV), anthraquinone-2,6-disulfonate ($E^{\circ\prime}$ = −180 mV), and *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine (TMPD, $E^{\circ\prime}$ = +270 mV). TRIQUAT was synthesized as described by Salmon and Hawkridge (1980), while the other dyes were purchased from Aldrich Chemical Co. and were used without further purification. 5-Deazaflavin was a gift from Dr. Vincent Massey at The University of Michigan.

Enzyme Purification. Recombinant methionine synthase from *E. coli* K-12 was purified and assayed as described previously (Banerjee et al., 1989).

Analytical Techniques. An EPR spectroelectrochemical titrator designed by Harder et al. (1989a) was employed in these studies. In brief, the titration cell requires small volumes (ca. 0.6 mL) of protein solution and permits convenient transfer of the sample between the electrochemical compartment and the EPR tube. Hence, oxygen leakage that would disturb the ambient potential during sample transfer is avoided. The electrochemical cell utilizes a typical three-electrode circuit with Ag/AgCl reference and counterelectrodes and a gold foil working electrode. Concentrated dye solutions (final concentration 500 μM) were added to the cell and degassed by cycling with vacuum and nitrogen for at least 1 h. Nitrogen was deoxygenated by passage over a heated copper catalyst. Under a high positive gas flow, 0.65 mL of methionine synthase in anaerobic buffer (20 mM KP_i/100 mM KCl, pH 7.2, unless mentioned otherwise) was added to the cell with a 1-mL syringe (gas tight, Hamilton) that had been purged with nitrogen. The cell was again cycled several times with vacuum/nitrogen. A potentiostat (ECO 551) was employed to poise the potential below the desired equilibrium potential. Subsequently, the potentiostat was turned off, and the solution was allowed to drift to equilibrium while a voltmeter across the working and reference electrodes measured the ambient potential. When equilibrium had been achieved as evidenced by a stable measured potential, the cell was tipped to transfer the solution to the EPR tube, the solution was then

transferred back to the electrochemical compartment, and the potential was recorded. The sample was then retransferred to the EPR tube and frozen in liquid nitrogen for EPR spectroscopy. EPR spectroscopy was performed on a Varian Model E115 spectrometer with an automatic frequency counter (EIP, Model 548A). The temperature was maintained at 100 K with a variable-temperature controller (Varian) or at 20 K with a Heli-Tran unit and an Air Products temperature controller. The digital readout on the Air Products unit had been calibrated with a gold thermocouple. Spectrometer settings are given in the figure legends. The spin concentrations were determined by double integration of the spectra with reference to a solution of 1 mM copper perchlorate as described by Fee (1978). The concentration of the base-on cob(II)alamin and base-off species were determined by measuring the height of the S-shaped $g = 2.3$ and $g = 2.5$ signals, respectively. The concentration of the cob(I)alamin or cob(III)alamin species was then determined by subtraction from the starting spectrum that corresponded to pure cob(II)alamin. Nernst plots were employed for data analysis using eq 2, where E is the measured potential, $E^{\circ\prime}$ is the equilibrium

$$E = E^{\circ\prime} + (0.059/n)(\log [\mathrm{ox}]/[\mathrm{red}]) \qquad (2)$$

midpoint potential, n is the number of electron equivalents transferred, and [ox]/[red] represents the ratio of the concentration of the oxidized and reduced species, respectively.

Spectrophotometric Analysis of the Reaction of Cob(I)alamin with AdoMet and CH_3-H_4folate. Reduction of methionine synthase by photochemically reduced 5-deazaflavin was accomplished in an anaerobic cuvette as follows. To 25 μM methionine synthase in 20 mM KP_i, pH 7.2, EDTA and 5-deazaflavin were added to 15 mM and 1 μM final concentrations, respectively. AdoMet (1.2 mM) was placed in the side arm, and the cell was made anaerobic by repeated cycles of vacuum and nitrogen. The cell was placed in an ice bath and irradiated with a Sun Gun lamp (650 W, 120 mV) with the voltage set at 80 V with a variable-voltage controller.

To monitor the reaction of cob(I)alamin with CH_3-H_4folate, the former was generated by mixing anaerobic methylated methionine synthase (5 μM) with homocysteine (1 mM). (6R,S)-CH_3-H_4folate (200 μM) was added rapidly, and the reaction was followed by measuring the disappearance of the 390-nm peak associated with cob(I)alamin.

Results

EPR Spectrum of Methionine Synthase. The EPR spectrum of an anaerobic solution of methionine synthase in 20 mM KP_i/100 mM KCl, pH 7.2, revealed the presence of base-on cob(II)alamin (Figure 1B) with a 105-G spacing of the high-field triplets, in contrast to the base-off form of the enzyme (Figure 1A) that had been seen previously. Since several differences existed in the conditions employed in both sample preparation and the spectral acquisition parameters between this and the earlier studies (Frasca et al., 1988), the influence of these variables on the spectral morphology was investigated.

