Cobalamin-Dependent Methyltransferases

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ABSTRACT

Cobalamin cofactors play critical roles in radical-catalyzed rearrangements and in methyl transfers. This Account focuses on the role of methylcobalamin and its structural homologues, the methylcorrinoids, as intermediaries in methyl transfer reactions, and particularly on the reaction catalyzed by cobalamin-dependent methionine synthase. In these methyl transfer reactions, the cobalt(I) form of the cofactor serves as the methyl acceptor. Biological methyl donors to cobalamin include N5-methyltetrahydrofolate, other methylamines, methanol, aromatic methyl ethers, acetate, and dimethyl sulfide. The challenge for chemists is to determine the enzymatic mechanisms for activation of these unreactive methyl donors and to mimic these amazing biological reactions.

Cobalamin has been referred to as "nature's most beautiful cofactor".[1] The highly substituted and reduced corrin macrocycle (Figure 1) proved a monumental challenge to structural elucidation[2] and to chemical synthesis.[3,4] The biological cofactors adenosylcobalamin and methylcobalamin share the common feature of a carbon−cobalt bond between the upper axial ligand and the cobalt; the existence of such metal−carbon bonds was first revealed when the X-ray structure of adenosylcobalamin was determined.[5]

Cobalamin cofactors play critical roles in several types of biological reactions. Perhaps best understood is the involvement of adenosylcobalamin in radical generation. Adenosylcobalamin, with its weak carbon−cobalt bond, can undergo homolytic cleavage to yield a 5′-deoxyadenosyl radical and cob(II)alamin. The 5′-deoxyadenosyl radical may then serve to generate a substrate radical by hydrogen atom abstraction, or to generate a protein radical that in turn abstracts a hydrogen atom from the substrate. Adenosylcobalamin is found in a wide variety of mutases, where rearrangement of a substrate radical is triggered by hydrogen atom abstraction, and in ribonucleotide triphosphate reductase, where generation of the substrate radical on C3′ of the ribose leads to expulsion of the hydroxyl substituent at C2′.

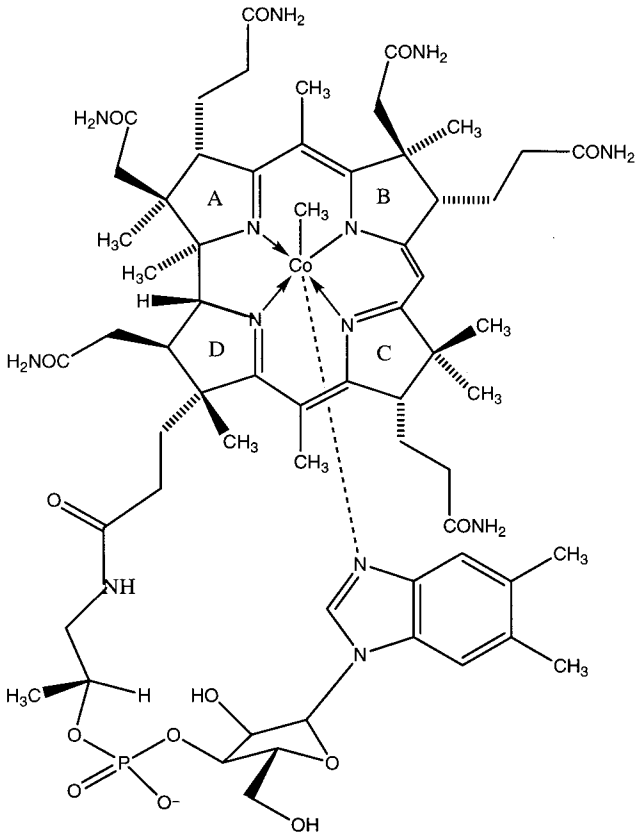
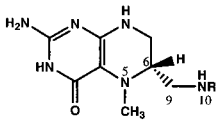
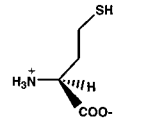


FIGURE 1. Structure of methylcobalamin. In adenosylcobalamin or coenzyme B_{12}, a 5′-deoxyadenosyl group, ligated to the cobalt via the methylene carbon at C5′, replaces the methyl group in methylcobalamin.

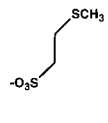
A second major role for cobalamin, and for its close relatives the corrinoids,[6] is in methyl transfer reactions. The best-studied reaction is that catalyzed by cobalamin-dependent methionine synthase.[7] This enzyme catalyzes the transfer of a methyl group from CH_3-H_4folate (**1**), a



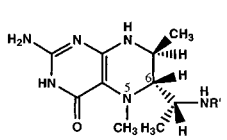
CH_3-H_4folate (1)



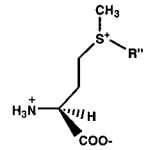
L-homocysteine (2)



methyl-coenzyme M (3)



CH_3-H_4methanopterin (4)



S-adenosylmethionine (5)

tertiary amine, to homocysteine (**2**), an alkanethiol. The cobalamin cofactor plays an integral role in methyl transfer, accepting methyl groups from CH_3-H_4folate and donating them to homocysteine. During catalytic turnover, the cofactor cycles between methylcobalamin and cob(I)alamin forms,[8] alternately demethylated by homocysteine and remethylated by CH_3-H_4folate (Figure 2).

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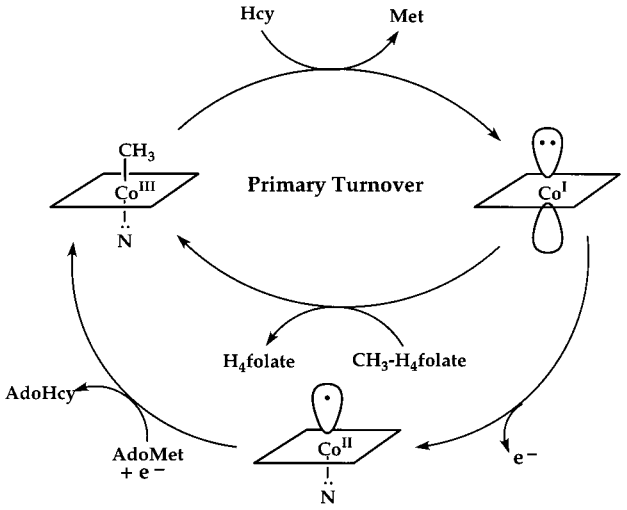


FIGURE 2. Reactions catalyzed by cobalamin-dependent methionine synthase. During primary turnover, the enzyme-bound cobalamin prosthetic group cycles between methylcobalamin and cob(I)alamin forms. The cob(I)alamin form is occasionally oxidized by molecular oxygen, yielding an inactive enzyme in the cob(II)alamin form. Return of this species to the catalytic cycle requires a reductive methylation, in which an electron must be supplied by an exogenous donor, and a methyl group is provided by the biological sulfonium, adenosylmethionine. In *E. coli*, the electron donor is reduced flavodoxin,[48] while in humans the electron is supplied by the homologous flavoprotein methionine synthase reductase.[49]

In acetogenic prokaryotes[9] and methanogenic Archaea,[10] corrinoid cofactors are involved in catalysis of a wide variety of methyl transfer reactions. The acetogenic organism *Moorella* (formerly *Clostridium*) *thermoaceticum* employs CH_3-H_4folate as the methyl donor to a corrinoid iron−sulfur protein, which in turn transfers the methyl group to acetyl CoA synthase. The cobalt(I) form of the corrinoid cofactor is the methyl acceptor. Acetogens and other prokaryotes also use aromatic methyl ethers[11] or aliphatic or aromatic chlorides[12] as methyl donors to cob(I)alamin.

