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A Protein Radical Cage Slows Photolysis of Methylcobalamin in Methionine Synthase from *Escherichia coli*

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Abstract—Methionine synthase from *Escherichia coli* is a B_{12}-dependent enzyme that utilizes a methylcobalamin prosthetic group. In the catalytic cycle, the methyl group of methylcobalamin is transferred to homocysteine, generating methionine and cob(I)-alamin, and cob(I)alamin is then remethylated by a methyl group from methyltetrahydrofolate. Methionine synthase occasionally undergoes side reactions that produce the inactive cob(II)alamin form of the enzyme. One such reaction is photolytic homolysis of the methylcobalamin C—Co bond. Binding to the methionine synthase apoenzyme protects the methylcobalamin cofactor against photolysis, decreasing the rate of this reaction by ≈50-fold. The X-ray structure of the cobalamin-binding region of methionine synthase suggests how the protein might protect the methylcobalamin cofactor in the resting enzyme. In particular, the upper face (methyl or β face) of the cobalamin cofactor is in contact with several hydrophobic residues provided by an α-helical domain, and these residues could slow photolysis by caging the methyl radical and favoring recombination of the CH_3•/cob(II)alamin radical pair. We have introduced mutations at three positions in the cap domain; phenylalanine 708, phenyl-alanine 714, and leucine 715 have each been replaced by alanine. Calculations based on the wild-type structure predict that two of these three mutations (Phe708Ala and Leu715Ala) will increase solvent accessibility to the methylcobalamin cofactor, and in fact these mutations result in dramatic increases in the rate of photolysis. The third mutation, Phe714Ala, is not predicted to increase the accessibility of the cofactor and has only a modest effect on the photolysis rate of the enzyme. These results confirm that the α-helical domain covers the cofactor in the resting methylcobalamin enzyme and that residues from this domain can protect the enzyme against photolysis. Further, we show that binding the substrate methyltetrahydrofolate to the wild-type enzyme results in a saturable increase in the rate of photolysis, suggesting that substrate binding induces a conformational change in the protein that increases the accessibility of the methylcobalamin cofactor. Copyright © 1996 Elsevier Science Ltd

Introduction

Vitamin B_{12}-dependent methionine synthase from *Escherichia coli* contains a methylcobalamin prosthetic group. The enzyme alternately catalyzes the transfer of a methyl group from methylcobalamin to homocysteine and from methyltetrahydrofolate (CH_3-H_4folate) to cob(I)alamin; these methyl transfers involve heterolytic cleavage and reformation of the C—Co bond (Fig. 1).[1,2] The cobalamin cofactor is prone to various side reactions, including oxidation of the cob(I)alamin (B_{12s}) cofactor and photolysis of the methylcobalamin cofactor, both generating the inactive cob(II)alamin (B_{12r}) form of the enzyme. Reactivation of the cob(II)alamin enzyme involves a reductive methylation that uses an electron from reduced *E. coli* flavodoxin and a methyl group from *S*-adenosylmethionine (AdoMet) to regenerate the methylcobalamin cofactor.[3,4] This reactivation reaction is slow relative to catalytic turnover of the enzyme, and conditions that generate increased levels of cob(II)alamin enzyme will lead to marked decreases in the net rate of methionine production. Thus the enzyme may have evolved to limit cob(II)alamin formation and to stabilize the active cob(I)alamin and methylcobalamin forms of the enzyme.

The crystal structure of a 27 kDa cobalamin-binding fragment of *E. coli* methionine synthase has recently been solved.[5] In this structure, the corrin ring system of the cobalamin cofactor is sandwiched between two protein domains (Fig. 2). An α/β (Rossmann) domain below the cofactor contributes a histidine ligand to the lower axial position of the cobalt and binds the dimethylbenzimidazole substituent of the cofactor deep within a hydrophobic pocket. The histidine ligand provides a means by which the protein can control the reactivity of the cobalt, and we have found that this ligand promotes heterolysis and inhibits photolytic homolysis of the C—Co bond.[6] An α-helical domain contributes residues that shield the upper β face of the cobalamin. This domain forms a 'cap' over the corrin ring and may allow the protein to control access to and from the reactive methyl group of the cofactor.[5,7] By

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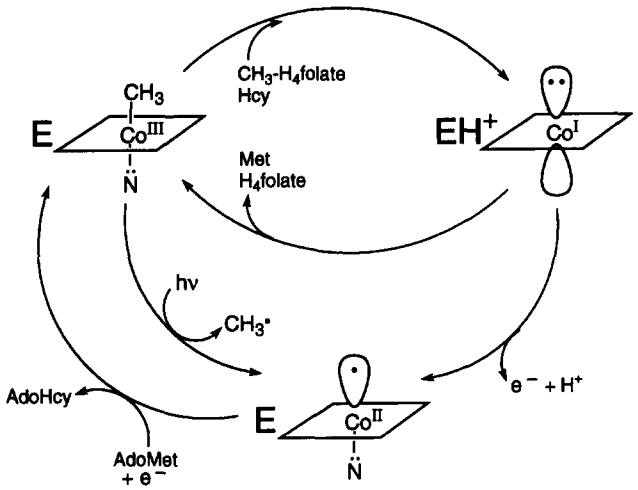

Figure 1. Reaction scheme for turnover and reactivation of methionine synthase. Methylcobalamin enzyme binds homocysteine (Hcy) and CH_3-H_4folate in a ternary complex prior to reacting with Hcy to generate the cob(I)alamin intermediate. This intermediate reacts with CH_3-H_4folate to regenerate the methylcobalamin form of the enzyme prior to product release. Oxidation of cob(I)alamin and photolysis of methylcobalamin enzyme both lead to formation of the inactive cob(II)alamin enzyme. Reactivation of cob(II)alamin enzyme requires a reductive methylation which utilizes AdoMet as methyl donor and reduced *E. coli* flavodoxin as electron donor.

controlling access to the cofactor, the protein may prevent deleterious side reactions, such as reaction with adventitious nucleophiles or homolytic loss of the methyl group.