In our earlier studies, methionine synthase had been prepared aerobically in 20 mM KP_i/20% glycerol, pH 7.2 (the buffer routinely employed for long-term storage of the enzyme), and spectra were recorded at 40 K. Under these conditions, the observed EPR spectrum resembled published spectra of base-off cobalamin, cobinamide, and porphyrin complexes containing Co^{2+} [Pilbrow (1982) and references cited therein; de deBolfo et al., 1976; Bayston et al., 1970]. Base-on and base-off cobalamin EPR spectra can be readily distinguished by the pattern and spacing of their high-field

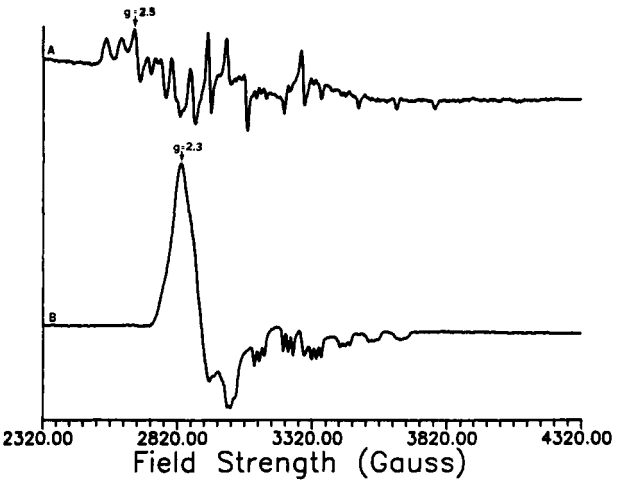


FIGURE 1: Component EPR spectra of methionine synthase in 20% glycerol obtained after deconvolution. EPR spectra of methionine synthase (164 μM) were recorded in 20 mM Tris/100 mM KCl, pH 7.2, containing 20% glycerol. Spectra (single scans) were recorded at 100 K, 40-mW microwave power, and 10-G modulation amplitude with a resolution of 1024 points. A pure base-on spectrum of a sample in 20 mM Tris/100 mM KCl, pH 7.2, was appropriately scaled and subtracted from the mixed spectrum to obtain the base-off component (A). The base-on component is shown in (B).

peaks, which result from the interaction of the unpaired electron in the d_{z^2} orbital with the cobalt nucleus and, in the case of a cobalamin in which the dimethylbenzimidazole substituent is coordinated to the cobalt nucleus in the lower axial position, with the nitrogen of dimethylbenzimidazole. Interaction of the electron with the cobalt nucleus (nuclear spin = $^7/_2$) results in hyperfine splitting to produce eight equally spaced lines. Superhyperfine splittings by the coordinated dimethylbenzimidazole result in further splitting of each of the eight lines into triplets. Thus, the base-on cobalamins show spectra with a series of high-field triplets with ca. 105-G spacing, while base-off cobalamins and cobinamides show spectra with a series of high-field singlets with ca. 140–160-G spacing (Bayston et al., 1970; Pilbrow, 1982).

In contrast to the present studies, which were under anaerobic conditions, previous studies were performed with aerobic solutions, and a variable amount of the cob(III)alamin superoxide complex was seen. Since the enzyme is purified aerobically, it is not surprising that the superoxo species is observed. Rather, it is remarkable that the enzyme so shelters the cob(II)alamin prosthetic group from oxidation that any cob(II)alamin enzyme is detected after aerobic isolation.

The overall spectral morphology of the enzyme (in 20 mM KP_i/100 mM KCl, pH 7.2) observed in the present study was found to be independent of temperature with the same $g = 2.3$ feature and high-field triplets seen at temperatures of 20 and 100 K. The presence or absence of salt (100 mM KCl in 20 mM KP_i, pH 7.2), the pH (5.5 in MES buffer or 7.2 in KP_i buffer), the enzyme concentration (over a 10-fold range in phosphate buffer), and the choice of the buffer (Tris, KP_i, MES, and bicine) had no influence on the base-on spectrum. Addition of air to the anaerobic samples resulted in rapid conversion to the superoxo species with no observable base-off intermediates. Formation of the superoxo species is reversible, as evidenced by its disappearance under anaerobic conditions, consistent with the reversible association of oxygen with cob(II)alamin reported by Hoffman et al. (1970).

