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Catalysis of Methyl Group Transfers Involving Tetrahydrofolate and B_{12}

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Abstract

This review focuses on the reaction mechanism of enzymes that use B_{12} and tetrahydrofolate (THF) to catalyze methyl group transfers. It also covers the related reactions that use B_{12} and tetrahydromethanopterin (THMPT), which is a THF analog used by archaea. In the past decade, our understanding of the mechanisms of these enzymes has increased greatly because the crystal structures for three classes of B_{12}-dependent methyltransferases have become available and because biophysical and kinetic studies have elucidated the intermediates involved in catalysis. These steps include binding of the cofactors and substrates, activation of the methyl donors and acceptors, the methyl transfer reaction itself, and product dissociation. Activation of the methyl donor in one class of methyltransferases is achieved by an unexpected proton transfer mechanism. The cobalt (Co) ion within the B_{12} macrocycle must be in the Co(I) oxidation state to serve as a nucleophile in the methyl transfer reaction. Recent studies have uncovered important principles that control how this highly reducing active state of B_{12} is generated and maintained.

I. Introduction to Methyltransferases and Their Cofactors

An organic chemist wishing to insert a methyl group into a compound might use methyltriflate or diazomethane. In biology, the methyl donors are much less explosive. Figure 10.1 shows some of the simple methyl donors that are found in nature like methanol, methylamines, methanethiols as well as the cofactors that are involved in methylation reactions like methyltetrahydrofolate (MTHF), *S*-adenosyl-*L*-methionine (SAM), and methyl-B_{12}. A methyltransferase catalyzes the transfer of a methyl group from one of the donors shown in Fig. 10.1, like MTHF or SAM, to an acceptor like homocysteine or the N6 group on adenine in DNA (Fig. 10.2).

Two cofactors figure prominently in methyltransferase chemistry: vitamin B_{12} (or cobalamin) and tetrahydrofolate (THF). The history of vitamin B_{12} dates back to its description as the antipernicious anemia factor (Minot and Murphy, 1926; Whipple and Robscheit-Robbins, 1925). Cobalamin was isolated by Smith and Folkers in 1948 (Rickes *et al.*, 1948; Smith, 1948) and its structure was crystallized (Rickes *et al.*, 1948) and its structure was determined in 1956 (Hodgkin *et al.*, 1956). Like heme, F_{430}, and chlorophyll,

vitamin B_{12} is a tetrapyrrolic cofactor with a central Co atom coordinated by the four equatorial pyrrole nitrogen ligands (Fig. 10.1). Extending from one of the pyrrole rings is a propanolamine-linked group that can serve as the lower axial ligand to Co. The lower ligand is dimethylbenzimidazole in cobalamins, while the upper axial ligand is a cyano-, methyl-, or 5′-deoxyadenosyl- group in vitamin B_{12},methylcobalamin (MeCbl[1]), and adenosylcobalamin (AdoCbl) or coenzyme B_{12}, respectively. The biological role of MeCbl as an essential coenzyme for a methyltransferase (Guest *et al.*, 1962) was revealed a few years after a role for AdoCbl as the coenzyme for glutamate mutase was discovered (Barker *et al.*, 1958).

Tetrahydrofolate (THF) is the reduced form of the vitamin folic acid, which was first recognized as the compound in brewer's yeast that can reverse anemia. Folic acid was isolated in a highly purified form from 4 tons of spinach leaves by Esmond Snell, Herschel Mitchell, and Roger Williams (Mitchell *et al.*, 1941). The vitamin was crystallized soon after (Pfiffner *et al.*, 1945; Stokstad, 1943) and synthesized in 1946 by Lederle Laboratories (Angier *et al.*, 1946). Besides serving as a cofactor in methyltransferase reactions, THF is the major one-carbon carrier in cells and is needed for protein and DNA synthesis and is important in nitrogen metabolism. Its essentiality in such key metabolic processes makes THF metabolism a key target for anticancer and antimicrobial drugs (M.P. Costi, S. Ferrari, 2001).

There are two classes of methyltransferases, which differ in their use of an activated versus an unactivated methyl group donor. One class of methyltransferases, exemplified by methionine synthase, use an unactivated methyl donor and an intermediate methyl carrier, cobalamin, which in the Co(I) oxidation state is an extremely potent nucleophile that can react with the methyl group of MTHF to generate an intermediate organometallic MeCbl intermediate. The methyl group is then transferred from MeCbl to the final acceptor, which for methionine synthase is homocysteine, generating methionine. The other class of methyltransferases uses an activated methyl donor in the form of SAM to directly methylate the N6 of adenine or the amino groups of lysine or arginine in histones, and so on. This review focuses on the first class of methyltransferases that utilize THF and B_{12}.

In all methyltransferase-catalyzed reactions, as shown in Fig. 10.3, transfer of the methyl group involves cleavage of a methyl-X bond, where “X” can be one of a variety of functional groups (N, S, Cl, O, etc.). Heterolysis can lead to two products: a methyl cation or a methyl anion. On the contrary, homolysis leads to a methyl radical. For methyl group transfers, heterolysis is most common, leading to the formation of a methyl carbocation equivalent.

II. Three Component Systems Required for B_{12}/THF-Dependent Methyltransferases

All B_{12}-dependent methyltransferases contain three components, as indicated in Fig. 10.4 and Table 10.1. In these systems, the “B” component binds the methyl donor, a “C” component binds B_{12}, and an “A” component binds the methyl group acceptor. An alternative nomenclature in use describes the B component as MT1 and the A component as MT2. Recent literature references for each methyltransferase system are given in Table 10.1. In each case, the methyl group is unactivated, that is, it is a secondary alcohol, an amine (primary, secondary, or tertiary), or a thiolate. The required electrophilic activation is accomplished by the “B” component, as described in more detail below. The “C” component supplies the supernuclophilic Co(I) state of B_{12}, which acts as an intermediary methyl group acceptor that interacts with the “B” component to accept the methyl group from the

substrate, forming MeCbl. The C Component then interacts with the A component to transfer the methyl group to the ultimate methyl group acceptor.