Alkylcobalamins are subject to photolytic homolysis of the carbon–cobalt bond upon exposure to visible light; this process has received considerable attention as a potential model for homolytic bond cleavage in adenosylcobalamin-dependent enzymes.[8–18] Free methylcobalamin undergoes slow photolysis under anaerobic conditions yielding cob(II)alamin, methane, and ethane, while under aerobic conditions the reaction is more rapid and yields aquocob(III)alamin and formaldehyde.[11,16] The photolysis process is complex and the observed macroscopic rate depends upon a number of competing chemical and physical events (Fig. 3). Absorption of ultraviolet or visible light[13,18] leads to the formation of an excited state methylcobalamin species[13,17] which can partition between relaxation through vibrational modes to the ground state (k_{-1}) or homolytic bond cleavage (k_2).[19] Alkylcobalamins are not luminescent, and therefore fluorescence and phosphorescence do not contribute significantly to the relaxation of the excited state.[19] Homolytic bond cleavage results in a CH_3•/cob(II)alamin radical pair surrounded by a cage of solvent molecules that inhibits diffusive separation of the radical pair (k_4) and, therefore, promotes recombination (k_{-2}).[19] While the radical pair is initially born in the singlet spin state, hyperfine coupling with the cobalt nucleus results in rapid intersystem crossover (k_3), leading to the formation of the triplet spin state before significant geminate recombination can occur.[12] The triplet radical pair cannot recombine unless a second intersystem crossover event (k_{-3}) occurs before the radical pair diffuses apart (k_4).[12] In addition, the initially tetrahedral methyl group flattens to a planar methyl radical, and this structural perturbation slows the rate of recombination.[12] In flash photolysis experiments, methylcobalamin is reformed from the triplet spin state radical pair at a rate of $\approx 2 \times 10^4\ s^{-1}$.[12] If the methyl radical diffuses out of the radical cage, it may eventually recollide with a cob(II)alamin radical (k_{-4}), potentially leading to reformation of methylcobalamin. Under some conditions, however, one or both of the radicals is intercepted (k_5) at the diffusion limited rate by oxygen or another radical quenching agent (e.g. thiols, nitroxides, and some organic solvents).[19] In the presence of an excess of quenching agent, the quantum yield (Φ=[alkyl radical trapped]/[photons absorbed]) is in the range of 0.3–0.5[8,9,18] and is relatively wavelength independent above a threshold energy corresponding to $\approx$550–600 nm.[13,18] In the absence of oxygen or other quenching agents, the quantum yield is much lower, with values in the order of 0.01 to 0.1,[8,9] suggesting that under these conditions, recombination of the methyl radical with the persistent cob(II)alamin radical effect-

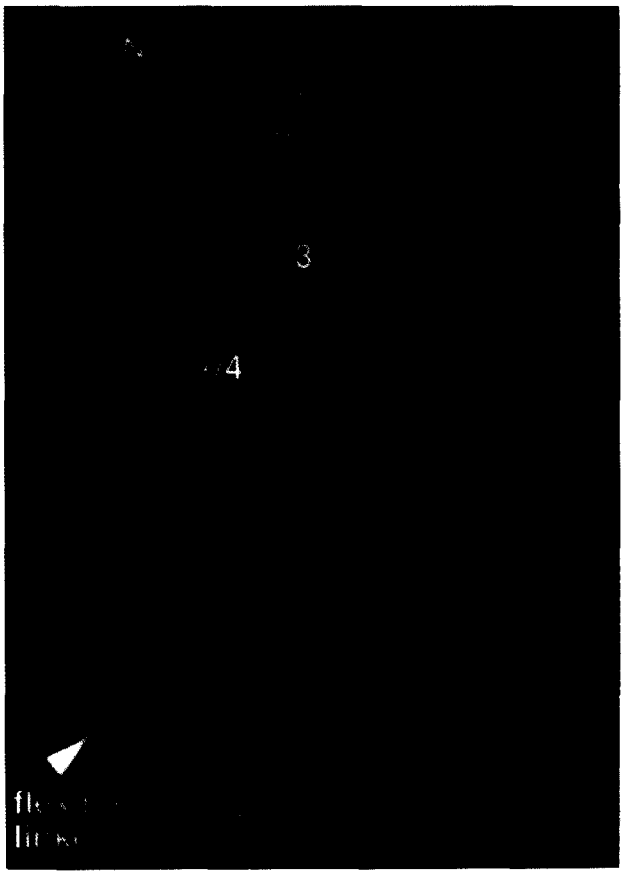

Figure 2. A ribbon depiction of the 27 kDa cobalamin-binding domain of methionine synthase. The corrin ring system of the methylcobalamin cofactor is bound between a lower α/β domain that contributes a histidyl ligand to the cobalt and an α-helical cap domain. The N-terminal α-helical domain is connected to the C-terminal α/β domain by a flexible peptide linker. Helices α3 and α4 and the intervening loop contain residues that contact the upper face of the cobalamin.[5]

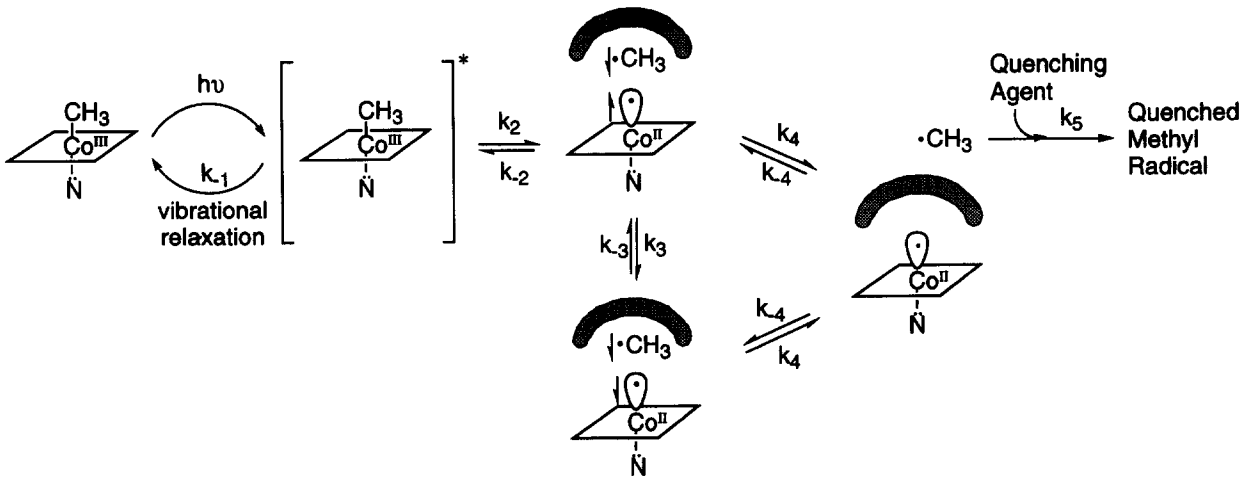

Figure 3. Reaction scheme for photolysis of methylcobalamin. Absorption of visible light (hν) leads to formation of an excited state that relaxes by partitioning between vibrational relaxation (k_{-1}) and cleavage of the C—Co bond (k_2). The CH_3•/cob(II)alamin radical pair is initially produced in the singlet spin state and is trapped within a cage that is composed of protein and/or solvent atoms; this cage slows the diffusion and separation of the radical pair. Hyperfine coupling with the cobalt nucleus results in rapid intersystem crossing to the triplet spin state (k_3). Eventually the radical pair diffuses apart (k_4). Under anaerobic conditions, the free cob(II)alamin cofactor efficiently recaptures the methyl radical, regenerating methylcobalamin cofactor (k_{-4} to k_{-1}). In the presence of an efficient radical quenching agent, such as oxygen, TEMPO, or certain protein residues, the methyl radical is irreversibly quenched (k_5) and the reaction is driven to completion.

ively competes with radical quenching via hydrogen abstraction from the cobalamin cofactor.[20]