A central pathway for energy generation in methanogens involves production of methane by reduction of methyl coenzyme M (**3**).[10] This pathway has been extensively studied in *Methanobacterium thermoautotrophicum*, where methyl groups are formed by reduction of CO_2 using electrons derived from H_2, resulting ultimately in the formation of CH_3-H_4methanopterin (**4**), a tertiary amine that is structurally similar to CH_3-H_4folate. CH_3-H_4methanopterin serves as the methyl donor to a corrinoid cofactor in a multisubunit protein complex, MtrA-H, and the methyl group is then transferred to the sulfur of coenzyme M. The reaction catalyzed by MtrA-H is chemically analogous to that catalyzed by methionine synthase, in that a corrinoid prosthetic group mediates methyl transfer from a tertiary amine to an alkanethiol, cycling during catalysis between methylcorrinoid and cobalt(I) corrinoid states. In another branch of the methanogenic Archaea, *Methanosarcina*, a variety of simple one-carbon compounds can serve as direct methyl donors to enzyme-bound corrinoid cofactors and thence to coenzyme M. These methyl donors include methanol, acetate, mono-, di-, and trimethylamines, and dimethyl sulfide.

The rationale for participation of cob(I)alamin or cobalt(I) corrinoids in methyl transfer reactions has remained obscure. An attractive mechanism would be applicable to the activation of each of the observed methyl donors to enzyme-bound corrinoids, e.g., methanol, chloromethane, phenyl methyl ether, simple methylamines, and CH_3-H_4folate. It was our hope that studies of methyl transfer in cobalamin-dependent methionine synthase would illuminate a broad spectrum of corrinoid methyl transfer reactions and reveal the unique properties of the highly reduced transition metal that facilitate methyl transfers from unactivated donors.

A Difficult Reaction

The catalytic cycle of MetH involves the two half-reactions shown in eqs 1 and 2, and in this section we will examine

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

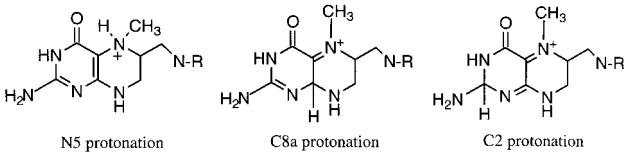
$$\text{methylcob(III)alamin} + \text{homocysteine} \rightarrow \text{cob(I)alamin} + \text{methionine} + \text{H}^+ \quad (2)$$

precedents for each of these half-reactions in model systems. The use of an unactivated tertiary amine such as CH_3-H_4folate as a methyl donor lacks precedent in the organic chemistry literature. The vast majority of biological methyl transfers employ sulfonium compounds such as *S*-adenosylmethionine (**5**) and *S*-methylmethionine as methyl donors. Transfers of methyl groups from adenosylmethionine to a wide variety of oxygen and nitrogen acceptors have been shown to proceed with inversion of stereochemistry at the transferred methyl group, consistent with an S_N2 mechanism for methyl transfer.[13] Adenosylhomocysteine and methionine are excellent leaving groups; the pK_a value for the conjugate acid of a neutral thiol is ~ -7,[14] and that for the conjugate acid of a thioether should be comparable. In contrast, the leaving group formed by methyl transfer from CH_3-H_4folate, a tetrahydrofolate anion, is the conjugate base of an acid that is expected to have a very high pK_a: the pK_a for ammonia dissociating to form the ammonia anion is 38![14]

By analogy with the use of methyl sulfonium compounds as activated methyl donors, quaternary amines can also serve as methyl donors, although the leaving groups are much less favorable. Quaternary ammonium salts function as methyl donors to cob(I)alamin, as does 5,5,6,7-tetramethyl-5,6,7,8-tetrahydropteridinium.[15] Pratt and his colleagues have determined a rate constant of 2×10^{-3} M^{-1} s^{-1} for the reaction of cob(I)alamin with $PhNMe_3^+$ at 25 °C.[16]

The overall conversion of CH_3-H_4folate to H_4folate shown in eq 1 requires proton transfer to N5 of the folate product, suggesting that CH_3-H_4folate is preactivated for displacement of the methyl group by protonation at N5. Absorption spectroscopy of CH_3-H_4folate in aqueous

Scheme 1. Possible Positions for Protonation of CH_3-H_4folate



solution reveals a pK_a of 5.05.[17] Since CH_3-H_4folate is an enamine, protonation could occur either on N5 or on the conjugated carbons 8a or 2 (Scheme 1). The DEPT 135° NMR pulse sequence has been employed to probe the position at which protonation of CH_3-H_4folate occurs in aqueous solution.[17] No protonation of C8a or C2 is seen at low pH, but ~2.4 ppm downfield chemical shifts of the resonances of both the N5-methyl and C6 carbons occur as the pH is lowered, consistent with protonation occurring at N5.

If a basic nucleophile is mixed with a protonated amine in aqueous solution, one would expect proton transfer rather than methyl transfer to result. Cob(I)alamin is very weakly basic, with pK_a < 1 for the conjugate acid,[18] hydridocobalamin. Thus, cob(I)alamin is uniquely suited to discriminate between H and CH_3 substituents at N5 of protonated CH_3-H_4folate. Furthermore, cob(I)alamin is a superb nucleophile—a so-called supernucleophile.[19] Indeed, formation of methylcorrinoids from Co(I) corrinoids and methanol or dimethylaniline under acidic conditions has been demonstrated, although rate constants have not been determined for these model reactions.[20]

The second methyl transfer reaction catalyzed by methionine synthase is from methylcobalamin to homocysteine, as shown in eq 2. Homocysteine is an alkanethiol with a microscopic pK_a of 9.0 when the amino group is protonated.[21] Hogenkamp and co-workers[22] have shown that the pH dependence of the rate of reaction of 2-mercaptoethanol with methylcobalamin is consistent with attack by a thiolate nucleophile. The reaction of the thiolate, however, is very slow, with a second-order rate constant of 2.2×10^{-2} M^{-1} s^{-1} at 43 °C and pH 11. Pratt was unable to measure the rate of demethylation of methylcobalamin by homocysteine thiolate in aqueous solution at pH 12 and 25 °C, although he determined a rate constant of 0.015 M^{-1} s^{-1} for the reaction of homocysteine thiolate with the cationic axial-base free form of methylcobalamin, methylcobinamide, and estimated that the rate of reaction of methylcobalamin was at least 1500-fold slower.[23]

In the next section, we will describe a strategy that has allowed us to compare rate constants for the reaction of enzyme-bound homocysteine with either free or enzyme-bound methylcobalamin and for the reaction of enzyme-bound CH_3-H_4folate with either free or enzyme-bound cob(I)alamin. The second-order rate constants determined using this strategy can be directly compared with those observed in model reactions, allowing us to estimate the acceleration of each individual methyl transfer achieved by methionine synthase.