Addition of glycerol (from 20% to 80% final concentration in anaerobic 20 mM Tris-HCl, pH 7.2) resulted in the appearance of the base-off species (Figure 1), and the relative concentrations of base-on and base-off forms were dependent

Table I: Relative Concentrations and the Midpoint Potentials of Base-On and Base-Off Cobalamin in Buffers Containing Different Glycerol Concentrations

	cobalamin		$E^{\circ\prime}$ (mV)		slope (mV)	
buffer[a] (% glycerol)	% base-on	% base-off	base-on	base-off	base-on	base-off
0	100		−541 ± 10		70 ± 7	
20	67	33	−542 ± 10	−576 ± 23	120 ± 12	121 ± 19
50		100		−572 ± 24		123 ± 7

[a] Buffer employed in this set of experiments was 20 mM Tris-HCl, pH 7.2, containing 100 mM KCl, and with the indicated amount of glycerol.

on the glycerol concentration (Table I). It is not clear how the glycerol concentration affects the equilibrium distribution of the two conformers; it may cause a decrease in the dielectric constant of the corrin environment and/or effect the solvent replacement of the dimethylbenzimidazole ligand. The ability of the methionine synthase bound cobalamin to be in either conformational state is, to our knowledge, unique and affords the attractive opportunity to measure the redox potentials of both base-on and base-off cobalamin bound to the same protein.

Reduction of Cob(II)alamin. Methylviologen and TRIQUAT were used as redox mediators in the titration of the cob(II)alamin/cob(I)alamin redox couple with 175 μM methionine synthase in 20 mM KP_i/100 mM KCl, pH 7.2. The initial concentration of the enzyme obtained by intergration of the EPR spectrum at an ambient potential of ca. −50 mV was 0.99 spin/mol of cobalt and therefore in excellent agreement with the enzyme concentration calculated from the absorbance spectrum. The reduction was monitored by the disappearance of the cob(II)alamin signal, since cob(I)alamin is diamagnetic. Analysis of the data by the Nerst equation gave an $E^{\circ\prime}$ of −526 ± 5 mV as shown in Figure 2. The slope of this plot is 70 ± 4 mV, indicating that approximately one electron equivalent is transferred. The reduction was found to be completely reversible.

The midpoint potential of the cob(II)alamin/cob(I)alamin couple was also measured in 20 mM bicine/100 mM KCl, pH 7.2, in order to establish whether the measured redox potential was a function of the buffer employed. On freezing a 10 mM KP_i solution to liquid nitrogen temperature, a ΔpH of −1.2 has been measured. The ΔpH for bicine recorded under the same conditions is only −0.1, and bicine is therefore one of the ideal buffers for low-temperature EPR titrations (Williams-Smith et al., 1977). The midpoint potential was found to be −517 ± 5 mV, which is within the limit of error of that determined in phosphate buffer.

Reduction of Cob(II)alamin at Different Glycerol Concentrations. The results from the reduction of enzyme-bound cob(II)alamin in buffered solutions containing different glycerol concentrations are summarized in Table I. The midpoint potential of the base-on cobalamin was unaffected by the presence of base-off cobalamin and vice versa.

The basis for the observed deviation from the expected 59-mV slope in the titrations performed in glycerol is not understood. In this solvent, slopes of 120 mV, which correspond to transfer of 0.5 electron equivalent to cobalt, were observed. Similar deviations from expected slopes were also seen in titrations in the presence of CH_3-H_4folate (see below), even though these were conducted in the absence of glycerol. Reduction was completely reversible in these experiments, ruling out the possibility that the system was not at equilibrium when the measurements were made. Typically, the potential of the sample was measured 1 h after the potentiostat had been set at the desired potential. Under these conditions, generally a 2–5-mV drift in the measured potential was observed before and after the EPR spectra were recorded. The enzyme is

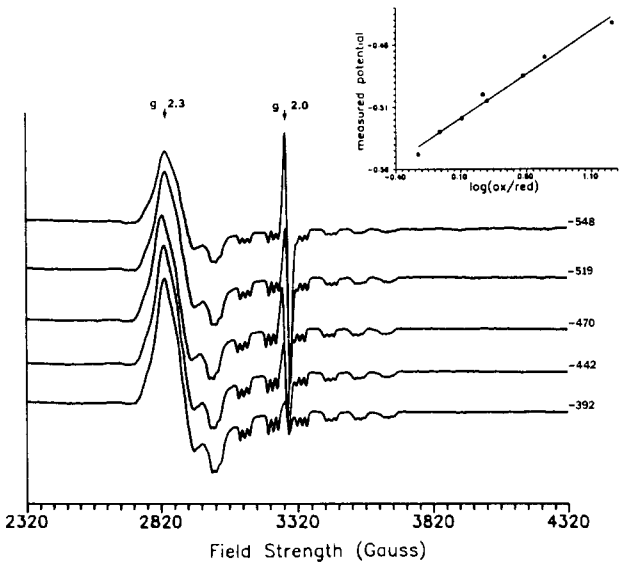


FIGURE 2: Determination of the cob(II)alamin/cob(I)alamin midpoint potential by EPR spectroelectrochemistry. Titration was performed with methionine synthase (175 μM) in 20 mM KP_i/100 mM KCl, pH 7.2, and spectra were recorded under conditions described in Figure 1. The $g = 2$ signal is due to dye radical. The measured potentials are indicated alongside the spectra. Inset: Nernst plot of the EPR titration.

monomeric in dilute solution, but reversible dimerization of the reduced protein may be responsible for the observed steep slopes (Clark, 1960).