The methylcobalamin cofactor is resistant to photolysis when it is bound to methionine synthase,[21] even in the presence of oxygen, and many hours of exposure to room light are required to detect significant cob(II)-alamin formation. Based upon the mechanism presented above, this increased stability could result from three factors. First, the kinetic partitioning of the excited state between relaxation and bond homolysis may have been altered by binding of methylcobalamin to the protein. This effect could result from more efficient relaxation (k_{-1} increased) or less efficient bond homolysis (k_2 decreased). The lower axial ligand to cobalt in methionine synthase is a histidine that is hydrogen bonded to an aspartate and thence to a serine,[5] and we have shown that this 'ligand triad' contributes to the stabilization of the wild-type enzyme against photolysis.[6] The increased stability may result from an increased C—Co bond strength in the protein-bound cofactor that leads to a decrease in the bond homolysis rate (k_2); we are pursuing other methods of measuring this bond strength to test this hypothesis more rigorously. A second factor that could affect the rate of photolysis is a change in the equilibrium between singlet and triplet spin states in the resulting radical pair. A third factor that could contribute to the increased stability of protein-bound methylcobalamin is a decrease in the rate of cage escape (k_4) that leads to an increase in the cage efficiency.[19] During the photolysis of free methylcobalamin, the radical pair is held within a cage of solvent molecules that inhibits diffusive separation of the radical pair. When methylcobalamin is bound to the protein, protein residues completely surround the methyl group.[5] These residues form a cage which provides a relatively unreactive environment that could slow escape of the methyl radical and favor radical recombination.

The crystal structure of the cobalamin-binding region of methionine synthase shows several residues that contact the upper face of the cobalamin and may help to prevent radical escape (Fig. 4).[5] In particular, phenylalanine 708 and leucine 715 are located above and to the side of the methyl group in the structure of bound methylcobalamin, and these residues limit solvent access to the upper face of the methylcobalamin cofactor. In fact, Phe708 and Leu715 are conserved in the known sequences of methionine synthase.[22] Other residues in the cap domain may contribute to this shielding effect by enforcing a degree of conformational rigidity on those residues that make direct contact with the cofactor; phenylalanine 714 is located adjacent to Leu715 and may be involved in promoting the shielding effect of this residue. Substitutions of alanine for Phe708 and Leu715 are predicted to increase the solvent accessibility of the methylcobalamin cofactor (Fig. 4C, D), and thus to increase the cage escape rate of the methyl radical. Substitution of alanine for Phe714 is expected to have a much smaller effect (Fig. 4B).

On the other hand, the packing of the cap domain might be an artifact arising from the crystallization of a proteolytic fragment. A single flexible loop connects the cap domain to the α/β domain that binds the lower face of the cobalamin (Fig. 2), and the cap domain could have changed position following proteolytic digestion of the holoenzyme. If this were the case, Phe708 and Leu715 would not contribute to the protein cage in the intact enzyme, and would not be expected to influence photolysis rates. The results presented in this paper demonstrate that these residues

are flanking the methyl group in the intact resting methylcobalamin enzyme, and that the cap domain structure is not an artifact.

We have generated the mutant enzymes Phe708Ala, Phe714Ala, and Leu715Ala, and have reported the steady-state activities of these proteins elsewhere.[22] In this paper, we discuss the effect of these mutations on the rate of photolysis of the methylcobalamin enzyme. We analyze the fate of the methyl radical when methylcobalamin methionine synthase is photolyzed under anaerobic and aerobic conditions. Finally, we report the effect of substrate binding on the photolysis rates of the wild-type and mutant enzymes.

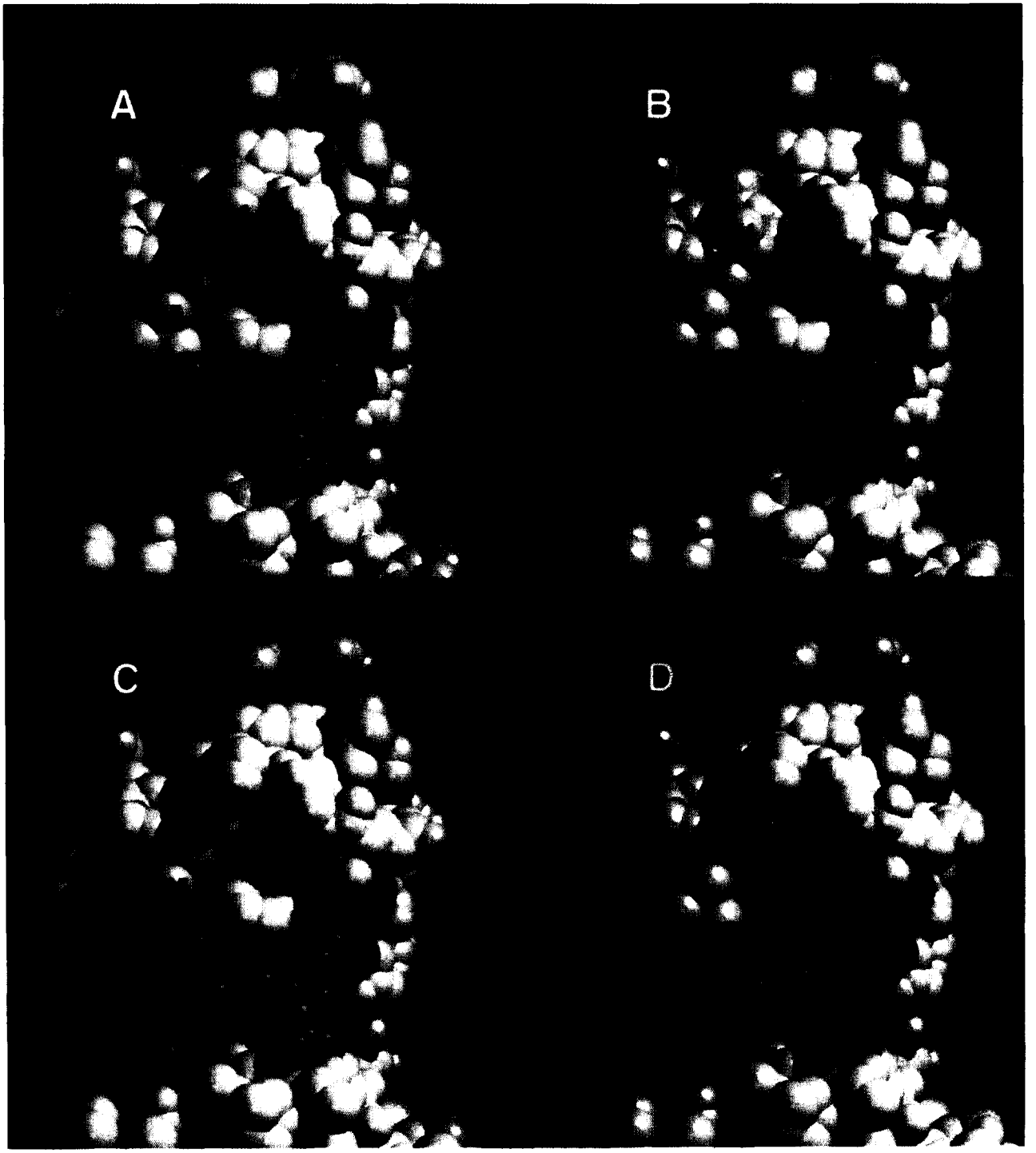

Figure 4. (A) Space-filling model of the cobalamin-binding region of wild-type methionine synthase. The protein is shown in gray, while the cofactor is blue, the methyl group is red, and the residues that were mutated are in yellow. (B–D) Hypothetical space-filling models of the mutant enzymes (B) Phe714Ala, (C) Leu715Ala, and (D) Phe708Ala. These 'mutations' were introduced by truncating the side chain of each residue at the β-carbon. No attempt has been made to energy minimize or refold the resulting structures, and the true structures may differ from those depicted.