III. Biological Systems Impacted by B_{12} and Folate-Dependent Methyltransferases

A. Methionine biosynthesis

B_{12}-dependent methyltransferases play an important role in microbial and eukaryotic metabolism. Biosynthesis of methionine in most microbes and eukaryotes depends upon the B_{12}- and THF-dependent enzyme, methionine synthase (MetH). Since methionine is the precursor of SAM, methionine synthase supports the many methyltransferases involved in methylation of DNA, proteins, and neurotransmittors (Fig. 10.2). An elevated level of homocysteine is linked to a number of pathological states, including premature heart disease and neural tube defects and methionine synthase plays an important role in controlling homocysteine homeostasis (Col *et al.*, 2007).

B. Methanogenesis

As shown in Fig. 10.5, growth of methanogens requires the conversion of substrates (CO_2, methylamines, methylthiols, and acetate) to methyl- SCoM. The nickel containing enzyme methyl-SCoM reductase then catalyzes the conversion of methyl-SCoM to methane. Table 10.1 lists six B_{12}-dependent methyltransferases that are important in growth of methanogenic archaea. Most of these methyltransferases catalyze the transfer of a methyl group from the methyl donor (Methyl-X) to CoM, thus providing methyl-SCoM needed for methane formation. These methanogenic methyltransferases use B_{12} (or an analog with a slight modification in the benzimidazole component) bound to the “C” component. Growth on acetate or H_2/CO_2 involves the generation of methyltetrahydromethanopterin (MTHMPT), which is converted to methyl-SCoM by the Mtr system. As described below, this is interesting membrane-bound system links the methyl transfer reaction to generation of a sodium ion gradient, which is utilized to make ATP (Gottschalk and Thauer, 2001).

C. Methanogenic methyltransferases and the 22nd amino acid

Study of the methanogenic methyltransferases has uncovered the 22nd amino acid, pyrrolysine, which is encoded by a UAG stop codon. This system is reminiscent of selenocysteine, encoded by the UGA codon. For pyrrolysine synthesis, there is an enzymatic system, encoded by the *pylBCD* genes to synthesize pyrrolysine, a dedicated pyrrolysyl-tRNA synthetase (encoded by *pylS*), which aminoacylates a specific amber-decoding tRNA (encoded by the *pylT* gene) with pyrrolysine (Blight *et al.*, 2004; Longstaff *et al.*, 2007). Thus, like the other 20 natural amino acids, pyrrolysine is co-translationally placed into the nascent polypeptide chain. The role of pyrrolysine in the methyltransferase reaction is discussed below.

D. Acetogenesis

A role for B_{12} in anaerobic CO_2 fixation was described in ~1965 (Ljungdahl *et al.*, 1966; Poston *et al.*, 1964). There are two B_{12}-dependent methyltransferases involved in the Wood-Ljungdahl pathway of CO_2 fixation (Fig. 10.6). The first, MTHF: corrinoid iron-sulfur protein (CFeSP) methyltransferase is encoded by the *acsE* gene and catalyzes transfer of the methyl group from MTHF to the Co(I)-B_{12} site in the CFeSP, which is encoded by the *acsCD* genes. In the subsequent reaction, the methylated CFeSP transfers the methyl group to a NiFeS cluster in acetyl-CoA synthase (ACS); thus, this is a metal (Co) to metal (Ni) methyl group transfer between two proteins. ACS then catalyzes the condensation of its Ni-bound methyl group with CO (generated by CO dehydrogenase, CODH) and coenzyme A to

generate acetyl-CoA. The series of organometallic intermediates is one of the novel features of the Wood-Ljungdahl pathway.

Two other methyltransferase systems that are shown in Fig. 10.6 couple to the Wood-Ljungdahl pathway. The MtvABC system transfers the methyl group of vanillate or other methoxylated aromatics to THF to generate MTHF (Engelmann *et al.*, 2001; Naidu and Ragsdale, 2001), while MtaABC catalyzes the synthesis of MTHF from the methyl group of methanol and THF (Das *et al.*, 2007). MtvB was shown to catalyze methyl transfer from the aromatic phenylmethyl ether to the corrinoid component of MtvC and MtvA catalyzed the MtvC-dependent methylation of THF (Naidu and Ragsdale, 2001). The sequence homology between the Mtv and Mta systems suggests that the A, B, and C components in the two systems share similar functions (Das *et al.*, 2007).

E. Other metabolic systems in which B_{12}- and folate-dependent methyltransferases play a key role

Growth of *Sphingomonas paucimobilis* SYK-6 on lignin-derived biaryls and monomers requires the *ligM* gene, which is homologous to *mtvB*, to catalyze the transfer of the methyl group from vanillate to THF. Since the *ligM* gene in this organism is located in the same gene cluster as two genes encoding THF-dependent enzymes, it is likely that lignin degradation is linked directly to one-carbon metabolism (Abe *et al.*, 2005). However, unlike the methyltransferase reactions described in the previous section, these remain to be characterized.

IV. Structure and Function of B_{12} in Methyltransferases

A. Binding of B_{12} to the enzymes

Three modes of B_{12} binding have been described: “dmb-on,” “dmb-off ”/“his-on,” and “base-off,” which refers to whether or not a lower axial ligand is coordinated to Co (Fig. 10.7). Thus, “dmb-on” indicates that the dimethylbenzimidazole group, which is appended to one of the tetrapyrrole rings, is ligated to Co, while “his-on” refers to the state in which the dmb ligand is replaced by a His residue donated by the protein. “Dmb-on” and “dmb-off ” have often been referred to as “base-off ” and “base-on”; however, this is inaccurate because the His ligand also acts as a base. It is more accurate to only refer to the “base-off” binding mode when there is no nitrogenous base ligand. Such a coordination mode was first revealed by spectroscopic studies of the CFeSP in the Wood-Ljungdahl pathway (Ragsdale *et al.*, 1987) and recently confirmed in the crystal structure (Svetlitchnaia *et al.*, 2006). In most cases, unambiguous definition of the ligation state, that is, “dmb-on”, “dmb-off”, “base-off ”, was first revealed by spectroscopic studies. For example, a “dmb-off” state was revealed for several proteins by electron paramagnetic resonance (EPR) spectroscopy (Ragsdale *et al.*, 1987) (Stupperich, 1990, #220) several years before a crystal structure revealed a “his-on”“dmb-off ” binding mode in the B_{12}-binding domain of methionine synthase (Drennan *et al.*, 1994). EPR spectroscopic studies have been successful in revealing the ligation mode in a number of other enzymes (Abend *et al.*, 1998, #6281; Yamanishi *et al.*, 1998, #2310; Lawrence *et al.*, 1999, #4157; Abend *et al.*, 1999, #3897; Ke *et al.*, 1999, #6277). The EPR spectrum of the Co(II) state of B_{12} is particularly diagnostic of the axial ligation state because the unpaired electron in the d_z^2 orbital exhibits strong interactions with the axial ligand. If this ligand is a nitrogen atom, with a nuclear spin (I) of 1, each of the eight hyperfine lines (due to splitting of the resonance by interactions with the Co nucleus with $I = 7/2$) in the EPR spectrum exhibit a three-line splitting. The “base-off ” mode of B_{12} binding is characterized by the presence of singlets instead of triplets at each of the eight resonant positions, as was observed in the EPR spectrum of the CFeSP (Ragsdale *et al.*, 1987). The “his-on” mode is clearly indicated by adding ^{15}N-His to the growth