Cobalamin-Dependent Methionine Synthase from *Escherichia coli*

Cobalamin-dependent methionine synthase exhibits the beautifully modular construction shown in Figure 3, with modules for binding and activation of homocysteine and

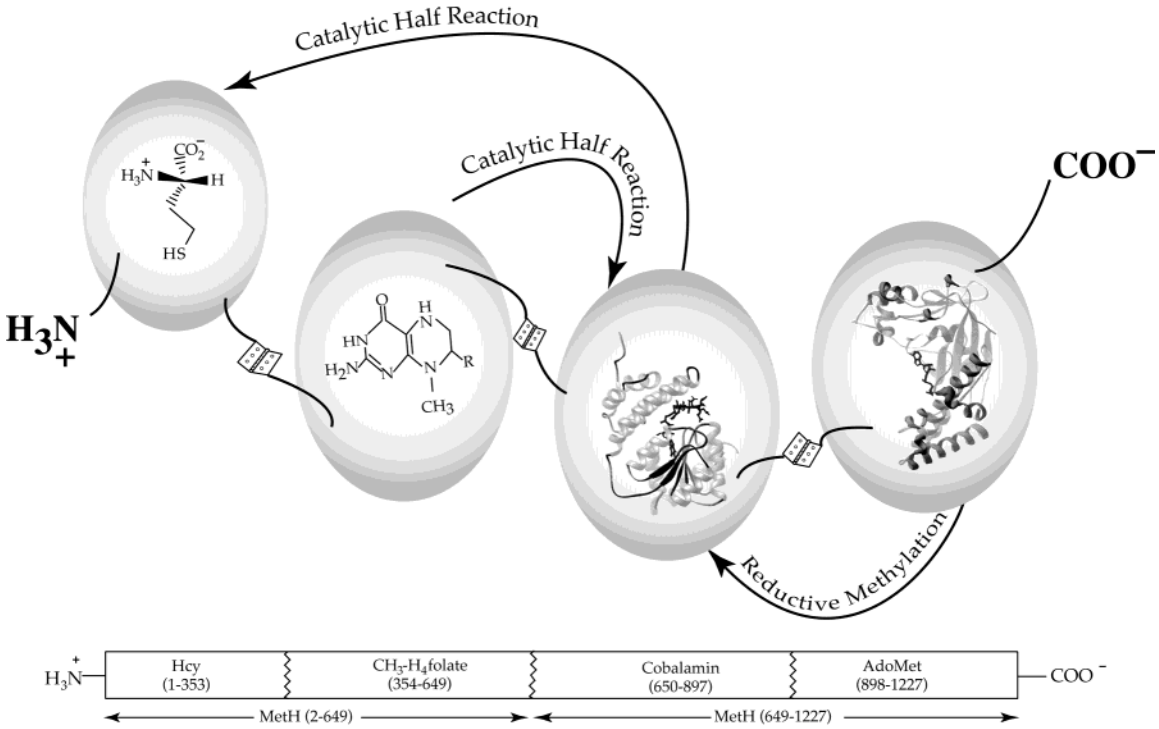


FIGURE 3. Diagram of the modular structure of cobalamin-dependent methionine synthase. The four modules are connected by flexible linker regions, shown as hinges, that allow the three substrate-binding modules to contact the cobalamin-binding module to transfer methyl groups to and from the cobalamin. The region of the amino acid sequence comprising each module is shown below the diagram.

Table 1. Second-Order Rate Constants for Methyl Transfer Reactions

reaction	rate constant for model reaction (M^{-1} s^{-1})	ref	rate constant for MetH(2−649) + cob_{exo} (M^{-1} s^{-1})	ref	rate constant for MetH(2−649) + E·cob (M^{-1} s^{-1})	ref
$CH_3N^+R_3$ + cob(I)	2×10^{-3} (25 °C)	16				
CH_3NR_2 + cob(I)			600 (37 °C)	27	70,000 (37 °C)	30
CH_3-Cob + RSH	2.2×10^{-2} (43 °C, pH 11)	22	2300 (37 °C)	27	135,000 (37 °C)	30

CH_3-H_4folate, for binding the cobalamin cofactor, and for reductive activation of the enzyme.[24−26] The overall rate of catalytic turnover appears to be determined primarily by conformational changes required to present different substrates to the enzyme-bound cobalamin, so steady-state kinetic measurements provide little information about the rates of the chemical steps.

Fragments of MetH retain some of their functional properties. Thus, a fragment containing the two substrate-binding modules, but lacking the B_{12}-binding and activation modules, MetH(2−649), retains the ability to transfer methyl groups from CH_3-H_4folate to exogenous cob(I)alamin and from exogenous methylcobalamin to homocysteine.[27] Each of these reactions is first order in added cobalamin and in MetH(2−649), and saturable in the other substrate, allowing comparison of the second-order rate constants with those for model reactions (Table 1). The ability to react with exogenous cobalamin appears to be a general feature of corrinoid methyltransferases.[28,29]

Substrates bound to MetH(2−649) also react with cobalamin bound to a fragment of MetH containing the B_{12} and activation modules, MetH(649−1227).[30] Turnover is first order in each fragment of MetH. Table 1 also presents second-order rate constants determined for methylation of enzyme-bound cob(I)alamin by CH_3-H_4-folate and for demethylation of enzyme-bound methylcobalamin by homocysteine. Methyl transfer from enzyme-bound methylcobalamin to enzyme-bound homocysteine is accelerated ~6 000 000-fold as compared to the model reaction and 60-fold as compared to the reaction of exogenous methylcobalamin with enzyme-bound homocysteine, while the methyl transfer from CH_3-H_4folate to cob(I)alamin is accelerated ~35 000 000-fold and 120-fold, respectively. In the discussion that follows, I will attempt to identify salient features of the modules that may contribute to the large observed rate accelerations.