Results

The exposure of the methylcobalamin cofactor in wild-type and mutant enzymes

Based upon the mechanism for photolysis depicted in Figure 3, the rate of photolysis of the methylcobalamin enzyme will depend in part on the ability of the protein to prevent methyl radical escape from the protein cage (k_4). As is apparent by visual inspection of the predicted structures in Figure 4, the mutations Phe708Ala and Leu715Ala increase the exposure of the methyl group, and presumably this leads to an increase in the cage escape rate of these mutants. These mutations may also increase the mobility of the cap residues and this may contribute to increased rates of cage escape. Finally, the mutant proteins may allow greater access of external radical quenching agents such as oxygen to the methyl radical within the protein cage. We would predict that the order of the rates of photolysis of these mutants should be Phe708Ala > Leu715Ala >> Phe714Ala ≈ wild type.

Mutations in the cap domain result in increased photolysis rates

Wild-type methionine synthase is isolated primarily in the methylcobalamin form, even though the purification procedure results in incidental exposure to room light for 2–3 days. While the Phe714Ala mutant enzyme was isolated in the methylcobalamin form, the Leu715Ala and Phe708Ala mutant enzymes were isolated in the cob(II)alamin form,[22] suggesting that the mutant enzymes were either deficient in their ability to protect methylcobalamin enzyme or were unable to remethylate cob(II)alamin enzyme. When the cob(II)-alamin mutant enzymes were incubated with AdoMet, flavodoxin, NADPH-ferredoxin (flavodoxin) oxidoreductase, and NADPH, conditions supporting reductive activation, they were converted to the methylcobalamin enzymes at rates comparable to the wild-type enzyme (J. Jarrett, unpublished data). This suggested that the mutant enzymes were unable to retain the methyl group, consistent with our hypothesis that these mutations would result in increased access to and from the upper face of the cobalamin.

We were able to follow the photolysis of methylcobalamin in a stopped-flow visible spectrophotometer upon exposure to a high-intensity xenon lamp. The wild-type and mutant proteins have nearly identical spectra, and therefore differences in the overlap between the absorption spectra and the xenon lamp emission spectrum should be minimal and should not contribute to differences in the photolysis rates.[17,18] At 25 °C in air-saturated buffer, the wild-type methylcobalamin enzyme is completely photolyzed in ≈2 min, resulting in conversion to cob(II)alamin enzyme (Fig. 5 and Table 1). The rates of photolysis of the Phe714Ala, Leu715Ala, and Phe708Ala mutant enzymes are increased by factors of 3, 30, and 62 as compared to the wild-type enzyme. In fact, the Phe708Ala mutant is photolyzed slightly faster (~1.3-fold) than the free

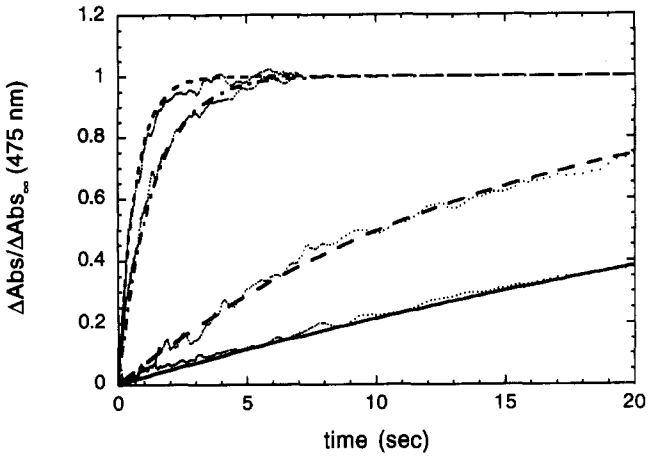

Figure 5. Photolysis of methylcobalamin enzymes. Normalized photolysis curves are shown for: wild-type (————), Phe714Ala (— — —), Leu715Ala (— — — —), and Phe708Ala (- - - -) enzymes. The methylcobalamin enzyme (≈10 μM) was photolyzed under aerobic conditions as described in the experimental methods and the normalized data fit to a single exponential curve. The rate constants derived from the fits are shown in Table 1, column 3.

cofactor. We interpret these results as indicating that the Phe708Ala and Leu715Ala mutants compromise the protein cage and increase the rate of cage escape (k_4 in Fig. 3).

Photolysis rates are not affected by the presence of quenching agents

Free methylcobalamin photolyzes very slowly in the absence of radical quenching agents such as oxygen, thiols, and nitroxides.[9,11] Recombination of the methyl radical with free cob(II)alamin is very efficient, and under anaerobic conditions the pathways available for quenching the methyl radical are not competitive. The addition of a quenching agent, such as oxygen or 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), results in increased rates of photolysis of the free cofactor by altering the partitioning of the escaped radical between recapture (k_{-4} in Fig. 3) or quenching (k_5); we observe increases of ≈25-fold in the photolysis rate of free

Table 1. Photolysis rate constants under anaerobic and radical-quenching conditions

Mutant enzyme	Rate constant for photolysis (s^{-1})		
	Anaerobic	Aerobic[a]	TEMPO[b]
Wild type	0.023	0.024	0.015
Phe714Ala	0.038	0.068	0.063
Leu715Ala	0.58	0.73	0.79
Phe708Ala	1.39	1.49	1.19
Methylcobalamin	0.045	1.16	1.08

[a]The enzyme in anaerobic potassium phosphate buffer was rapidly mixed with air-saturated potassium phosphate buffer at 25 °C producing a solution which was ≈100 μM oxygen.
[b]The enzyme was rapidly mixed with TEMPO (250 μM after mixing) in anaerobic potassium phosphate buffer at 25 °C.