medium, which replaces the natural abundance histidine (mostly ^{14}N) in enzymes. Since ^{15}N has a nuclear spin of ½, doublet instead of triplet superhyperfine lines are observed at each of the eight resonant positions in the EPR spectrum. Observation of the triplet spectrum in B_{12} proteins labeled with ^{15}N-His suggests a "dmb-on" binding mode.

Crystallographic studies of methyltransferases have revealed the elegant molecular details of how proteins bind B_{12}. The crystal structures of the cobalamin-binding component of several methyltransferases are available, including the B_{12}-binding domain of methionine synthase in several states (Bandarian *et al.*, 2002, 2003; Drennan *et al.*, 1994), MtaC (Das *et al.*, 2007) (Fig. 10.8) and the CFeSP (AcsCD) from *M. thermoacetica* (Svetlitchnaia *et al.*, 2006) (Fig. 10.9), and the MtaBC complex from *Methanosarcina barkeri* (Hagemeier *et al.*, 2006). In all of these methyltransferase structures, cobalamin is bound within a Rossman *α/β* fold (Fig. 10.10). The "C" components that have a "dmb-off/his-on" mode of B_{12} binding share a sequence motif DX**H**XXGX_{41}SXL$X_{26–28}$GG in which the His is the lower axial ligand. The Asp, His, and Ser residues in this sequence are referred to as the catalytic triad and facilitate formation of the "base-off " conformation by protonating the His ligand, (Ludwig and Matthews, 1997). In the *M. thermoacetica* MtaC, the Asp and His are present in the catalytic triad (Fig. 10.6); however, it appears that a Thr residue replaces Ser. The dmb side chain is deeply embedded and is responsible for much of the binding energy that tethers the cobalamin to the protein. A "dmb-on" structure is not available in the methyltransferase class, which is somewhat surprising since several AdoCbl-dependent isomerases share the "dmb-on" binding mode, including diol dehydratase (Abend *et al.*, 1998; Shibata *et al.*, 1999; Yamanishi *et al.*, 1998), ribonucleotide reductase (Lawrence *et al.*, 1999; Sintchak *et al.*, 2002), and ethanolamine ammonia lyase (Abend *et al.*, 1999; Ke *et al.*, 1999).

Most of the methyltransferases share the "dmb-off/his-on" binding mode, while the CFeSP involved in transferring the methyl group to the ACS component in the Wood-Ljungdahl pathway is in the "base-off " state, as revealed by spectroscopic studies (Ragsdale *et al.*, 1987) and confirmed by crystallographic studies (Svetlitchnaia *et al.*, 2006) of the CFeSP (Fig. 10.9). A related protein in the methanogenic acetyl-CoA decarbonylase synthase complex also is in the "base-off " state (Jablonski *et al.*, 1993). Proteins that bind B_{12} in the "base-off " state lack the DXH . . . signature sequence. Instead, they contain a relatively hydrophobic helix below the plane of the cobalamin (SVLTAWAA) (Fig. 10.9). A coordinating water molecule in the upper axial position is replaced by a methyl group during catalysis. The EPR spectrum of the CFeSP in H_2 ^{17}O exhibits ^{17}O-induced hyperfine broadening, providing conclusive demonstration that H_2O coordinates to the metal center in one of the open axial positions (Stich *et al.*, 2006).

B. Generation and maintenance of the active Co(I) state of B_{12}

Cobalt in B_{12} can exist in the (I), (II), and (III) states. Cobalt cycles between the Co(I) and methyl-Co(III) states during catalysis. In the Co(I) state, Co has a d^8 configuration. In B_{12} and related corrinoids, Co(I) is a supernucleophile (Schrauzer and Deutsch, 1969; Schrauzer *et al.*, 1968) and is weakly basic, with a pK_a below 1 for the Co(I)-H complex (Tackett *et al.*, 1963). Protein-bound Co(I) is also highly reducing with a standard reduction potential for the Co(II)/(I) couple below −500 mV (Banerjee *et al.*, 1990b). These properties make Co(I) fairly unstable and subject to inactivation. For example, in cobalamin-independent methionine synthase, the Co(I) center undergoes oxidative inactivation to the 2^+ state once in every 100–2,000 turnovers (Drummond *et al.*, 1993; Fujii *et al.*, 1977). The Co(II)/Co(I) reduction potential of the CFeSP-bound corrinoid in the anaerobic microbial system (AcsA-E), is −504 mV (Harder *et al.*, 1989), and the Co(I) intermediate is oxidized to the catalytically inactive Co(II) state once every 100 turnovers (Menon and Ragsdale, 1999).

Once oxidative inactivation occurs, the B_{12}-dependent methyltransferases require reductive activation to reenter the catalytic cycle. This difficult reduction of the inactive Co(II) to the Co(I) state is accomplished by different systems. In cobalamin-independent methionine synthase, the unfavorable one-electron reduction is coupled to the highly exergonic demethylation of SAM, forming MeCbl (Banerjee *et al.*, 1990a). In *E. coli*, flavodoxin is the electron donor, while, in humans, the donor is methionine synthase reductase (Olteanu and Banerjee, 2001). The reductive methylation reaction is necessary for reactivation of the Co(II) state because the quinone/ hydroquinone and hydroquinone/semiquinone couples of the *E. coli* flavodoxin have a significantly more positive redox potential (~ −250 mV and −450 mV, respectively (Vetter and Knappe, 1971)), than the Co(II)/(I) couple of methionine synthase (−526 mV) (Banerjee *et al.*, 1990c) (Olteanu, 2004, #6302). Before reductive activation, the His ligand dissociates from the Co center to generate the “base-off ” conformation. The rationale for generating the “base-off ” state is that the nitrogen ligand would donate electron density to the Co center, making the reduction more difficult. In methionine synthase, binding of flavodoxin, the redox partner responsible for reductive activation, leads to dissociation of the His ligand (Hoover *et al.*, 1997). Removal of the dmb ligand also is an intermediate step in the electrochemical reduction of Co(II) to the Co(I) state of B_{12} in solution (Lexa and Savéant, 1976). Thus, methyltransferases appears to have evolved a mechanism to facilitate the reductive activation that can be understood based on the principles of inorganic chemistry and electrochemistry.