The homocysteine-binding module of MetH contains a zinc ion that is essential for binding and activation of homocysteine.[26] The zinc ion is ligated to three cysteines from this module; addition of homocysteine results in displacement of a fourth oxygen or nitrogen ligand and direct coordination of the sulfur of homocysteine to the zinc (Figure 4).[31] Homocysteine binding at neutral pH is associated with release of protons to solution, consistent with binding of homocysteine as a thiolate rather than a thiol.[32] All the enzymes that catalyze methyl transfer from methylcorrinoids to thiols that have been examined contain zinc, and where the role of zinc has been investigated, the thiolate nucleophile binds as a ligand to the zinc, suggesting an important role for zinc in catalyzing methyl transfer to thiols.[33−35]

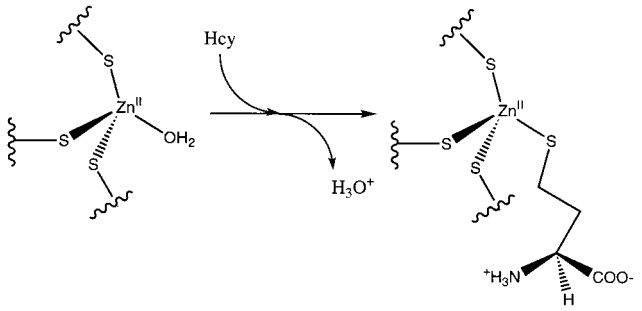


FIGURE 4. Binding of homocysteine to methionine synthase. As isolated from cells, a zinc ion is coordinated by cysteine residues 247, 310, and 311 in the homocysteine-binding module of MetH. The fourth ligand is an oxygen or nitrogen, presumably water, and is displaced on binding homocysteine.[31] Binding of homocysteine is accompanied by proton release, presumably associated with ionization of the thiol to form a thiolate.[32]

Corrinoid-dependent methyl transferases also share a unique mode of corrinoid binding. When the X-ray structure of the cobalamin-binding module of MetH was determined, it revealed an unexpected conformational change in the methylcobalamin cofactor. As illustrated in Figure 1, cobalt in the free cofactor is octahedrally coordinated by four nitrogen ligands from the corrin macrocycle, by the methyl group in the upper axial position, and by a dimethylbenzimidazole nucleotide substituent of the corrin ring in the lower axial position. On binding to MetH, the dimethylbenzimidazole substituent dissociates and His759 from the protein occupies the lower axial coordination site on cobalt (Figure 5). His759 is hydrogen bonded to Asp757, which in turn is hydrogen bonded to Ser810, and these residues are conserved in all cobalamin-dependent methionine synthases. All corrinoid-dependent methyl transferases bind the corrinoid cofactor with displacement of the dimethylbenzimidazole ligand; in most cases the imidazole side chain of a histidine residue from the protein is the new ligand, but the methylcorrinoid in the iron/sulfur corrinoid protein from *M. thermoaceticum* lacks a nitrogen ligand.[36]

Protonation State of CH_3-H_4folate in Binary Complexes with MetH

If methyl transfer from CH_3-H_4folate to cob(I)alamin proceeds by an S_N2 mechanism, one might expect activation of the methyl donor to precede group transfer, presumably by protonation of CH_3-H_4folate at N5. It is therefore pertinent to determine the protonation state of the substrate when bound to its module of MetH.

We have recently used the fragment of MetH that contains the homocysteine- and CH_3-H_4folate-binding modules, MetH(2−649), to measure the absorbance

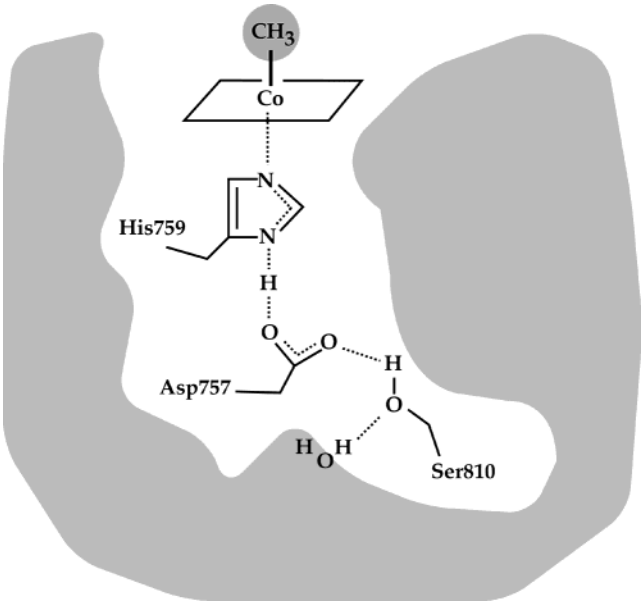
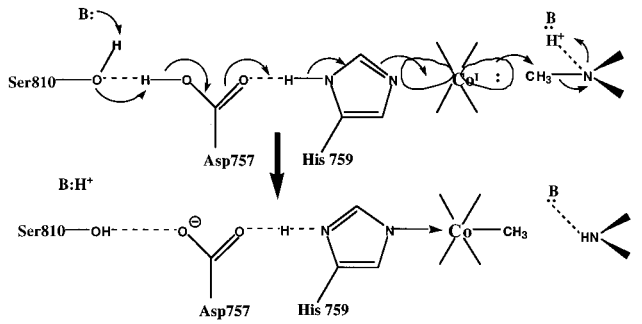


FIGURE 5. The ligand triad. The X-ray structure of the cobalamin-binding module of methionine synthase revealed that the dimethylbenzimidazole ligand, which is the lower axial ligand to cobalamin in free methylcobalamin, has been replaced by a histidine ligand from the protein, His759.[50] This histidine is linked by hydrogen bonds to Asp757 and thence to Ser810, and all three residues are strictly conserved in all MetH proteins. The ligand triad has been shown to take up a proton from solvent when enzyme in the cob(II)alamin form is reduced to cob(I)alamin, or when methylcobalamin is demethylated by homocysteine, and to release a proton when cob(I)alamin is remethylated by CH_3-H_4folate.[32]

changes associated with CH_3-H_4folate binding.[17] When the binding of CH_3-H_4folate to MetH(2−649) was examined as a function of pH, it was found that the fragment does not bind protonated CH_3-H_4folate, and that at low pH, binding is associated with proton release rather than proton uptake.[17] Thus, formation of a binary enzyme−substrate complex decreases the pK_a at N5 of bound CH_3-H_4folate below its pK_a in solution of 5.05, as might be expected if the substrate is introduced into a hydrophobic environment on binding to the protein. These findings are very difficult to reconcile with an S_N2 mechanism requiring activation of the methyl donor prior to group transfer, although they do not preclude proton transfer to N5 occurring only in MetH(2−649)·CH_3-H_4folate·cob(I)alamin ternary complexes.