Table 2. Products of the photolysis of ([3]H-*methyl*)-methylcobalamin enzyme

Photolysis conditions	Protein-associated radioactivity cpm (% initial)	Radioactivity in the solvent[a] cpm (% initial)	Unaccounted for radioactivity[b] cpm (% initial)
Anaerobic	4.7×10^5 (23%)	5.5×10^5 (27%)	1.01×10^6 (50%)
Aerobic	3.9×10^5 (20%)	1.45×10^6 (71%)	1.9×10^5 (9%)

[a]This includes water-soluble ^{3}H-labeled products due to oxidative quenching of the tritiated methyl radical such as formaldehyde and methanol.[11]
[b]This includes all products which are insoluble in water and freely diffuse into the atmosphere, including methane and ethane, as well as any radioactivity which does not elute from the Sephadex G-75 column.

methylcobalamin in the presence of an excess of either TEMPO or oxygen. A corresponding increase is not observed for methylcobalamin bound to either the wild-type or mutant enzymes (Table 1). The addition of oxygen or TEMPO results in rate increases of only one- to two-fold relative to the rate of anaerobic photolysis. These results suggest that during anaerobic photolysis, after the methyl radical leaves the protected environment of the protein cage (k_4 in Fig. 3), it does not return to recombine with cob(II)alamin as is observed with the free cofactor ($k_{-4} \approx 0$). Thus the protein acts to alter the partitioning of the escaped methyl radical between reentry into the cage (k_{-4}) and quenching (k_5). The protein cage may pose as much a barrier to radical reentry as it does to escape, leading to a decrease in k_{-4} relative to photolysis of free methylcobalamin. In addition, protein residues outside of the cage may act as efficient radical quenching agents, leading to an increase in k_5.

Protein residues in the cap domain prevent solvent access to the methyl group of the cofactor and appear to slow the rate of methyl radical escape from the protein cage; these same residues may limit the access of quenching agents into the protein cage. One might expect that oxygen would have limited access to the methyl radical within the protein environment, and that quenching would occur without the need for radical escape. If this were possible, then photolysis in the presence of oxygen would be expected to be much faster than in the presence of a sterically hindered quenching agent such as TEMPO. In fact, photolysis in the presence of oxygen is only $\approx$1.2-fold faster than in the presence of TEMPO (Table 1). This suggests that the rate at which oxygen can quench the methyl radical within the protein environment is slower than the rate of escape of the methyl radical from the protein cage, even in the mutant enzymes.

The protein is labeled during photolysis of ([3]H-*methyl*)-methylcobalamin enzyme

To test whether protein residues can quench the methyl radical during photolysis, we prepared wild-type methylcobalamin enzyme that was labeled with tritium in the photolabile methyl group. Samples of labeled enzyme were photolyzed under anaerobic or aerobic conditions and the protein was separated from low molecular weight tritiated products by gel filtration. Under both aerobic and anaerobic conditions, $\approx$20% of the tritium label is associated with the protein fraction (Table 2). Upon either precipitation of the protein in 10% trichloroacetic acid or denaturation of the protein in sodium dodecyl sulfate/2-mercaptoethanol, $\approx$50% of the protein-associated counts are retained in the protein, indicating covalent incorporation of the tritium label into the protein. Under anaerobic conditions, 50% of the label is lost during the work up, while under aerobic conditions only 9% of the label is unaccounted for. These observations suggest that under anaerobic conditions, hydrogen atom abstraction to form volatile methane is a significant mode of radical quenching. Since the reaction of the methyl radical with water does not contribute significantly to radical quenching with the free cofactor,[11] the protein is likely to be the source of hydrogen atoms for methane formation. Thus the protein does appear to be a very efficient radical trap, accounting for $\approx$70% of the total labeled products formed during anaerobic photolysis. Interestingly, gel electrophoresis demonstrates that the protein backbone is not significantly cleaved during anaerobic photolysis ($<$10%). This indicates that the methyl radical does not appear to abstract hydrogens bonded to backbone α-carbons, but may instead abstract hydrogens from certain amino acid side chains.

Under aerobic conditions, the majority of the radioactivity (70%) ends up as soluble low molecular weight products. The major end product of the reaction of methyl radical with oxygen is formaldehyde, while methanol and formic acid are minor products.[11] Since the presence of oxygen leads to a reduction in the loss of label during the work up, the reaction of the methyl radical with oxygen is presumably competitive with hydrogen atom abstraction from the protein to form methane. These studies, coupled with the kinetic studies shown in Table 1, suggest that although the preferred mode of aerobic quenching of the methyl radical is via reaction with oxygen to form formaldehyde, the protein is able to quench the methyl radical efficiently under anaerobic conditions. As long as the rate of quenching (k_5) is faster than the rates of escape (k_4) and recapture (k_{-4}), then the rate of quenching and the precise nature of the quenching reaction have no effect on the overall rate of photolysis. Thus the rate of photolysis of enzyme-bound methylcobalamin is relatively unaffected by the presence of exogenous quenching agents such as oxygen or TEMPO.

Photolysis of methylcobalamin enzyme is influenced by the presence of substrates

The limited accessibility of the cofactor found in the crystal structure of the cobalamin-binding fragment and confirmed by our photolysis experiments with the intact holoenzyme is somewhat puzzling since the chemistry catalyzed by methionine synthase is likely to require an in-line nucleophilic attack of homocysteine on the methyl group of methylcobalamin and of cob(I)alamin on the methyl group of CH_3-H_4folate (Fig. 1). The substrates homocysteine and CH_3-H_4folate, which are thought to bind to the N-terminal 70 kDa region of the enzyme (Fig. 6),[23] need to be positioned above the cofactor and this would require a conformational change upon binding one or both substrates that partially displaces the cap domain and brings the substrates into position over the cofactor. This conformational change might be expected to alter the solvent accessibility of the cofactor and therefore alter the cage escape rate of the methyl radical and the net photolysis rate.

We determined the rates of photolysis of wild-type enzyme in the presence of saturating amounts of CH_3-H_4folate, or AdoMet, or AdoMet + flavodoxin. CH_3-H_4folate and AdoMet do not react with enzyme in the methylcobalamin form; the effect of homocysteine could not be tested since it would result in rapid nucleophilic demethylation of the enzyme. Addition of AdoMet or AdoMet + flavodoxin decreases the rates of photolysis slightly (Fig. 7). However, when CH_3-H_4folate binds to the methylcobalamin enzyme, the photolysis rate is increased ≈3-fold (Figs 7 and 8). This effect is concentration dependent (Fig. 8) and gives an apparent K_d of 7 μM for (6*S*)-CH_3-H_4folate that compares well with the previously reported K_d of 5 μM determined by equilibrium dialysis.[24] The increase in the rate of photolysis in the presence of CH_3-H_4folate could be a result of two different effects: (1) an increase in the exposure of the methyl group similar to that introduced by the cap mutations described above, or (2) a decrease in the strength of the bond between the cobalt and the histidyl ligand.[6] Although our experiments do not distinguish between these possibilities, the first explanation is consistent with our current model of the ordered binding of substrates to the enzyme.[2] Methionine synthase reacts in a ternary complex with its substrates, and CH_3-H_4folate binding