Reduction of Cob(II)alamin in the Presence of AdoMet. Since the same midpoint potentials were measured in both bicine and KP_i buffers, further experiments were performed in KP_i, which is routinely used during purification and for assaying the enzyme. To investigate the reactivity of enzyme-bound cob(I)alamin toward AdoMet and to assess whether methylation by AdoMet is reversible, 1.2 mM AdoMet was added to 100 μM methionine synthase in a cell containing the enzyme, anthraquinone-2,6-disulfonate, benzylviologen, and methylviologen. The potential was poised at −220 mV, which is approximately 300 mV more positive than the midpoint potential of the cob(II)alamin/cob(I)alamin couple. The solution rapidly turned from orange-brown to red ($t_{1/2}$ <20 min). This color change was accompanied by a disappearance of the cob(II)alamin EPR signal. The reaction was apparently irreversible on the time scale of this experiment as poising at more positive potentials did not result in the return of the cob(II)alamin EPR signal. The UV/visible absorption spectrum of this sample was consistent with the presence of methylcobalamin and showed peaks at 525 and 374 nm. Furthermore, addition of homocysteine to this enzyme resulted in conversion to cob(I)alamin.

A time course for the methylation reaction was studied at a poised potential of −82 mV (Figure 3) and yielded an approximate $t_{1/2}$ of 225 min for the disappearance of the cob(II)alamin signal. At −82 mV, which is 444 mV more positive than the $E^{\circ\prime}$ determined in the absence of the AdoMet, the

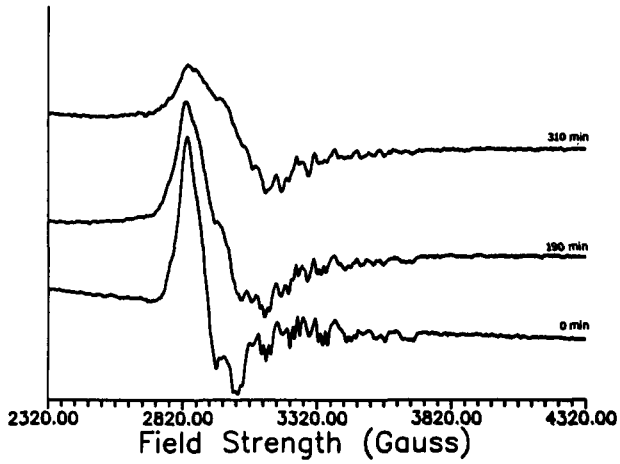


FIGURE 3: Disappearance of the cob(II)alamin spectrum with time at −82 mV, in the presence of AdoMet. 400 μM AdoMet was added to 100 μM methionine synthase in 20 mM KP_i/100 mM KCl, pH 7.2, and the potential was poised at −82 mV. Spectra were recorded under conditions described in Figure 1.

equilibrium distribution of the oxidized and reduced species is predicted to be 3×10^7 in favor of cob(II)alamin and yields an estimate of the $\Delta G^{\circ\prime}$ for transmethylation of >−9 kcal/mol. From these data, the bimolecular rate constant for the reaction of AdoMet with the enzyme-bound cob(I)alamin was estimated to be approximately 1×10^6 M^{-1} s^{-1}.

Reduction of Cob(II)alamin in the Presence of CH_3-H_4-folate. In an attempt to understand the basis for the inability of CH_3-H_4folate to serve as a methyl donor in the various in vitro activation systems that are employed, the effect of CH_3-H_4folate on the midpoint potential of the cob(II)alamin/cob(I)alamin couple was examined; 1.6 mM (6*R*,*S*)-CH_3-H_4folate was added to 100 μM enzyme, and reduction was monitored in the presence of benzylviologen, methylviologen, and TRIQUAT. Unlike the reduction in the presence of AdoMet, titration in the presence of CH_3-H_4folate was reversible, indicating that an equilibrium between cob(II)alamin, cob(I)alamin, and methylcobalamin is established. These results are completely consistent with the observation that H_4folate is able to demethylate methylcobalamin (Stavrianopoulos & Jaenicke, 1967; Taylor & Weissbach, 1969a,b; Banerjee, Ballou, and Matthews, unpublished results). Analysis of the data by the Nernst equation gave a midpoint potential of −450 ± 13 mV and a slope of 121 ± 10 mV (corresponding to transfer of 0.5 electron equivalent to cobalt). Hence, CH_3-H_4folate shifts the equilibrium between the oxidized and reduced forms of cobalamin by a factor of 19 and yields an estimate of $\Delta G^{\circ\prime}$ of −0.09 kcal/mol.