While SAM-dependent reductive methylation is used to return Co to the catalytic cycle in methionine synthase, in the methanol- (Daas *et al.*, 1996b) and dimethylamine- (Wassenaar *et al.*, 1998) methyltransferases, an ATP-dependent activating protein is involved. ATP-dependent activation also appears to be required for the aromatic O-demethylase from some acetogens (Kaufmann *et al.*, 1998).

In the *M. thermoaceticum* MTHF:CFeSP methyltransferase involved in the Wood-Ljungdahl pathway, the inactive Co(II) state of the CFeSP is already “base-off ” in the resting enzyme, which represents a “ready” state for electron transfer to form a four-coordinate Co(I) state (Harder *et al.*, 1989; Ragsdale *et al.*, 1987; Stich *et al.*, 2006; Wirt *et al.*, 1993, 1995). The direct electron donor is the [4Fe-4S] cluster of the AcsC subunit of the CFeSP (Menon and Ragsdale, 1999). This cluster has a reduction potential of −523 mV (Harder *et al.*, 1989), which is nearly isopotential with the Co(II)/Co(I) couple of the CFeSP-bound B_{12} and can accept electrons from a low-potential ferredoxin or directly from enzymatic systems that couple to ferredoxin, including CO/CODH, H_2/hydrogenase, or pyruvate/pyruvate ferredoxin oxidoreductase (Menon and Ragsdale, 1999). These low potential electron donors have a reduction potential similar to that of the Co(II)/(I) couple, which probably explains why this system does not require coupling to ATP or reductive methylation as in the systems described above.

C. The importance of the “dmb-off”/“dmb-on” equilibrium

Kinetic and thermodynamic studies of alkylated corrinoids have shown that the nature of the lower ligand strongly influences the rate and mode of Co–C bond breaking (Hogenkamp *et al.*, 1965; Kräutler, 1987; Pratt, 1999). Since most of the methyltransferase “C” components contain a Co-N-His bond replacing the Co-N-dmb ligation in the free cofactor, enzymatic (Dorweiler *et al.*, 2003) and model (Fasching *et al.*, 2000) studies have been conducted to determine if this ligand switch conferred a special mechanistic advantage. On the basis of studies of methyl transfer from MTHF to Co(I) in methionine synthase, it was concluded that the replacement of 5,6-dimethylbenzimidazole by imidazole has little effect on the kinetics of the methyl transfer reaction. This is in accord with studies of B_{12} models by Kräutler, which confirm the expectation that imidazole and benzimidazole are comparable as axial ligands (Fasching *et al.*, 2000). However, it was noted that the imidazole nitrogen in

imidazolylcobamide allows pH-dependent control of reactivity by protonation/deprotonation of the axial ligand, since the pK_a of the coordinating nitrogens in imidazole and benzimidazole are 7.0 (Datta and Grzybowski, 1966) and 5.5 (Catalan *et al.*, 1983; Lane and Quinlan, 1960), respectively. Accordingly, the pK_a of the imidazole ligand is 1.4 pH units higher than that of 5,6-dimethylbenzimidazole (pK_a = 2.9) in the methyl- Co(III) states (Fasching *et al.*, 2000).

Complete removal of the axial donor ligand to Co is expected to markedly affect the methyl transfer reaction. The presence of an N-donor ligand in the lower axial ligand position inhibits heterolytic cleavage, whereas O-donor ligands, such as water, do not exhibit this inhibition. For example, MeCbl reacted with homocysteinethiolate at least 1500-fold more slowly than methylcobinamide (Norris and Pratt, 1996). This axial ligand effect appears to be recapitulated in the *M. thermoacetica* CFeSP in which transfer of the methyl group from free methyl-cobinamide (lacking the lower axial dimethylbenzimidazole ligand) to the nickel center in ACS occurs at a rate ~10^3 times faster than that with free MeCbl (Seravalli *et al.*, 2001). Furthermore, in methyltransferases with a "dmb-off/ his-on" conformation, such as methionine synthase, the His ligand to the Co ion is removed to facilitate reduction of the inactive Co(II) enzyme. A similar axial His ligand dissociation from the Co(II) center has been observed upon binding of the ATP-dependent activating protein to the methanogenic MtaABC system from *M. barkeri* (Daas *et al.*, 1996a). A "catalytic triad" consisting of His759, Asp757, and Ser810 in methionine synthase, which is conserved among the "dmb-off/his-on" proteins, is involved in removal of this axial ligand by protonating the N-atom that ligates to Co, as illustrated by proton uptake associated with reduction of Co(II) to Co(I) (Hoover *et al.*, 1997). The role of ligand dissociation on methionine synthase takes on special significance since this coordination state change is accompanied by a global conformational change in the protein (Hoover *et al.*, 1997). There was only a minor affect of the axial ligation on the rates of methyl transfer between exogenous cobalamin cofactor and folate bound to the N-terminal domains of methionine synthase (residues 2–649) (Dorweiler *et al.*, 2003). In fact, based on the minor effect of the axial ligand on the methyl transfer from MTHF to Co(I) in methionine synthase, it was concluded that the primary role of the ligand triad is to control the conformational equilibria during catalysis, rather than to control axial ligation (Dorweiler *et al.*, 2003). These major conformational changes are an exciting hallmark of the catalytic cycle of methionine synthase.