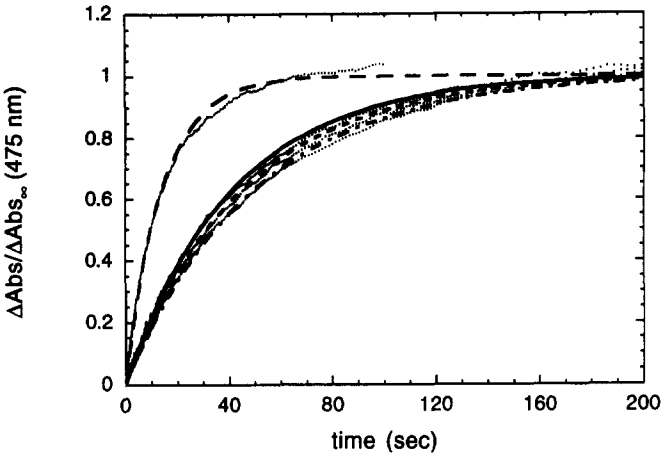

Figure 7. Effect of substrates on the photolysis of wild-type enzyme. The wild-type enzyme was photolyzed under aerobic conditions in the presence of: no substrates (————), AdoMet (200 μM) (– – – –), flavodoxin (30 μM) + AdoMet (200 μM) (— — — —), and CH_3-H_4folate (500 μM) (— — —). The methylcobalamin enzyme was rapidly mixed with an air-saturated solution of each substrate and photolysis was monitored as described in the Experimental section. The data are normalized and fit to a single exponential curve.

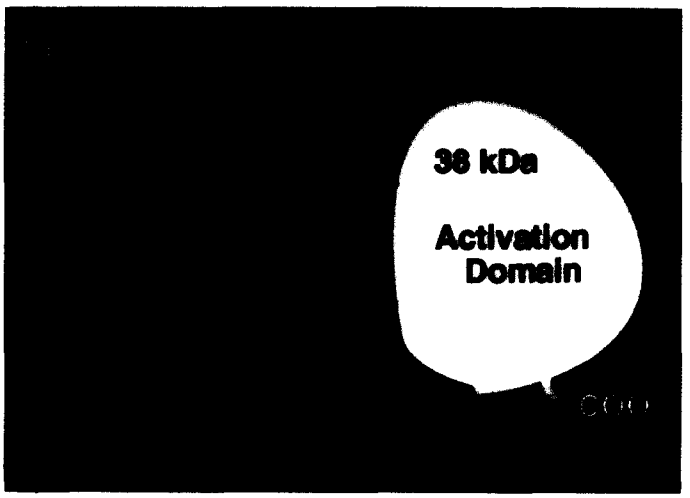

Figure 6. A cartoon of methionine synthase showing the relationships of functional domains deduced from studies of proteolytic fragments.[23] The central cobalamin-binding fragment is expected to interact with homocysteine and CH_3-H_4folate bound to the 70 kDa N-terminal region during turnover and with AdoMet bound to the 38 kDa C-terminal activation domain during reactivation of cob(II)alamin enzyme.

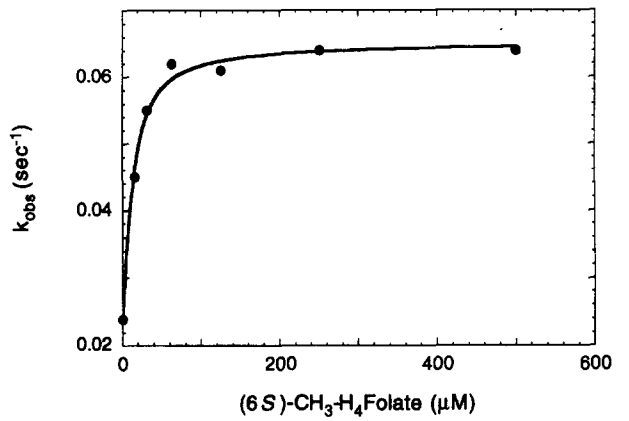

Figure 8. The dependence of the wild-type enzyme photolysis rate on the concentration of CH_3-H_4folate. The methylcobalamin enzyme was rapidly mixed with increasing concentrations of CH_3-H_4folate in air-saturated potassium phosphate buffer and photolysis was monitored as described in the Experimental section. Each set of data was fit to a single exponential curve and the rate constants derived from these fits are plotted as a function of the concentration of (6*S*)-CH_3-H_4folate. The data are fit to a quadratic binding curve using an enzyme concentration of 10 μM and give an apparent K_d of 7 μM for (6*S*)-CH_3-H_4folate.

must precede homocysteine binding. The binding of CH_3-H_4folate may induce a conformational change that creates a homocysteine binding pocket with access to the methylcobalamin cofactor. If this binding pocket were not filled, then the protein cage would be compromised and the rate of photolysis would increase.

Discussion

The mutation of residues in the α-helical domain that caps the cobalamin cofactor has allowed us to study the role of this domain in the intact enzyme. The mutations Phe708Ala and Leu715Ala were predicted to increase access to and from the upper face of the cobalamin. During protein purification, we observed that the methylcobalamin cofactor was less stable in these mutant enzymes.[22] While AdoMet-dependent reductive activation is not impaired (J. Jarrett, unpublished data), continuous-wave photolysis experiments demonstrated that the rate of photolysis is dramatically increased in these mutants. The photolysis rates we observe for the mutant proteins approximately correlate with changes in exposure of the methyl group of the cofactor as shown in Figure 4. Mutation of a single residue, Phe708Ala, results in an increase in the rate of photolysis to approximately that of the free cofactor.

The photolysis of methylcobalamin is a complex process whose rate is influenced by several intrinsic and environmental factors, including the intensity and wavelength of incident light, the absorption efficiency (extinction coefficient) at the wavelength of exposure, the vibrational relaxation efficiency of the molecule, the strength of the bond subject to cleavage, and the efficiency of the molecular cage surrounding the radical pair (Fig. 3). In particular for alkylcobalamins, the partition between recombination (k_{-2}) and cage escape (k_4) can be rate-limiting. The cage efficiency is related to the viscosity of the surrounding medium, with high viscosity solvents favoring radical recombination.[19] In methionine synthase, protein residues from the cap domain form the cage in which photolysis of methylcobalamin occurs, and this protein cage provides an environment which is more viscous than the surrounding aqueous medium.

In order for the cap domain residues to participate in an effective cage, it is important that these residues be relatively unreactive towards the methyl radical. The primary reaction of the methyl radical with protein residues is hydrogen atom abstraction and the driving force for this reaction can be estimated by comparing the bond dissociation energy (BDE) of the C—H bond in methane (105 kcal/mol)[25] with the BDE of the bond that is broken by hydrogen atom abstraction. Only the hydrogen atoms of the phenyl ring of Phe708 are in close proximity to the methyl group of the cofactor (Fig. 4), but the BDE of the hydrogen atoms of this aromatic side chain are ≈111 kcal/mol. Thus, hydrogen atom abstraction is unlikely inside the protein cage. However, once the methyl radical leaves the protective environment of this cage, hydrogen atom abstraction from protein residues may occur in the absence of oxygen. Hydrogen atoms that are more prone to abstraction include the thiol hydrogen of cysteine (BDE ≈ 87 kcal/mol), the hydrogens bonded to the β-carbons of serine and threonine (BDE ≤ 93 kcal/mol), and the hydrogens bonded to the tertiary carbons of valine and leucine (BDE ≈ 96 kcal/mol). This analysis suggests that positioning of a phenylalanine above the methyl group is critical to the protective nature of the protein cage.