Reaction of AdoMet and CH_3-H_4folate with Cob(I)alamin. Methionine synthase was subjected to reduction by photochemically reduced 5-deazaflavin in an anaerobic cuvet. Maximal reduction of the enzyme-bound cob(II)alamin was seen after 30 min of irradiation and resulted in a mixture of cob(II)alamin (ca. 70%, λ_{max} = 470 nm) and cob(I)alamin (ca. 30%, λ_{max} = 390 nm). AdoMet was then tipped into the anaerobic solution from a side arm. As can be seen in the difference spectrum (Figure 4), only cob(I)alamin disappears on addition of AdoMet, and methylcobalamin is formed (λ_{max} = 525 nm; Frasca et al., 1988). This clearly demonstrates the ability of enzyme-bound cob(I)alamin to react with AdoMet. Similarly, reaction of CH_3-H_4folate with cob(I)alamin resulted in the formation of methylcobalamin (data not shown).

Oxidation of Cob(II)alamin. Oxidation of cob(II)alamin to cob(III)alamin was also monitored by EPR spectroelectrochemistry, by following the conversion of the paramagnetic

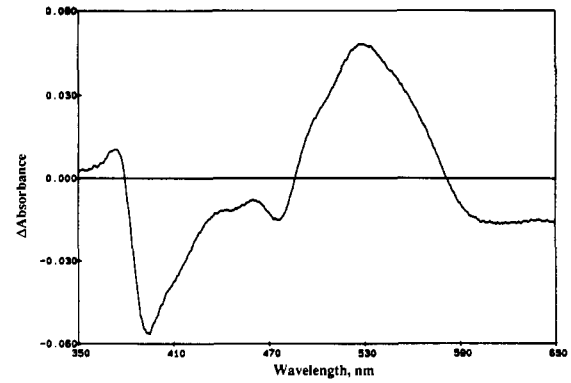


FIGURE 4: Difference spectrum showing conversion of methionine synthase bound cob(I)alamin to methylcobalamin. The spectrum of methionine synthase (partially reduced by 5-deazaflavin) before addition of AdoMet was subtracted from the spectrum obtained after addition of AdoMet to a mixture of cob(I)alamin and cob(II)alamin. Absorbance is given in optical density units on the *Y* axis.

cob(II)alamin to diamagnetic cob(III)alamin. TMPD was employed to mediate reduction of 147 μM methionine synthase in this titration. From the Nernst plot the oxidation–reduction potential was calculated to be +273 ± 4 mV. The slope of the plot was 59 ± 5 mV. This titration was repeated in 20 mM bicine/100 mM KCl, pH 7.2, and the same value for the midpoint potential was obtained.

The effect of AdoMet binding on cob(II)alamin oxidation was examined by addition of 600 μM AdoMet to 100 μM enzyme. The $E^{\circ\prime}$ of the cob(III)alamin/cob(II)alamin couple in the presence of AdoMet was found to be +272 ± 5 mV, and it was concluded that AdoMet binding does not perturb the midpoint potential of this couple.

Discussion

The UV/visible and absorbance EPR spectra of methionine synthase as isolated are characteristic of cob(II)alamin. The paramagnetic nature of cob(II)alamin permits convenient EPR spectroelectrochemical titration studies, as both the +1 and +3 states are diamagnetic. Hence, oxidation or reduction of cob(II)alamin can be quantitated easily by the diminution of the EPR signal.

In this study, a base-on cob(II)alamin EPR signal of the enzyme-bound prosthetic group was observed, whereas in an earlier study a base-off cob(II)alamin signal was seen (Frasca et al., 1988). The choice of a buffer containing 20% glycerol and the aerobic conditions employed in the previous study appear to have contributed to the observation of the base-off and superoxo species. Lexa and Saveant (1977) have reported that a 440-nm shoulder is associated with the electronic absorbance spectra of base-on form but not with the base-off form of free cob(II)alamin. Although such a shoulder *is* observed in the visible spectrum of methionine synthase as isolated, it is quite a subtle feature that may just as well be related to perturbations induced when cobalamin is bound to the enzyme as to the coordination state of dimethylbenzimidazole. It is difficult to distinguish exclusively on the basis of the visible spectrum between the base-on and base-off conformers.