Other effects of the protein, besides the N-donor ligand, on the methyl transfer reaction are observed in methyltransferases. In the CFeSP, the Co–OH_2 bond is lengthened by ~0.2 A, which is proposed to further enhance reduction of the Co(II) species (Stich *et al.*, 2006). Evidence for this enhancement is suggested by the 100-fold faster methylation of ACS by the methylated CFeSP than by free $MeCbi^+$. This rate enhancement is likely due to a combination of favorable protein–protein interactions between ACS and CFeSP and enzyme-induced elongation of the lower axial ligand bond (Stich *et al.*, 2006). Lengthening the Co–OH_2 bond in the methyl-Co(III) state would stabilize the unoccupied Co $3d_z^2$ orbital, increasing mixing of this orbital with the corrin-based occupied frontier orbitals, effectively making the Co center more "Co(I)-like," which would facilitate heterolytic Co–C bond cleavage. Furthermore, partially dissociating the lower water ligand would discourage homolytic bond cleavage given the strong preference of Co(II)corrinoids to retain an axial ligand.

V. Activation of the Methyl Group Donors

A. Binding of the methyl group donor to the Mt“x”B (MTII) component

The substrates that donate the methyl groups in biology vary in size from methanol (32 Da) to MTHF (443 Da for the monoglutamate derivative). Thus, the size of the substrate-binding site must vary markedly among different methyltransferases. The electronic properties also differ, since the methyl group is bonded to a hydroxy, phenoxy, thiol, or amine (primary, secondary, or tertiary) group. Regardless, in all methyltransferases, the domain/protein that binds the methyl group donor folds into an *α/β* triosephosphate isomerase (TIM) barrel structure, as observed in the crystal structures of the methanol-binding protein MtaB (Hagemeier *et al.*, 2006), the homocysteine and MTH-binding domains of methionine synthase (Evans *et al.*, 2004), the methylamine-binding protein MtmB (Hao *et al.*, 2002), the MTHF:CFeSP methyltransferase (Doukov *et al.*, 2000, 2007), and even the MTHF-binding domain of the corrinoid-independent methionine synthase (MetE) (Pejchal and Ludwig, 2005). In all of these proteins, the methyl donor binds within the cavity formed by the TIM barrel, as shown in the electrostatic surface rendering of the structure of the MTHF:CFeSP methyltransferase (Fig. 10.11). The red surface at the MTHF-binding site indicates the negative charge, which complements positively charges on the substrate. MTHF (or THF) is strongly cemented within this cavity (Kd<10 μM) by hydrogen bonds that are conserved between MTHF: CFeSP methyltransferase and methionine synthase (Fig. 10.12). The MTHF-binding site also resembles the binding site for the related pterin substrate (dihydropteroate) in dihydropteroate synthase (Achari *et al.*, 1997; Hampele *et al.*, 1997), with conserved residues D75, N96, and D160, forming a “pterin hook” (Doukov *et al.*, 2000).

Based on the structure of the MtaBC complex and on biochemical studies, it was proposed that methanol binds between a Zn ion in the TIM barrel of MtaB and the cobalamin site in MtaC (Fig. 10.13) (Evans *et al.*, 2004). In the case of the methylamine methyltransferase (MtmB), a novel amino acid, pyrrolysine, is located within the cavity of the TIM barrel (Fig. 10.14) (Hao *et al.*, 2002). Biochemical and crystallographic results indicate that the methylamine substrate forms a covalent complex with pyrrolysine as part of the activation mechanism (Fig. 10.15), as discussed in more detail below.

B. General acid catalysis, Lewis acid catalysis, and covalent catalysis to accomplish electrophilic activation of the methyl group donor

Among the methyltransferases, a similar principle underlies the activation of the methyl group that will be transferred: to donate positive charge to the heteroatom attached to the methyl group, leading to electrophilic activation of the methyl group. There is a solid chemical basis for this mechanism. Quaternary amines, with a full positive charge on nitrogen, do not require activation, as shown by studies of methyl transfer from a variety of quaternary ammonium salts to Co(I)-cobaloxime (Hilhorst *et al.*, 1994), trimethylphenylammonium cation to cob(I)alamin (Pratt *et al.*, 1994), and dimethylaniline at low pH to Co(I)-cobyrinate (Zheng *et al.*, 1999). In addition, methyl transfer is catalyzed by Lewis acids, such as Zn(II) (Wedemeyer-Exl *et al.*, 1999). Among the various methyltransferases, different mechanisms are used to accomplish electrophilic activation of the methyl group, including general acid catalysis in which proton transfer occurs to the N5 group on the pterin of MTHF, Lewis acid catalysis involving binding of methanol to a Zn(II) site, and covalent catalysis in which an adduct is formed between the methyl donor and a pyrrolysine residue.

In the methyltransferases that transfer the methyl group of MTHF to cobalamin, protonation of the N5 group of MTHF leads to electrophilic activation of the methyl group (Fig. 10.16).

This mechanism has been most thoroughly studied in methionine synthase and the *M. thermoacetica* MTHF: CFeSP methyltransferase. Proton uptake has been measured with pH indicators, by transient kinetics, and nuclear magnetic resonance (NMR) (Seravalli *et al.*, 1999; Smith and Matthews, 2000). The protonation step also has been studied by following the pH dependencies of the steady state and transient reaction kinetics of the MTHF:CFeSP methyltransferase (Zhao *et al.*, 1995) and methionine synthase (Matthews, 2001) and by studies of variants that are compromised in acid–base catalysis (Doukov *et al.*, 2007). Thus, in both methionine synthase and MTHF:CFeSP methyltransferase, the rate of reaction of CH_3-H_4folate with the protein-bound or exogenous cobalamin increases as the pH is lowered (with a pK_a of 5.6–6.0) and the reverse reactions exhibit the opposite pH-rate profile (Matthews, 2001; Zhao *et al.*, 1995). Furthermore, binding of CH_3-H_4folate to MTHF:CFeSP methyltransferase is coupled to proton uptake from solution with a pH profile similar to that of the methyl transfer reaction (Seravalli *et al.*, 1999), suggesting protonation in the binary complex. Surprisingly, when a similar experiment was performed with the MetH(2–649) fragment, proton release rather than proton uptake is observed (Smith and Matthews, 2000). Thus, it is clear that general acid catalysis facilitates the methyl transfer reaction, but whether this occurs in the binary or ternary complex, or perhaps in the transition state for the methyl transfer reaction remains undecided. Perhaps methionine synthase and MTHF:CFeSP methyltransferase differ in this aspect of catalysis.