The cap domain also prevents access to the cofactor in the resting form of the enzyme (Figures 2 and 6). Substrate binding must induce conformational changes in the enzyme that selectively allow homocysteine and CH_3-H_4folate to approach the cofactor during turnover. The binding of CH_3-H_4folate to the wild-type methylcobalamin enzyme results in a three-fold increase in the rate of photolysis. This effect is both concentration-dependent and saturable and suggests that binding of substrate induces conformational changes in the protein. Since the enzyme turns over in a ternary complex with both substrates, both substrates may need to be present for complete conversion to the catalytically active conformation. Unfortunately, photolysis cannot be examined in the presence of homocysteine, which rapidly demethylates the methylcobalamin cofactor.

Photolysis of the enzyme-bound methylcobalamin cofactor is inefficient and ultimately destructive to the organism. In the presence of trace levels of oxygen, conditions under which methionine synthase is normally expressed, the methyl radical generated by photolysis results in protein damage via hydrogen atom abstraction, covalent modification, and reaction of protein residues with formaldehyde. Furthermore, the cob(II)alamin enzyme must be reactivated in a reaction requiring the methyl group of AdoMet and an electron and therefore consuming energy. The prevention of this process is presumably beneficial to organisms that are exposed to visible light and the cap domain of methionine synthase may have evolved in response to this pressure. However, since *E. coli* is an enteric bacterium, it is unlikely that it is exposed to sufficient light to result in substantial photolysis in vivo. Why is the cap domain present in methionine synthase in these bacteria? Another role that the cap domain could fulfill is to prevent adventitious reactions. For instance, free methylcobalamin is demethylated by thiols (e.g. 2-mercaptoethanol) at a significant uncatalyzed rate (10^{-4} M^{-1} s^{-1} at pH 7).[26] The cap domain may limit access of intracellular nucleophiles to the cofactor. Another possible side reaction is oxidation of the cob(I)alamin intermediate to the inactive hydroxocob(III)alamin enzyme, and the cap domain may slow the access of oxygen to the free cob(I)alamin form of the enzyme. By limiting access to the cofactor, the enzyme is able to synthesize methionine with minimal energy wasted in nonproductive side reactions.

Experimental

Materials

2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO) was purchased from Aldrich. AdoMet and homocysteine were purchased from Sigma. ^{3}H-(*methyl*)-AdoMet was from Amersham. (6*R*,*S*)-CH_3-H_4Folate was purchased from Schircks Laboratories. All buffers were titrated to pH 7.2, unless otherwise specified.

Wild-type methionine synthase was overexpressed in strain DH5αF′/p4B6.3[27] and purified as recently described.[22] The mutant enzymes Phe708Ala, Phe714Ala, and Leu715Ala were overexpressed in the strain XL1-Blue containing the plasmids pMMA-14, pMMA-15 and pMMA-16, respectively. These plasmids contain a synthetic module encoding the 27 kDa cobalamin-binding region embedded within the wild-type *metH* gene. The construction of this semi-synthetic gene, the introduction of mutations in this gene, and the overexpression and purification of the mutant proteins have been described previously.[22] *E. coli* flavodoxin[28,29] and NADPH-ferredoxin (flavodoxin) oxidoreductase[30,31] were also overexpressed and purified as previously described.

Methionine synthase as purified contains a mixture of methylcobalamin, cob(II)alamin, and cob(III)alamin forms of the prosthetic group. This mixture can be converted to a homogeneous sample of the methylcobalamin enzyme by electrochemical reduction in the presence of AdoMet.[6,32] Briefly, the enzyme (≈100 μM) is made anaerobic and poised in an electrochemical cell at −450 mV (vs SHE) in the presence of methyl viologen (500 μM) and AdoMet (500 μM) for 45 min at 20 °C. The excess reagents are removed by gel filtration chromatography on a Superose-12 column (1 × 30 cm, Pharmacia).

Photolysis of methylcobalamin enzyme

Kinetic measurements of the photolysis of methylcobalamin enzyme were recorded on a HiTech stopped-flow UV/visible spectrophotometer. A xenon arc lamp was used for simultaneous continuous-wave photolysis and spectral observation of the cobalamin cofactor. The light from the xenon arc lamp (80 W, 78 V) was conducted through a fiber optic cable to the flow cell of the stopped-flow instrument. The light which passed through the sample was directed into a monochromator (2 nm resolution) and the wavelength of interest was selected and detected with a photomultiplier tube operating at −450 V. This instrument also allows spectra of the sample to be recorded (3-s scan time). The instrument was made anaerobic by equilibrating overnight with an oxygen scrubbing solution containing protocatechuic acid and protocatechuate dioxygenase. The xenon lamp is prone to day-to-day variations in absolute light intensity that resulted in variations in photolysis rates of ±20%, so critical comparisons were made from data collected on the same day. Although absolute rates of photolysis were not exactly reproducible from one day to the next, the same trends were observed.

The enzyme (20 μM) in 10 mM potassium phosphate buffer was made anaerobic in a glass tonometer by repeated evacuation and equilibration with argon. To initiate photolysis, the enzyme was rapidly mixed with a second solution containing either anaerobic buffer, TEMPO in anaerobic buffer, air-saturated buffer, or air-saturated buffer containing CH_3-H_4folate (50–1000 μM), AdoMet (400 μM), or AdoMet (400 μM) + oxidized flavodoxin (60 μM). After mixing, the sample was passed into the flow cell of the stopped-flow spectrophotometer, where exposure to the light from the xenon lamp produced photolysis. Due to the instability of intensity of the xenon lamp, the kinetic traces obtained were relatively noisy. To improve the quality of the data, the data prior to 8 msec were discarded, and a 5-point-smoothing function was applied to the remainder of the data. These data were normalized by division with the total change in absorbance to give the data shown in Figures 5 and 7 and were fit to a single exponential curve to generate the rate constants listed in Table 1 and in the text.

Photolysis of [^{3}H-*methyl*]-methylcobalamin methionine synthase

Methionine synthase (50 nmol) was methylated with unlabeled AdoMet, purified by gel filtration, and then treated with homocysteine and dithiothreitol under anaerobic conditions as previously described.[6] This treatment resulted in a homogeneous sample of cob(II)alamin enzyme with no remaining unlabeled methylcobalamin and no bound AdoMet. The cob(II)alamin protein (≈40 nmol in 2 mL of 10 mM potassium phosphate at pH 7.2) was treated with (^{3}H-*methyl*)-AdoMet (100 nmol in 0.5 mL of 1 mM HCl, 6.5×10^5 dpm/nmol), flavodoxin (0.5 μM final concentration), NADPH-ferredoxin (flavodoxin) oxidoreductase (0.5 μM), and NADPH (1 mM) in an anaerobic cuvette under argon, and the reaction was followed spectrally. After 2 h at room temperature, the reaction mixture was applied to a Sephadex G-75 column (Pharmacia, 1 × 10 cm) equilibrated in 50 mM potassium phosphate buffer and eluted with 30 mL of the same buffer. A small sample of each fraction (5 μL of 1 mL total) was counted; the protein eluted in fractions 5–6 while the unreacted AdoMet eluted in fractions 12–15. The protein fractions were pooled, yielding 25 nmoles of [^{3}H-*methyl*]-methylcobalamin protein that was separated into 0.5 mL portions and frozen at −80 °C.