The room temperature EPR spectrum of the enzyme (in 20 mM KP_i/10 mM KCl, pH 7.2), though poorly resolved, is of the base-on species (data not shown). The current investigation strongly suggests that the physiological or mechanistically relevant form of the coenzyme is the base-on conformation.

The finding that the enzyme stabilizes base-off cob(II)alamin, making it more difficult to reduce than the base-on state of cob(II)alamin, on the enzyme is a surprising one. This

contrasts with the measurements made for the midpoint potentials of the cob(II)alamin/cob(I)alamin couple in solution (Lexa & Saveant 1976) where the base-off cobalamin has a midpoint potential that is 110 mV more positive than the base-on form. While base-off cob(II)alamin was presumed to be 5-coordinate by Lexa and Saveant (1976), evidence for a pentacoordinate Co(II) center has been provided by Kräutler et al. (1987). They have determined the crystal structure of perchloratoheptamethylcob(II)yrinate, and found that an axially bound perchlorate occupies the fifth position. In aqueous solution base-off cob(II)alamin is presumed to have a water molecule as the fifth ligand. Substitution of this water ligand by dimethylbenzimidazole is expected to render the reduction more difficult due to the more strongly electron-donating character of dimethylbenzimidazole. The net stabilization of base-off cobalamin seen on methionine synthase corresponds to a $\Delta G^{\circ\prime}$ of −3.23 kcal/mol. It is conceivable that the glycerol-induced conformational change brings a strongly electron-donating group in the proximity of the reducing center and thereby renders reduction more difficult than that of the base-on cobalamin.

Alternatively, the solvent-induced conformational change to the base-off form may result from replacement of the nitrogen of dimethylbenzimidazole by a protein ligand that has no nuclear spin (such as oxygen or sulfur) and is therefore undetectable by EPR. Candidate ligands that might be more electron donating than dimethylbenzimidazole include cysteinyl, glutamyl, and aspartyl residues on the protein.

The observation that the two species in 20% glycerol titrate independently and have midpoint potentials that are identical with those determined under conditions where each is present as the only species suggests that the two are not in equilibrium in the time scale of these experiments. If the two species were in rapid equilibrium, at any given potential more of the base-on species would be reduced relative to the base-off form, and this would lead to a reequilibration of the remaining oxidized enzyme. This would result in an apparent $E^{\circ\prime}$ for the base-off species in 20% glycerol that would be more positive than that measured in 50% glycerol, where only the base-off form is present. This is clearly not observed. These results also argue against the base-off form seen in these titrations being an obligatory intermediate on the pathway for reduction of base-on cob(II)alamin on methionine synthase.

Conversion between the base-on and base-off coordination states of cob(II)alamin appears to be very accessible on methionine synthase. Hogenkamp (1968) first suggested an accessible base-off conformation for propylated holoenzyme on the basis of the observed shift of the visible absorbance maximum to shorter wavelengths associated with propylation (Taylor & Weissbach, 1967b). In addition, Fujii and Huennekens (1979) reported a temperature-dependent shift of the methylated holoenzyme (methylated by CH_3-H_4folate), in which the 520-nm peak seen at 18 °C moved to 455 nm at 40 °C. These changes are consistent with a shift in equilibrium between base-on and base-off conformers as observed in our studies.

Measurements of the cob(II)alamin/cob(I)alamin midpoint potentials of both free and enzyme-bound cobalamin have yielded values in the range of −610 mV for the base-on species and −500 mV for the base-off species (Lexa & Saveant, 1976). Base-on cob(I)alamin of methionine synthase with an $E^{\circ\prime}$ of −526 mV for the cob(II)alamin/cob(I)alamin couple is therefore somewhat stabilized by interaction with the protein. On the basis of the similarity in the measured redox potentials of cobamides bound to two corrinoid proteins to the potentials measured for the cofactor free in solution, it was contended by Harder et al. (1989a) that the midpoint potential of the cobalamin redox couples is modulated primarily by the coordination of cob(II)alamin with the benzimidazole base. Electron donation by the nitrogen of dimethylbenzimidazole renders reduction of cob(II)alamin less favorable and drives the midpoint potential lower by ca. 125 mV. In the case of methionine synthase, the protein environment clearly plays a role in altering the midpoint potential and therefore facilitating reduction of base-on cob(II)alamin. Such a situation is not without precedent. The flavoproteins constitute just such a family of proteins where bound flavin coenzymes exhibit a wide range of equilibrium redox potentials.