The product H_4folate contains a proton at N5; what is the timing of proton uptake with respect to group transfer? The second-order rate constant characterizing the reaction of CH_3-H_4folate bound to MetH(2−649) with exogenous cob(I)alamin increases as the pH is lowered in association with an apparent pK_a of 5.9 ± 0.1 (April Smith and Rowena Matthews, unpublished data). In the reverse direction, methyl transfer from methylcobalamin to H_4folate, the pH dependence is reversed, and the second-order rate constant decreases as the pH is lowered. These observations are consistent with general acid/general base catalysis occurring in ternary MetH(2−649)·CH_3-H_4folate·cob(I)alamin complexes; if the general acid catalyst is unprotonated at a given pH, collisions between cob(I)alamin and enzyme-bound CH_3-H_4folate do not result in product formation, while collisions betwen enzyme-bound H_4-folate and methylcobalamin result in formation of CH_3-H_4folate and cob(I)alamin. Such observations suggest that proton transfer and methyl transfer are coordinated, perhaps with the general acid catalyst initially hydrogen bonded to N5 of CH_3-H_4folate (Scheme 2). As the methyl group bond to N5 is weakened, N5 will become progressively more basic, until the proton is transferred from the general acid to N5. Similar pH−rate profiles were determined for the reversible transfer of methyl groups from CH_3-H_4folate to cob(I)alamin catalyzed by the corrinoid iron/sulfur protein methyl transferase AcsE from *M. thermoaceticum*.[28]

Scheme 2. Coordinated Group and Proton Transfer in an S_N2 Mechanism[a]



[a] A general acid catalyst on the enzyme is hydrogen bonded to N5 of CH_3-H_4folate in the ground state. As cob(I)alamin attacks and the methyl−N5 bond is weakened, the proton is transferred from the general acid catalyst to H_4folate. This scheme also depicts a role for the ligand triad. As cob(I)alamin is converted to methylcobalamin, a proton is released to solvent and histidine of the ligand triad coordinates to the cobalamin in the lower axial position.

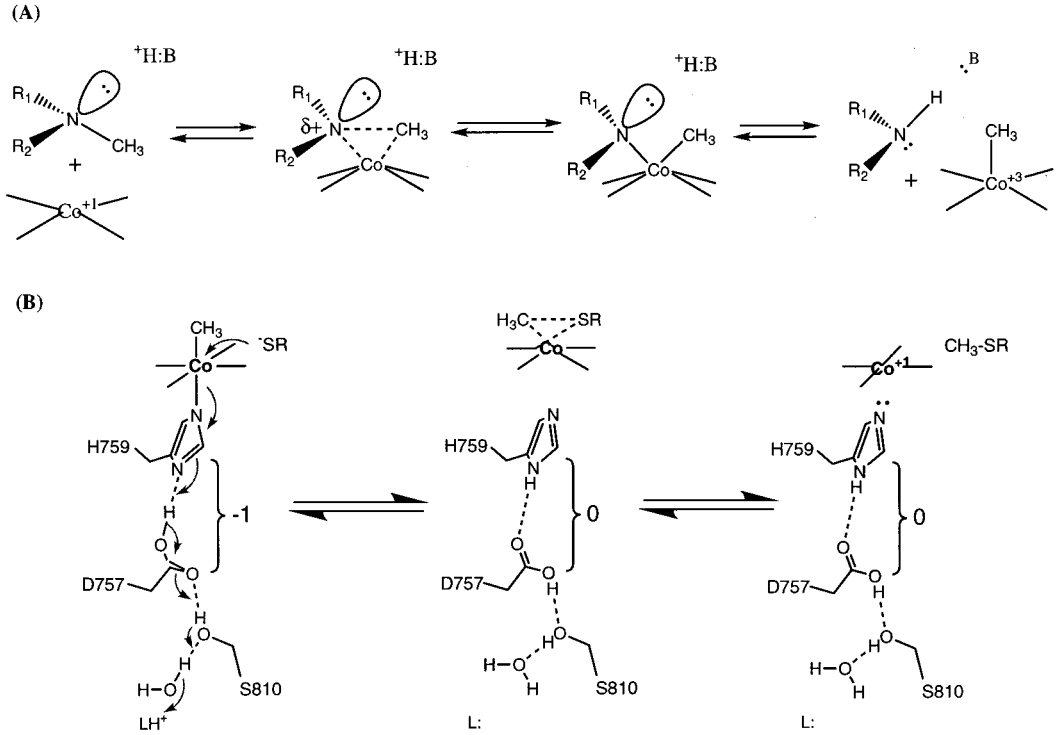
Coordinated group and proton transfers have not previously been observed in S_N2 reactions, where preactivation of the leaving group is the norm. However, some of the unactivated methyl donors that serve as substrates for methylation of corrinoid proteins cannot be preactivated by protonation or Lewis acid catalysis, e.g., aromatic methyl ethers. Thus, it is tempting to propose that our unexpected observation that CH_3-H_4folate is not protonated in binary enzyme−substrate complexes at neutral pH reflects a more general feature of corrinoid-dependent methyl transferase reactions.

Mechanisms for Methyl Transfer to and from Cobalamin

Three possible mechanisms for the methyl transfers catalyzed by methionine synthase can be envisioned: transfers by an S_N2 mechanism, transfers involving oxidative addition of the CH_3−X bond to the cobalt of the corrinoid, or transfers involving single electron transfer. In this section, I will discuss each mechanism in turn and the constraints on each mechanism imposed by our knowledge of the enzyme-catalyzed reactions.

Transfers involving an S_N2 mechanism have the virtues of simplicity and chemical precedent. Extensive physical organic studies of such reactions have established that the methyl group being transferred is partially bonded both to the incoming nucleophile and to the departing leaving

Scheme 3. Oxidative Addition Mechanisms: (A) for Methyl Transfer to Cobalamin from CH_3-H_4folate and (B) for the Reaction of Homocysteine with Methylcobalamin[a]



[a] Histidine of the ligand triad dissociates to allow formation of the cis adduct at the upper axial face of the cobalamin and dissociation is accompanied by protonation of the triad.

group. Our studies showing that CH_3-H_4folate is not protonated in binary enzyme−substrate complexes and that MetH(2−649) will not bind protonated CH_3-H_4folate suggest that if an S_N2 mechanism is employed for methyl transfer, N5 of the leaving group must be hydrogen bonded to a general acid catalyst, permitting proton transfer to accompany group transfer (Scheme 2). However, the structure of the CH_3-H_4folate−corrinoid iron/sulfur protein methyl transferase AcsE, which is homologous to the CH_3-H_4folate-binding module of MetH, did not reveal any obvious general acid catalysts positioned appropriately for hydrogen bonding to N5.[37]

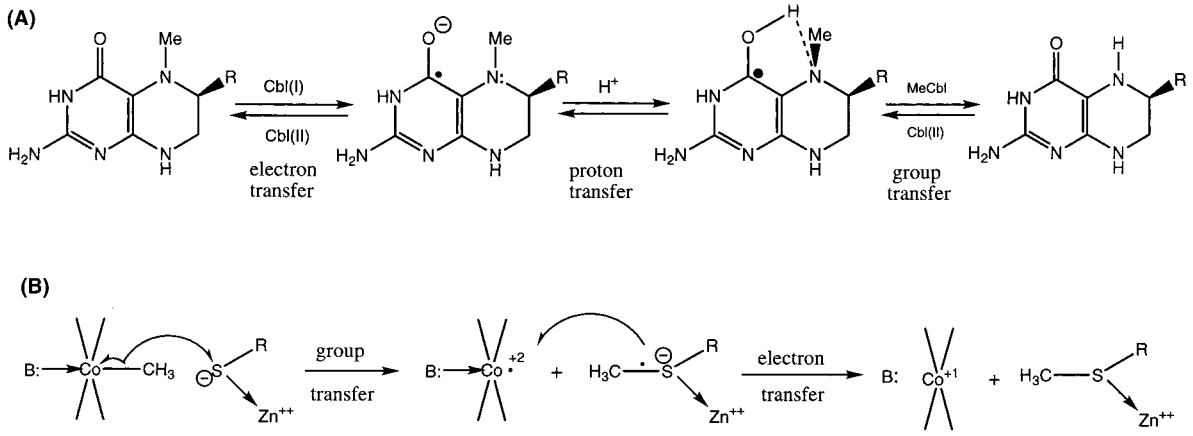
The stereochemical course of the overall reaction catalyzed by cobalamin-dependent methionine synthase, in which net retention is observed for the overall reaction,[38] is certainly consistent with successive S_N2 transfers of the methyl group to and from the cobalamin cofactor. The overall retention of methyl group stereochemistry in the transfer of a methyl group from CH_3-H_4folate to acetyl CoA by the acetyl CoA synthase complex is also consistent with successive S_N2 transfers in the reaction catalyzed by AcsE.[39]