The crystal structure of the MTHF:CFeSP methyltransferase revealed no obvious proton donor within H-bonding distance of the N5 position of CH_3-H_4 folate and the only amino acid located near enough to N5 to participate in H-bonding is the side chain of Asn199 (Fig. 10.16) (Doukov *et al.*, 2007). Although Asn is not chemically suitable to be a proton donor, it is conserved in all methyltransferases. The recent crystal structures of the binary complexes of CH_3-H_4folate bound to methionine synthase (Evans *et al.*, 2004) and the MTHF:CFeSP methyltransferase (Doukov *et al.*, 2007) reveal very similar environments around N5 of the pterin (Fig. 10.16). An important role for the Asn residue is indicated by its movement from a distant position to within H-bonding distance of the N5 atom upon CH_3-H_4folate binding, where it becomes part of an extended H-bonding network that includes several water molecules that are also conserved in the methionine synthase structure.

The lack of a discernable proton transfer pathway is seen in a number of enzymes, including methionine synthase (Evans *et al.*, 2004), dihydrofolate reductase (Rod and Brooks, 2003), and purine nucleoside phosphorylase (Fedorov *et al.*, 2001). Figure 10.16 shows the similarity in the H-bonding patterns among these proteins. Given the provocative location of Asn199 in MTHF:CFeSP methyltransferase and its potential role in transition state stabilization in the transmethylation reaction, an Asn199 variant was prepared by site-directed mutagenesis and the properties of this variant were compared to those of the wild-type protein by kinetic and structural studies to evaluate the contribution of this residue to catalysis (Doukov *et al.*, 2007). These experiments are consistent with the involvement of an extended H-bonding network in proton transfer to N5 of the folate that includes Asn199, a conserved Asp (Asp160), and a water molecule. This situation is reminiscent of purine nucleoside phosphorylase, which involves protonation of the purine N7 in the transition state and is accomplished by an extended H-bonding network that includes water molecules, a Glu residue, and an Asn residue (Kicska *et al.*, 2002). Similarly, in MTHF: CFeSP methyltransferase, an N199A variant exhibits only ~20-fold weakened affinity for CH_3-H_4folate, but a much more marked 20,000–40,000-fold effect on catalysis, suggesting that Asn199 plays an important role in stabilizing a transition state or high-energy intermediate for methyl transfer (Doukov *et al.*, 2007). Thus, we speculate that the conformation of Asn in the transition state for methyl transfer resembles that of the Asn243 in the phosphorylase in which the carboxamide oxygen would accept the H-bond from N5-H, and the

carboxamide nitrogen would donate a H-bond to O_4 (Fig. 10.17). This dual H-bonding function could rationalize the placement of Asn at this key position in the methyltransferases.

In the methyl group transfer from methanol to CoM, catalyzed by the methanogenic MtaB, activation by protonation of the hydroxyl group is not feasible since the pK_a of $CH_3OH_2^+$ is −1.5 (Olah, 1993). Biochemical experiments provided strong evidence that activation involves Lewis acid catalysis by a Zn ion, which donates positive charge to the oxygen of the hydroxyl group of the substrate (Sauer and Thauer, 1997). The biochemical studies are complemented by model studies, which demonstrate Zn(II)-catalyzed methylation of cob(I)alamin by methanol (Schnyder *et al.*, 1998). The catalytic metal-binding site of MtaB is deep within a funnel at the C-terminus, where the Zn(II) is ligated by two sulfurs from Cys residues and one carboxylate O from Glu (Fig. 10.18) (Hagemeier *et al.*, 2006). Zn and the Co in the cobalamin are only 7.7 Å apart. It was proposed that the Lewis acid activation of methanol occurs by coordination of the hydroxyl group of methanol to the empty fourth coordination site at Zn(II). This binding mode is similar to that observed in alcohol dehydrogenase and carbonic anhydrase, both of which use Zn-based Lewis acid catalysis for substrate activation. There are other charged residues in the second coordination sphere that may also provide H-bonds and a suitable electrostatic environment for the electrophilic activation of the methyl group, facilitating attack on the methyl group by the Co center.

Figure 10.15 summarizes the proposed mechanism of covalent catalysis by pyrrolysine in activation of the methyl group of mono-, di-, and trimethylamines (Hao *et al.*, 2002). Recent structures of MtmB in the presence of hydroxylamine and *N*-methyl-hydroxylamine demonstrate the adduct between the amine and C-2 of pyrrolysine (Hao *et al.*, 2004). Thus, as shown in Fig. 10.15, nucleophilic attack of the methylamine substrate on the imino group of pyrrolysine generates a substituted methyl ammonium adduct at C-2. The positive charge on nitrogen is expected to lead to electrophilic activation of the methyl group, facilitating attack by Co(I), which would generate methyl-Co(III) and leave a covalent amine adduct on pyrrolysine. Proton transfer associated with elimination of the amine as ammonia would regenerate pyrrolysine for the next round of catalysis.

Thus, there are at least three ways that enzymes activate the methyl donor in methyltransferases: general acid catalyzed protonation of the N5 of pterins in MTHF (and probably in MTHMPT) through a H-bonding network, Lewis acid catalysis using a Zn active site near the cobalamin, and covalent catalysis using a novel amino acid.

VI. Activation of the Methyl Group Acceptors: Zn Thiolates and NiFeS Clusters

The methyl group acceptors listed in Table 10.1 are amines (THF, THMPT), thiols (CoM or homocysteine), and metal ions (a NiFeS cluster). Transfer of the methyl group to THF or THMPT is simply the reverse of the methylation of cobalamin by MTHF or MTHMPT and will not be discussed here, where the focus will be on methylation of homocysteine by methionine synthase, of CoM by several of the “A” components, or of the Ni center in the A-Cluster of ACS.

A. Methylation of thiol acceptors

A general theme for enzymes catalyzing alkyl transfers to thiols is that they possess a catalytic Zn site, which is considered to be important in enhancing the nucleophilicity of the thiol at neutral pH (Matthews and Goulding, 1997). The role of Zn appears to be activation of the thiol group by decreasing its pK_a value, since a proton is released upon binding of homocysteine to methionine synthase (Goulding and Matthews, 1997). Although the

nucleophilicity of a Zn-bound thiol is less than that of a free thiolate, the pK_a of the free thiol of CoM or homocysteine is too high to allow significant amounts of the thiolate to be present, and the Zn-bound thiolate is much more nucleophilic than a thiol. Thus, metal ion-catalyzed activation poises homocysteine or CoM for nucleophilic attack on an intermediate methyl donor, such as MeCbl.