Our studies with the coupled reduction/methylation reactions clarify the absolute requirement for AdoMet as a methyl donor in the in vitro activation systems that are employed. As discussed before, the driving force for the seemingly difficult reduction of methionine synthase bound cob(II)alamin has been postulated to come from the covalent capture of the reactive cob(I)alamin intermediate by AdoMet (Fujii & Huennekens, 1977). The free energy change for the methylation of homocysteine by AdoMet can be calculated from the measured enthalpy changes accompanying methyl group transfer from AdoMet and other sulfonium compounds. The enthalpy changes accompanying methyl group transfer from *S*-methylmethionine and AdoMet to the common acceptor, homocysteine, are −6.7 and −13.2 kcal/mol, respectively (Mudd et al., 1966). Hence, AdoMet is destabilized by −6.5 kcal/mol with respect to methylmethionine. If one assumes that there is zero entropy change in methyl transfer from methylsulfonium of AdoMet to methionine, the relative free energy of hydrolysis of AdoMet can be obtained from the $\Delta G^{\circ\prime}$ for the transfer of the methyl group from methylmethionine to homocysteine, which is −10.5 kcal/mol [Durell et al., 1962; corrected by Mudd et al. (1966)]. Hence, the estimated $\Delta G^{\circ\prime}$ for the methylation of homocysteine by AdoMet is −17 kcal/mol (Fujii & Huennekens, 1979). Since the carbon–cobalt bond of the methylcobalamin is significantly weaker than the carbon–sulfur bond of methionine, the $\Delta G^{\circ\prime}$ associated with the demethylation of AdoMet by cob(I)alamin must be correspondingly smaller.

The free energy decrease associated with the demethylation of the methylsulfonium of AdoMet by cob(I)alamin can shift the equilibrium of the redox couple by a factor of at least 3×10^7. Hence, the reduction of cob(II)alamin in the presence of AdoMet is expected to be very facile in the redox range available with the R/F/NADPH reducing system, i.e., ca. −350 mV. The observation that the rate of AdoMet-dependent methylation is dependent on the redox potential is consistent with an electron transfer preceding methylation and suggests that cob(I)alamin is the species that reacts with AdoMet. In addition, we have demonstrated spectrophotometrically the ability of enzyme-bound cob(I)alamin (generated by photochemical reduction) to rapidly demethylate AdoMet. It is reasonable to assume then that reaction of AdoMet with the enzyme that is reduced either photochemically or electrochemically involves cob(I)alamin, even though our electrochemical studies do not exclude the possibility that the redox potential dependence of the methylation rate may be due to inefficient dye mediation at high potentials. The methylation of cob(I)alamin by AdoMet has an equilibrium that strongly favors products, and the reaction is therefore effectively irreversible. Thus, AdoMet appears to facilitate the reduction by trapping any cob(I)alamin that is formed as methylcobalamin.

CH_3-H_4folate by comparison shifts the equilibrium of the redox couple by a factor of 19, so that the apparent midpoint potential of the cob(II)alamin/cob(I)alamin couple is around −450 mV. The requirement for AdoMet in the in vitro assay system is therefore thermodynamic, since both methyl donors are able to react with the same intermediate, namely, cob(I)alamin.

It should be borne in mind that the activation step is only occasionally required during turnover. Once primed, the enzyme shuttles between the methylcobalamin and cob(I)alamin oxidation states as depicted in Scheme I. It is the occasional cob(II)alamin formed by adventitious oxidation of the reactive cob(I)alamin intermediate that would require the activation step. The frequency of such accidental oxidations in vivo is not known. The rate of consumption of reduced pyridine nucleotide and AdoMet is only about 1% of the rate of formation of methionine under in vitro steady-state turnover conditions (Foster et al., 1964; Stavrianopoulos & Jaenicke, 1967; Fujii et al., 1977).

A question that these studies raise is how the enzyme occludes AdoMet as the methyl donor during turnover. One possibility is that the enzyme forms a ternary complex during catalysis and that CH_3-H_4folate binding is favored in such a complex. Results from both steady-state and pre-steady-state kinetic studies support the participation of a ternary complex (Banerjee, Ballou, and Matthews, unpublished results). Alternatively, CH_3-H_4folate may have a much higher affinity for the methylated form of the enzyme (which is presumably its resting state) than AdoMet.

In conclusion, these studies demonstrate how methionine synthase has alleviated the barrier to reduction at a low redox potential by coupling the reduction to a methylation reaction with a large free energy decrease. Such coupled reductions may well be employed by other enzymatic systems to "ratchet down" the potential of redox sinks. Two examples where such a mechanism is plausible are nitrogenase and pyruvate formate-lyase, both of which require delivery of electrons at fairly low potentials. Reduction of the molybdenum–iron protein of nitrogenase by the iron protein requires ATP cleavage (Eady et al., 1978), whereas reductive activation of pyruvate formate-lyase is accompanied by a stoichiometric cleavage of AdoMet into methionine and 5′-deoxyadenosine (Knappe & Blaschkowski, 1975).

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REFERENCES

Banerjee, R. V., Frasca, V., Johnston, N. L., Ballou, D. P., Datta, P., & Matthews, R. G. (1988) *Biochemistry 27*, 3101.