An oxidative addition mechanism for methyl transfer from CH_3-H_4folate to cob(I)alamin is shown in Scheme 3A. Because this oxidative addition mechanism predicts that proton uptake occurs simultaneously with or following C−N bond cleavage, it could be applicable to the reactions of a wide variety of unactivated methyl donors to cobalamin, including methanol and aromatic methyl ethers. In this mechanism, the cob(I)alamin cofactor is proposed to form a three-centered bond with the CH_3−N5 moiety of CH_3-H_4folate: the empty 4s orbital of the cobalt would hybridize with the symmetry-matched σ orbital of the C−N bond, while a filled $3d_{xz}$ or $3d_{yz}$ orbital of the metal would be of appropriate symmetry to hybridize with the empty σ^* orbital of the C−N bond. These interactions would weaken the N5−methyl bond of CH_3-H_4folate, and the cobalt would serve as a Lewis acid to stabilize the H_4folate leaving group. As the C−N bond is cleaved, the pK_a of N5 of the folate substrate would increase from its value below 6 in the enzyme−CH_3-H_4-folate binary complex to a value above 30 in the H_4folate product. We predict that general acid catalysis would be required to permit the cleavage of the Co−N bond to form the H_4folate product.

Monovalent cationic transition metals in the gas phase undergo oxidative insertion into H−H, H−C, C−C, and C−X bonds.[40] Oxidative addition to the N−H bond of ammonia has been observed with cobalt(I) in the gas phase,[41] where a transient $[H-Co-NH_2]^+$ adduct can be detected using guided ion beam mass spectrometry. The bond between Co and nitrogen in this intermediate is surprisingly strong, with an estimated bond dissociation energy of 62 kcal/mol, and further decomposition of this intermediate by explusion of $^{\bullet}NH_2^-$ occurs only at very high energies. In contrast to the strong gas-phase Co^+−N bond, the bond dissociation energy of the gas-phase Co^+-CH_3 bond is weaker, at 51 kcal/mL.[42]

Crabtree[43] has defined the requirements for oxidative addition to a C−H bond by a metal−ligand system, and these same priniciples should govern insertion into a C−N bond as well: the system must be coordinatively unsatur-

Scheme 4. Single Electron Transfer Mechanisms: (A) for Methyl Transfer to Cobalamin from CH_3-H_4folate and (B) for Reaction of Homocysteine with Methylcobalamin



ated, there must be strong metal−carbon and metal−hydrogen (or metal−nitrogen) bonds, the metal−ligand system must be sterically uncongested, and a filled metal orbital must be available to interact with the σ^* orbital of the bond to be broken. Cob(I)alamin is, of course, coordinately unsaturated, in that it is thought to be four-coordinate.[44] However, unambiguous evidence for the formation of cis complexes of cobalamin is lacking, although π complexes of cob(III)alamin with olefins have been proposed.[45] The cobalt−nitrogen bond formed on cleavage of the N5−methyl bond is expected to be stronger than the Co−methyl bond by analogy with studies in the gas phase; the bond dissociation energy for homolysis of the Co−methyl bond of six-coordinate methylcobalamin is ~37 kcal/mL.[46] Cob(I)alamin does, indeed, have filled 3d orbitals of appropriate symmetry to interact with the σ^* orbital of the methyl−nitrogen bond. Finally, and perhaps most critically, the metal−ligand system must be sterically uncongested. One may, indeed, question whether cob(I)alamin, with its bulky corrin macrocycle, can be considered sterically uncongested. A critical issue for an oxidative addition mechanism is whether CH_3-H_4folate can be positioned close enough to the corrin ring to allow the orbital overlaps necessary for methyl transfer.

A clear point of distinction between an S_N2 mechanism and one involving oxidative addition is the relative orientation of the cobalamin vis-à-vis the methyl−nitrogen bond of CH_3-H_4folate. The oxidative addition mechanism would require that the C−N bond to be cleaved be parallel to the plane of the corrin ring. Thus, determination of the structures of complexes between enzyme-bound CH_3-H_4folate and cobalamin may distinguish between these alternatives.

The geometry of the proposed transition state for oxidative insertion suggests that methyl transfer from CH_3-H_4folate to cob(I)alamin would occur with retention of stereochemistry. Although there are few examples where the stereochemistry of oxidative addition to C−X bonds has been examined, indirect evidence suggests that reductive eliminations (the reverse of oxidative additions) that form C−C, C−H, and C−X bonds always proceed with retention of sterochemistry at carbon.[47] In this context, we note that the observation of retention of configuration at carbon in the overall transfer of a methyl group from CH_3-H_4folate to homocysteine requires that both half-reactions exhibit the same stereochemistry. That is, if an oxidative addition mechanism occurs with retention of configuration when the methyl group is transferred from CH_3-H_4folate to cob(I)alamin, a reductive elimination must be proposed for transfer of the methyl group from methylcobalamin to homocysteine (Scheme 3B).

Stereochemical analysis of the course of the individual half-reactions catalyzed by methionine synthase should distinguish between oxidative addition and S_N2 mechanisms for methyl transfer. However, such analysis is challenging due to complications caused by the rapid scrambling of chiral methyl groups in mixtures of free cob(I)alamin and methylcobalamin (Duilio Arigoni, personal communication). It will be necessary to carry out the methyl transfer with enzyme-bound cobalamin, where the protein serves as a cage to prevent scrambling, and to remove the chiral methyl group from methylcobalamin as an anion rather than a cation. We are currently collaborating with Bernard Golding to determine the stereochemistry of the individual half-reactions of methionine synthase.