Labeled protein (~6.3 nmol) was diluted to 1 mL in 50 mM potassium phosphate buffer. One sample was made anaerobic by repeated vacuum/argon cycles over 1 h, while a second sample was allowed to rest at room temperature exposed to air, in order to saturate the sample with oxygen. Each sample was photolyzed in a sealed cuvette by immersing in a beaker filled with ice water and exposing to a 600 W tungsten lamp for 20 s. A spectrum of each sample showed complete conver-

sion to cob(II)alamin. The samples were equilibrated at room temperature for 5 min to allow complete quenching of the radical species and then applied to a Sephadex G-75 column as described above. The protein was separated from soluble low molecular weight products and products which were water insoluble and volatile (i.e. methane, ethane). A small sample from each fraction (10 μL of 1 mL) was counted to determine the total counts in the protein and soluble low molecular weight fractions.

To determine the fraction of acid-labile protein-associated counts, unlabeled bovine serum albumin (0.1 mg in 50 μL) was added to a portion of the labeled protein fraction (50 μL) and this mixture was added to 10% trichloroacetic acid. The precipitated protein was pelleted (14000 × *g* for 10 min at 4 °C) and the soluble fraction was counted. The protein pellet was resuspended in 500 μL water and was added to scintillation fluid and counted.

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References

1. Zydowsky, T. M.; Courtney, L. F.; Frasca, V.; Kobayashi, K.; Shimizu, H.; Yuen, L.-D.; Matthews, R. G.; Benkovic, S. J.; Floss, H. G. *J. Am. Chem. Soc.* **1986**, *108*, 3152.

2. Banerjee, R. V.; Frasca, V.; Ballou, D. P.; Matthews, R. G. *Biochemistry* **1990**, *29*, 11101.

3. Banerjee, R. V.; Harder, S. R.; Ragsdale, S. W.; Matthews, R. G. *Biochemistry* **1990**, *29*, 1129.

4. Fujii, K.; Huennekens, F. M. *J. Biol. Chem.* **1974**, *249*, 6745.

5. Drennan, C. L.; Huang, S.; Drummond, J. T.; Matthews, R. G.; Ludwig, M. L. *Science* **1994**, *266*, 1669.

6. Jarrett, J. T.; Amaratunga, M.; Drennan, C. L.; Scholten, J. D.; Sands, R. H.; Ludwig, M. L.; Matthews, R. G. *Biochemistry* **1996**, *35*, 2464.

7. Drennan, C. L.; Matthews, R. G.; Ludwig, M. L. *Current Opinions in Structural Biology* **1994**, *4*, 919.

8. Chagovetz, A. M.; Grissom, C. B. *J. Am. Chem. Soc.* **1993**, *115*, 12152.

9. Chen, E.; Chance, M. R. *Biochemistry* **1993**, *32*, 1480.

10. Endicott, J. F.; Ferraudi, G. J. *J. Am. Chem. Soc.* **1977**, *99*, 243.

11. Hogenkamp, H. P. C. *Biochemistry* **1966**, *5*, 417.

12. Lott, W. B.; Chagovetz, A. M.; Grissom, C. B. *J. Am. Chem. Soc.* **1995**, *117*, 12194.

13. Mok, C. Y.; Endicott, J. F. *J. Am. Chem. Soc.* **1978**, *100*, 123.

14. Pratt, J. M. *J. Chem. Soc.* **1964**, 5154.

15. Pratt, J. M.; Whitear, B. R. D. *J. Chem. Soc. (A)* **1971**, 252.

16. Schrauzer, G. N.; Sibert, J. W.; Windgassen, R. J. *J. Am. Chem. Soc.* **1968**, *90*, 6681.

17. Schrauzer, G. N.; Lee, L. P.; Silbert, J. W. *J. Am. Chem. Soc.* **1970**, *92*, 2997.

18. Taylor, R. T.; Smucker, L.; Hanna, M. L.; Gill, J. *Arch. Biochem. Biophys.* **1973**, *156*, 521.

19. Koenig, T. W.; Hay, B. P.; Finke, R. G. *Polyhedron* **1988**, *7*, 1499.

20. Daikh, B. E.; Finke, R. G. *J. Am. Chem. Soc.* **1992**, *114*, 2938.

21. Taylor, R. T.; Weissbach, H. *Arch. Biochem. Biophys.* **1968**, *123*, 109.

22. Amaratunga, M.; Fluhr, K.; Jarrett, J. T.; Drennan, C. L.; Ludwig, M. L.; Matthews, R. G.; Scholten, J. D. *Biochemistry* **1996**, *35*, 2453.

23. Drummond, J. T.; Huang, S.; Blumenthal, R. M.; Matthews, R. G. *Biochemistry* **1993**, *32*, 9290.

24. Taylor, R. T.; Hanna, M. L. *Arch. Biochem. Biophys.* **1972**, *151*, 401.

25. Lide, D. R., Ed. *Handbook of Chemistry and Physics* CRC Press: New York, 1995.

26. Hogenkamp, H. P. C.; Bratt, G. T.; Sun, S.-z. *Biochemistry* **1985**, *24*, 6428.

27. Banerjee, R. V.; Johnston, N. L.; Sobeski, J. K.; Datta, P.; Matthews, R. G. *J. Biol. Chem.* **1989**, *264*, 13888.

28. Bianchi, V.; Eliasson, R.; Fontecave, M.; Mulliez, E.; Hoover, D. M.; Matthews, R. G.; Reichard, P. *Biochem. Biophys. Res. Commun.* **1993**, *197*, 792.

29. Osborne, C.; Chen, L.-M.; Matthews, R. G. *J. Bacteriol.* **1991**, *173*, 1729.

30. Eliasson, R.; Pontis, E.; Fontecave, M.; Gerez, C.; Harder, J.; Jornvall, H.; Krook, M.; Reichard, P. *J. Biol. Chem.* **1992**, *267*, 25541.

31. Bianchi, V.; Reichard, P.; Eliasson, R.; Pontis, E.; Krook, M.; Jornvall, H.; Haggard-Ljungquist, E. *J. Bacteriol.* **1993**, *175*, 1590.

32. Luschinsky, C. L.; Drummond, J. T.; Matthews, R. G.; Ludwig, M. L. *J. Mol. Biol.* **1992**, *225*, 557.

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