Banerjee, R. V., Johnston, N. L., Sobeski, J. K., Datta, P., & Matthews, R. G. (1989) *J. Biol. Chem. 264*, 13888–13895.

Bayston, J. H., King, N. K., Looney, F. D., & Winfield, M. E. (1970) *Biochemistry 9*, 2162–2172.

Clark, W. M. (1960) in *Oxidation-Reduction Potentials of Organic Systems*, pp 163–183, Williams & Wilkins, Baltimore, MD.

deBolfo, J. A., Smith, T. P., Boas, J. F., & Pilbrow, J. R. (1976) *J. Chem. Soc., Dalton Trans.*, 1495–1500.

Durell, J., Rawitscher, M., & Sturtevant, J. M. (1962) *Biochim. Biophys. Acta 56*, 552–558.

Eady, R. R., Lowe, D. J., & Thorneley, R. N. (1978) *FEBS Lett. 95*, 211–213.

Fee, J. A. (1978) *Methods Enzymol. 49*, 512–528.

Foster, M. A., Dilworth, M. J., & Woods, D. D. (1964) *Nature (London) 201*, 39–42.

Frasca, V., Banerjee, R. V., Dunham, W. R., Sands, R. H., & Matthews, R. G. (1988) *Biochemistry 27*, 8458–8465.

Fujii, K., & Huennekens, F. M. (1974) *J. Biol. Chem. 249*, 6745–6753.

Fujii, K., & Huennekens, F. M. (1979) in *Biochemical Aspects of Nutrition* (Yagi, K., Ed.) pp 173–183, Japan Scientific Societies, Tokyo.

Fujii, K., Galivan, J. H., & Huennekens, F. M. (1977) *Arch. Biochem. Biophys. 178*, 662–670.

Guest, J. R., Friedman, S., & Foster, M. A. (1962) *Biochem. J. 84*, 93P–94P.

Harder, S. R., Lu, W.-P., Feinberg, B. A., & Ragsdale, S. W. (1989a) *Biochemistry 28*, 9080–9087.

Harder, S. R., Feinberg, B. A., & Ragsdale, S. W. (1989b) *Anal. Biochem. 181*, 283–287.

Hoffman, B. M., Diemente, D. L., & Basolo, F. (1970) *J. Am. Chem. Soc. 92*, 61–65.

Hogenkamp, H. P. C. (1968) *Annu. Rev. Biochem. 37*, 225–248.

Knappe, J., & Blaschkowski, H. P. (1975) *Methods Enzymol. 41*, 508–518.

Kräutler, B., Keller, W., Hughes, M., Caderas, C., & Kratky, C. (1987) *J. Chem. Soc., Chem. Commun.*, 1678–1680.

Lexa, D., & Saveant, J.-M. (1976) *J. Am. Chem. Soc. 98*, 2652–2658.

Lexa, D., & Saveant, J.-M. (1983) *Acc. Chem. Res. 16*, 235–243.

Lexa, D., Saveant, J.-M., & Zickler, J. (1977) *J. Am. Chem. Soc. 99*, 2786–2790.

Mangum, J. H., & Scrimgeour, K. G. (1962) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 21*, 242.

Matthews, R. G., Jencks, D. A., Frasca, V., & Matthews, K. D. (1986) in *Chemistry and Biology of Pteridines* (Cooper, B. A., & Whitehead, V. M., Eds.) Walter de Gruyter and Co., Berlin.

Mudd, S. H., Klee, W. A., & Ross, P. D. (1966) *Biochemistry 5*, 1653–1660.

Pilbrow, J. R. (1982) in B_{12} (Dolphin, D., Ed.) Vol. 1, pp 432–462, Wiley-Interscience, New York.

Salmon, R. T., & Hawkridge, F. M. (1980) *J. Electroanal. Chem. 112*, 253–264.

Stavrianopoulos, J., & Jaenicke, L. (1967) *Eur. J. Biochem. 3*, 95–106.

Taylor, R. T., & Weissbach, H. (1967a) *J. Biol. Chem. 242*, 1502–1508.

Taylor, R. T., & Weissbach, H. (1967b) *J. Biol. Chem. 242*, 1509–1516.

Taylor, R. T., & Weissbach, H. (1969a) *Arch. Biochem. Biophys. 129*, 728–744.

Taylor, R. T., & Weissbach, H. (1969b) *Arch. Biochem. Biophys. 129*, 745–766.

Vetter, H., & Knappe, J. (1971) *Z. Physiol. Chem. 352*, 433–446.

Williams-Smith, D. L., Bray, R. C., Barber, M. J., Tsopanakis, A. D., & Vincent, S. P. (1977) *Biochem. J. 167*, 593–600.