The final mechanism that can be proposed involves single electron transfer (SET). In formulating an SET mechanism, it is important to bear in mind that the methyl transfer between CH_3-H_4folate and cob(I)alamin is completely reversible, so irreversible electron transfers are precluded. In the reverse direction, where the methyl group is transferred from methyl cobalamin to H_4folate, the electron must be able to reduce cob(II)alamin to cob(I)alamin, with a midpoint potential vs the standard hydrogen electrode of −626 mV![44] Bernard Golding has suggested the mechanism shown in Scheme 4A for methyl transfer between CH_3-H_4folate and cob(I)alamin, and a similar proposal for the methyl transfer between methylcobalamin and homocysteine is shown in Scheme 4B. The initial electron transfer from cob(I)alamin to CH_3-H_4-folate may be thermodynamically unfavorable; model studies of the reduction potential of tetrahydropterins

would be needed to assess the feasibility of such a proposal. However, viewing the mechanism in the reverse direction, the CH_3-H_4folate anion should be able to reduce cob(II)alamin to cob(I)alamin.

Because methyl radicals are so unstable, we would predict that the radical is never "free", and thus that the reaction would proceed with inversion of stereochemistry. However, the requirement for in-line geometry of the donor, the methyl group, and the acceptor may be relaxed in an SET mechanism, and the transition state for methyl transfer may be looser, perhaps permitting some racemization to occur. The stereochemical analysis of the methionine synthase reaction revealed significant racemization accompanying the methyl transfers,[38] although it was not clear whether racemization was intrinsic to the mechanism or resulted from the workup of the methionine product.

An attractive feature of the SET mechanism proposed for the homocysteine half-reaction is that it rationalizes the coordination of the thiol to zinc. Lewis acid activation would be required to stabilize the methionine radical formed by group transfer.

It should be apparent by now that there are attractive and unattractive features of each of the proposed mechanisms, and that much further research is needed to distinguish between them. Fifty years after elucidation of the structure of cobalamin, and forty years after the discovery that methylcobalamin functions in methylation of homocysteine, there remains much to learn about the role of cobalamin in methyl transfers.

References

(1) Stubbe, J. Binding site revealed of nature's most beautiful cofactor. *Science* **1994**, *266*, 1663−1664.
(2) Hodgkin, D. C.; Kamper, J.; Mackay, M.; Pickworth, J. Structure of vitamin B_{12}. *Nature* **1956**, *178*, 64−66.
(3) Eschenmoser, A. Chemical synthesis of corrinoids: current problems and recent advances. In *Vitamin B_{12}*; Zagalak, B., Friedrich, W., Eds.; W. de Gruyter: Berlin, 1979; pp 89−117.
(4) Woodward, R. B. Synthetic vitamin B_{12}. In *Vitamin B_{12}*; Zagalak, B., Friedrich, W., Eds.; W. de Gruyter: Berlin, 1979; pp 37−87.
(5) Lenhert, P. G.; Hodgkin, D. C. Structure of the 5,6-dimethylbenzimidazolylcobamide coenzyme. *Nature* **1961**, *192*, 937−938.
(6) Corrinoids either lack a dimethylbenzimidazole nucleotide substituent or employ an alternate nucleotide.
(7) Matthews, R. G. Cobalamin-dependent methionine synthase. In *Chemistry and biochemistry of B_{12}*; Banerjee, R., Ed.; John Wiley: New York, 1999; pp 681−706.
(8) Banerjee, R. V.; Frasca, V.; Ballou, D. P.; Matthews, R. G. Participation of cob(I)alamin in the reaction catalyzed by methionine synthase from *Escherichia coli*: a steady-state and rapid reaction kinetic analysis. *Biochemistry* **1990**, *29*, 11101−11109.
(9) Ragsdale, S. W. The acetogenic corrinoid proteins. In *Chemistry and biochemistry of B_{12}*; Banerjee, R., Ed.; John Wiley: New York, 1999; pp 633−653.
(10) Sauer, K.; Thauer, R. K. The role of corrinoids in methanogens. In *Chemistry and biochemistry of B_{12}*; Banerjee, R., Ed.; John Wiley: New York, 1999; pp 655−679.
(11) Kaufmann, F.; Wohlfarth, G.; Diekert, G. *O*-Demethylase from *Acetobacterium dehalogenans:* Cloning, sequencing, and active expression of the gene encoding the corrinoid protein. *Eur. J. Biochem.* **1998**, *257*, 5125−521.
(12) Wohlfarth, G.; Diekert, G. Reductive dehalogenases. In *Chemistry and biochemistry of B_{12}*; Banerjee, R., Ed.; John Wiley: New York, 1999; pp 871−893.
(13) Streitwieser, A., Jr. Stereochemistry of the primary carbon. I. Optically active *n*-butanol-1-*d*. *J. Am. Chem. Soc.* **1953**, *75*, 5014−5018.
(14) March, J. *Advanced Organic Chemistry*; John Wiley & Sons: New York, 1992; pp 250−252
(15) Hilhorst, E.; Iskander, A. S.; Chen, T. B. R. A.; Pandit, U. Model studies of the cobalamin-dependent methionine synthase reactions. *Tetrahdedron Lett.* **1993**, *34*, 4257−4260.
(16) Pratt, J. M.; Norris, P. R.; Hamza, M. S. A.; Bolton, R. Methyl transfer from nitrogen to cobalt: model for the B_{12}-dependent methyl transfer enzymes. *J. Chem. Soc., Chem. Commun.* **1994**, 1333−1334.
(17) Smith, A. E.; Matthews, R. G. The protonation state of methyltetrahydrofolate in a binary complex with cobalamin-dependent methionine synthase. *Biochemistry* **2000**, *39*, 13880−13890.
(18) Tackett, S. L.; Collat, J. W.; Abbott, J. C. The formation of hydridocobalamin and its stability in aqueous solution. *Biochemistry* **1963**, *2*, 919−923.
(19) Schrauzer, G. N.; Deutsch, E. Reactions of cobalt(I) supernucleophiles. The alkylation of vitamin B_{12s}, cobaloximes (I), and related compounds. *J. Am. Chem. Soc.* **1969**, *91*, 3341−3350.
(20) Zheng, D.; Darbre, T.; Keese, R. Methanol and dimethylaniline as methylating agents of Co(I) corrinoids under acidic conditions. *Inorg. Biochem.* **1999**, *73*, 273−275.
(21) Reuben, D. M. E.; Bruice, T. C. Reaction of thiol anions with benzene oxide and malachite green. *J. Am. Chem. Soc.* **1976**, *98*, 114−121.
(22) Hogenkamp, H. P. C.; Bratt, G.; Sun, S.-Z. Methyl transfer from methylcobalamin to thiols. A reinvestigation. *Biochemistry* **1985**, *24*, 6428−6432.
(23) Norris, P. R.; Pratt, J. M. Methyl transfer reactions of protein-free Co Corrinoids. *BioFactors* **1996**, *5*, 240−241.
(24) Banerjee, R. V.; Johnston, N. L.; Sobeski, J. K.; Datta, P.; Matthews, R. G. Cloning and sequence analysis of the *Escherichia coli metH* gene encoding cobalamin-dependent methionine synthase and isolation of a tryptic fragment containing the cobalamin-binding domain. *J. Biol. Chem.* **1989**, *264*, 13888−13895.
(25) Drummond, J. T.; Huang, S.; Blumenthal, R. M.; Matthews, R. G. Assignment of enzymatic function to specific protein regions of cobalamin-dependent methionine synthase from *Escherichia coli*. *Biochemistry* **1993**, *32*, 9290−9295.
(26) Goulding, C. W.; Matthews, R. G. Cobalamin-dependent methionine synthase from *Escherichia coli*: involvement of zinc in homocysteine activation. *Biochemistry* **1997**, *36*, 15749−15757.
(27) Goulding, C. W.; Postigo, D.; Matthews, R. G. Cobalamin-dependent methionine synthase is a modular protein with distinct regions for binding homocysteine, methyltetrahydrofolate, cobalamin, and adenosylmethionine. *Biochemistry* **1997**, *36*, 8082−8091.
(28) Zhao, S.; Roberts, D. L.; Ragsdale, S. W. Mechanistic studies of the methyltransferase from *Clostridium thermoaceticum*: Origin of the pH dependence of the methyl group transfer from methyltetrahydrofolate to the corrinoid/iron−sulfur protein. *Biochemistry* **1995**, *34*, 15075−15083.
(29) Weiss, D. S.; Gartner, P.; Thauer, R. K. The energetics and sodium ion dependence of N5-methyltetrahydromethanopterin:coenzyme M methyltransferase studied with cob(I)alamin as methyl acceptor and methylcob(III)alamin as methyl donor. *Eur. J. Biochem.* **1994**, *226*, 799−809.
(30) Bandarian, V.; Matthews, R. G. Quantitation of rate enhancements attained by the binding of cobalamin to methionine synthase. *Biochemistry* **2001**, *40*, 5056−5064.
(31) Peariso, K.; Goulding, C. W.; Matthews, R. G.; Penner-Hahn, J. The role of zinc in binding of homocysteine to cobalamin-dependent methionine synthase. *J. Am. Chem. Soc.* **1998**, *120*, 8410−8416.
(32) Jarrett, J. T.; Choi, C. Y.; Matthews, R. G. Changes in protonation associated with substrate binding and cob(I)alamin formation in cobalamin-dependent methionine synthase. *Biochemistry* **1997**, *36*, 15739−15748.
(33) LeClerc, G. M.; Grahame, D. A. Methylcobamide:coenzyme M methyltransferase isozymes from *Methanosarcina barkeri*. *J. Biol. Chem.* **1996**, *271*, 18725−18731.
(34) Sauer, K.; Thauer, R. K. Methanol:coenzyme M methyltransferase from *Methanosarcina barkeri*: zinc dependence and thermodynamics of the methanol:cob(I)alamin methyltransferase reaction. *Eur. J. Biochem.* **1997**, *249*, 280−285.
(35) Paul, L.; Krzycki, J. Sequence and transcript analysis of a novel *Methanosarcina* methyltransferase II homologue and its associated corrinoid protein homologous to methionine synthase. *J. Bacteriol.* **1996**, *178*, 6599−6607.
(36) Ragsdale, S. W.; Lindahl, P. A.; Münck, E. Mossbauer, EPR, and optical studies of the corrinoid/iron−sulfur protein involved in the synthesis of acetyl coenzyme A by *Clostridium thermoaceticum*. *J. Biol. Chem.* **1987**, *262*, 14289−14297.
(37) Doukov, T.; Seravelli, J.; Stezowski, J. J.; Ragsdale, S. W. Crystal structure of a methyltetrahydrofolate- and corrinoid-dependent methyltransferase. *Structure* **2000**, *8*, 817−830.