A subset of these methyltransferases, including methionine synthase, MtaA, MtbA, and MtsA share a Cys-X-His-X*n*-Cys motif, where Zn coordinates to the His and two Cys residues (Gencic *et al.*, 2001; Krüer *et al.*, 2002; Tallant *et al.*, 2001; Zhou *et al.*, 1999). In methioine synthase, the ligation of homocysteine directly to a Zn site was shown by XAS studies using selenohomocysteine (Peariso *et al.*, 2001). The crystal structure of the homocysteine domain of methionine synthase reveals an $(\alpha\beta)_8$ TIM barrel, like that of the MTHF-binding domain. Although they exhibit little sequence identify, both cobalamin-dependent (MetH) and cobalamin–independent (MetE) methionine synthases contain Zn-binding sites. The Zn site in cobalamin-independent methionine synthase contains two Cys and one His ligands, while in cobalamin-dependent methionine synthase (Fig 10.19), it consists of three Cys ligands ((Evans *et al.*, 2004; Peariso *et al.*, 1998, 2001) and references therein). Homocysteine binds similarly to a $(Cys)_3Zn$ site within the TIM barrel in betaine–homocysteine methyltransferase (BHMT) (Evans *et al.*, 2002) .

The “A” components of a variety of methanogenic methyltransferases also contain catalytic Zn sites that are responsible for binding CoM or homocysteine, including the MtaA component of the methanol:CoM methyltransferase system, which is responsible for methyl transfer from methyl-Co(III) on MtaC to CoM (Gencic *et al.*, 2001). MtsA (Tallant *et al.*, 2001) and MtbA (Krüer *et al.*, 2002), which are MeCbl:CoM methyltransferases. Other Zn enzymes that bind thiols during their catalytic mechanism include the E.coli Ada protein (Myers *et al.*, 1994; Wilker and Lippard, 1997), S-methylmethionine:homocysteine methyltransferase (Thanbichler *et al.*, 1999), epoxyalkane:coenzyme M transferase (Allen *et al.*, 1999; Ensign and Allen, 2003), and protein farnesyl transferase (Huang *et al.*, 1997; Strickland *et al.*, 1998).

B. Methylation of the NiFeS cluster of ACS

Acetyl-CoA Synthase (ACS), encoded by the *acsB* gene in *M. thermoacetica*, is part of a complex that catalyzes the conversion of CO_2, CoA, and a methyl group to acetyl-CoA, as shown on the right hand side of Fig. 10.6. Several reviews that focus on the structure, function, and mechanism of ACS are available (Brunold, 2004; Drennan *et al.*, 2004; Lindahl, 2004; Ragsdale, 2006; Riordan, 2004). The other component of this complex is CODH, which is encoded by the *acsA* gene, and catalyzes the reduction of CO_2 to CO. CODH and ACS contain internal channels that interlink to form a 70 Å channel that sequesters CO and facilitates its delivery to the ACS active site (Tan *et al.*, 2005; Doukov *et al.*, 2008). This channel has been identified biochemically (Maynard and Lindahl, 1999; Seravalli and Ragsdale, 2000) and by X-ray crystallography (Darnault *et al.*, 2003; Doukov *et al.*, 2002, 2008).

The catalytic strategy of ACS is to use a Ni active site, the A-Cluster, to form organometallic intermediates. The A-Cluster consists of a [4Fe-4S] cluster bridged to a binuclear NiNi center, which contains a Ni site (Ni_p) that is thiolate bridged to another Ni ion in a thiolato- and carboxamidotype N_2S_2 coordination environment (Darnault *et al.*, 2003; Doukov *et al.*, 2002; Ragsdale *et al.*, 1985; Svetlitchnyi *et al.*, 2004). Although the details are under discussion, the basic mechanism of ACS involves metal-centered catalysis that includes the following bioorganometallic intermediates: methyl-Ni, Ni-CO, and acetyl-Ni. The mechanism involves transfer of a methyl group from the MeCbl state of the CFeSP to a Ni center, which also binds CO and CoA; then, ACS catalyzes condensation of the C–C

and C–S bonds to form acetyl-CoA (Ragsdale and Wood, 1985). The methyl transfer reaction could occur by either a radical or an S_N2-type nucleophilic mechanism. Model studies of the reaction between methyl-Co^{3+} (CH_3-Co^{3+} dimethylglyoximate) and a Ni^{1+} macrocycle provide precedent for a methyl radical transfer (Ram and Riordan, 1995; Ram *et al.*, 1997). A radical methyl transfer would require homolysis of the CH_3–Co bond of the methylated CFeSP, which Martin and Finke pointed out was not favorable because reduction of CH_3-Co^{3+} requires redox potentials (< -1 V) that are too low for physiological electron donors (Martin and Finke, 1990). Rapid kinetic studies and stereochemical studies using a chiral methyl donor also indicate that the transmethylation reaction involves an S_N2-type nucleophilic attack of Ni on the methyl group of the methylated CFeSP (CH_3–Co^{3+}) to generate methyl-Ni and Co^{1+}(Lebertz *et al.*, 1987; Menon and Ragsdale, 1998, 1999). Thus, it is likely that the metal-to-metal methyl transfer reaction is similar to the other B_{12}-dependent methyltransferase reactions described above. In fact, kinetic studies indicate that the Ni (I) site on ACS is as strong a nucleophile as is the Co^{1+} site in the CFeSP (Tan *et al.*, 2003).

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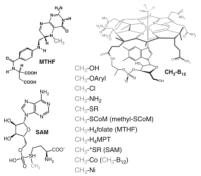

Figure 10.1.
Methyl donors in biology.

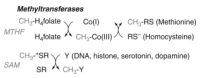

Figure 10.2.
Two types of methyltransferases.

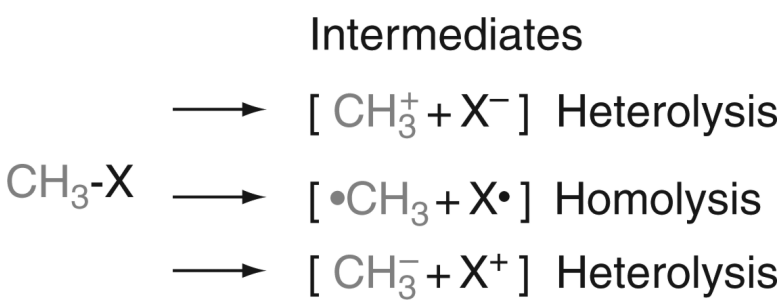

Figure 10.3.
Three ways to cleave a methyl-X bond.