(38) Zydowsky, T. M.; Courtney, L. F.; Frasca, V.; Kobayashi, K.; Shimizu, H.; Yuen, L.-D.; Matthews, R. G.; Benkovic, S. J.; Floss, H. G. Stereochemical analysis of the methyl transfer catalyzed by cobalamin-dependent methionine synthase from *Escherichia coli* B. *J. Am. Chem. Soc.* **1986**, *108*, 3152−3153.
(39) Lebertz, H.; Simon, H.; Courtney, L. F.; Benkovic, S. J.; Zydowsky, L. D.; Lee, K.; Floss, H. D. Stereochemistry of acetic acid formation from 5-methyltetrahydrofolate by *Clostridium thermoaceticum*. *J. Am. Chem. Soc.* **1987**, *109*, 3173−3174.
(40) Armentrout, P. B. C−H bond activation by gas-phase atomic metal ions. In *Selective hydrocarbon activation: principles and progress*; Davies, J. A., Watson, P. L., Greenberg, A., Liebman, J. F., Eds.; VCH: New York, 1990; pp 467−533.
(41) Clemmer, D. E.; Armentrout, P. B. Investigation of isomeric intermediates: Co^+-NH_3 and $H-Co^+-NH_2$. *J. Am. Chem. Soc.* **1989**, *111*, 8280−8281.
(42) Armentrout, P. B.; Georgiadis, R. Periodic trends in gas-phase M−H and M−C bond energies. *Polyhedron* **1988**, *7*, 1573−1581.
(43) Crabtree, R. H. The organometallic chemistry of alkenes. *Chem. Rev.* **1985**, *85*, 245−268.
(44) Lexa, D.; Saveant, J.-M. The electrochemistry of vitamin B_{12}. *Acc. Chem. Res.* **1983**, *16*, 235−243.
(45) Silverman, R. B.; Dolphin, D. Model Studies for coenzyme B12-dependent enzyme-catalyzed rearragements. Evidence for cobalt(III)−olefin π complexes. *J. Am. Chem. Soc.* **1976**, *98*, 4626−4633.
(46) Martin, B. D.; Finke, R. G. Co−C homolysis and bond dissociation energy studies of biological alkylcobalamins: methylcobalamin, including a $>10^{15}$ $Co-CH_3$ homolysis rate enhancement at 25 °C following one electron reduction. *J. Am. Chem. Soc.* **1990**, *112*, 2419−2420.
(47) Flood, T. C. Stereochemistry of reactions of transition metal−carbon sigma bonds. In *Topics in inorganic and organometallic stereochemistry*; Geoffroy, G. L., Ed.; Wiley: New York, 1981; Vol. 12.
(48) Fujii, K.; Huennekens, F. M. Activation of methionine synthetase by a reduced triphosphopyridine nucleotide-dependent flavoprotein system. *J. Biol. Chem.* **1974**, *249*, 6745−6753.
(49) LeClerc, D.; Wilson, A.; Dumas, R.; Gafuik, C.; Song, D.; Watkins, D.; Heng, H. H. Q.; Rommens, J. M.; Scherer, S. W.; Rosenblatt, D. S.; Gravel, R. A. Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. *Proc. Natl. Acad. Sci., U.S.A.* **1998**, *95*, 3059−3064.
(50) Drennan, C. L.; Huang, S.; Drummond, J. T.; Matthews, R. G.; Ludwig, M. L. How a protein binds B_{12}: A 3.0 Å x-ray structure of the B_{12} binding domains of methionine synthase. *Science* **1994**, *266*, 1669−1674.

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