Figure 10.4.
B_{12}-dependent methyltransferases.

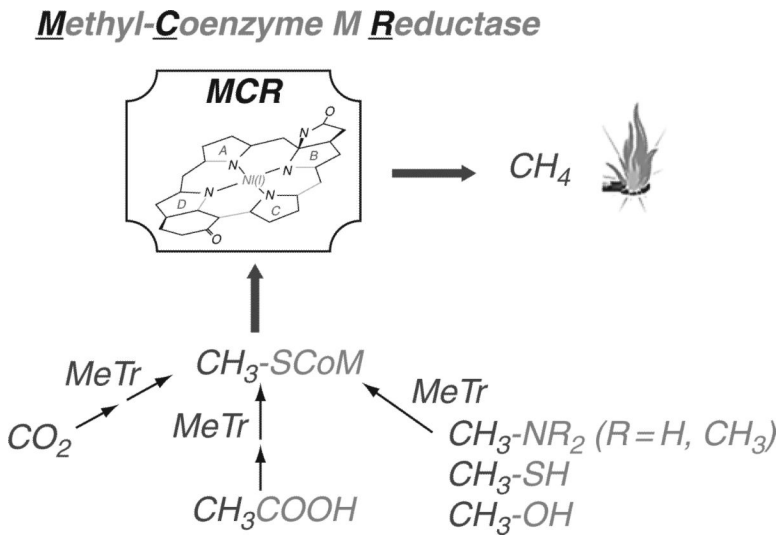

Figure 10.5.
Methanogenesis.

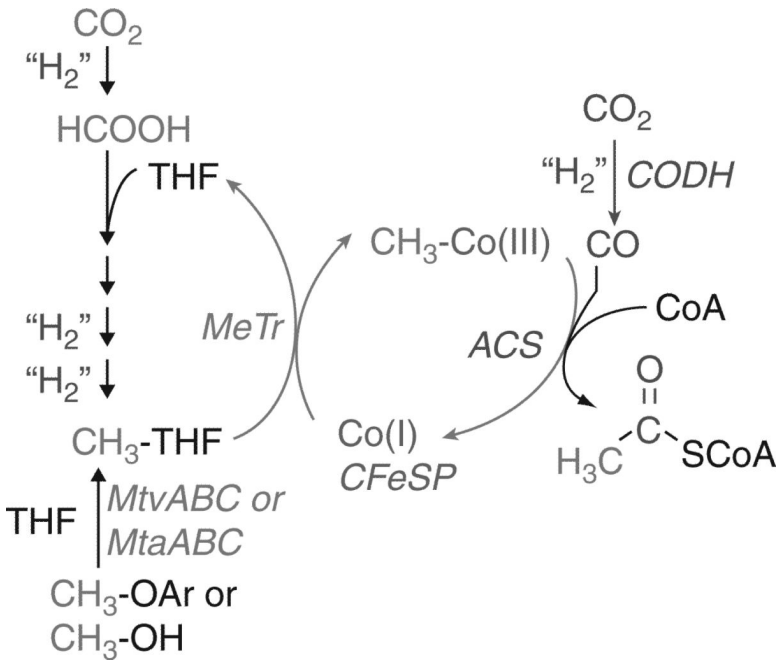

Figure 10.6.
Methyltransferases in acetogenesis.

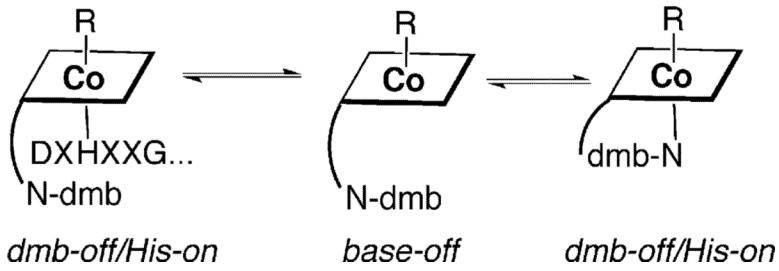

Figure 10.7.
Three modes of B_{12} binding.

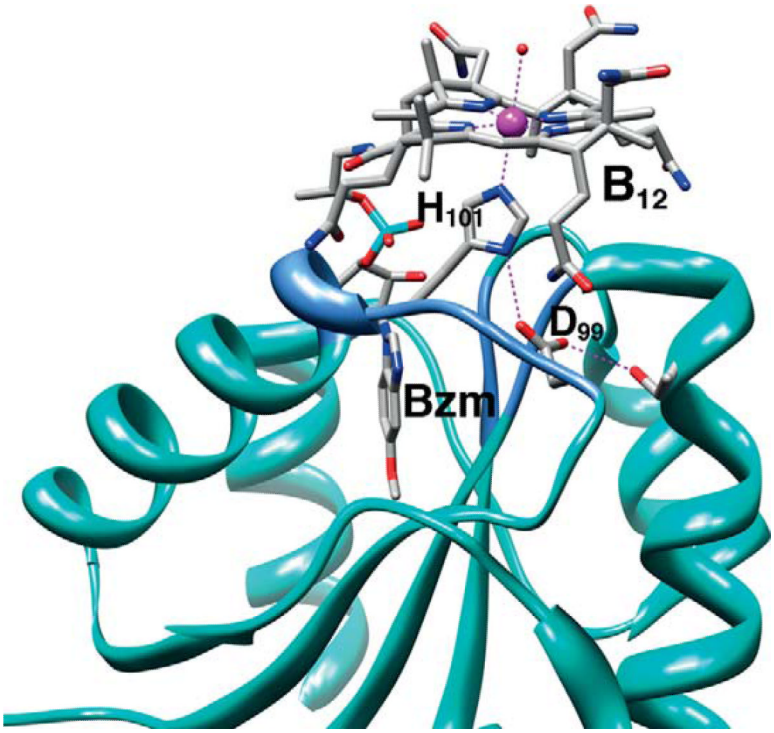

Figure 10.8.
B_{12}-binding site in *M. thermoacetica* MtaC (Das *et al.*, 2007). The region containing the conserved DXH motif (see text) is shown in cornflower blue. Generated from PDB ID# 1Y80 using Chimera.

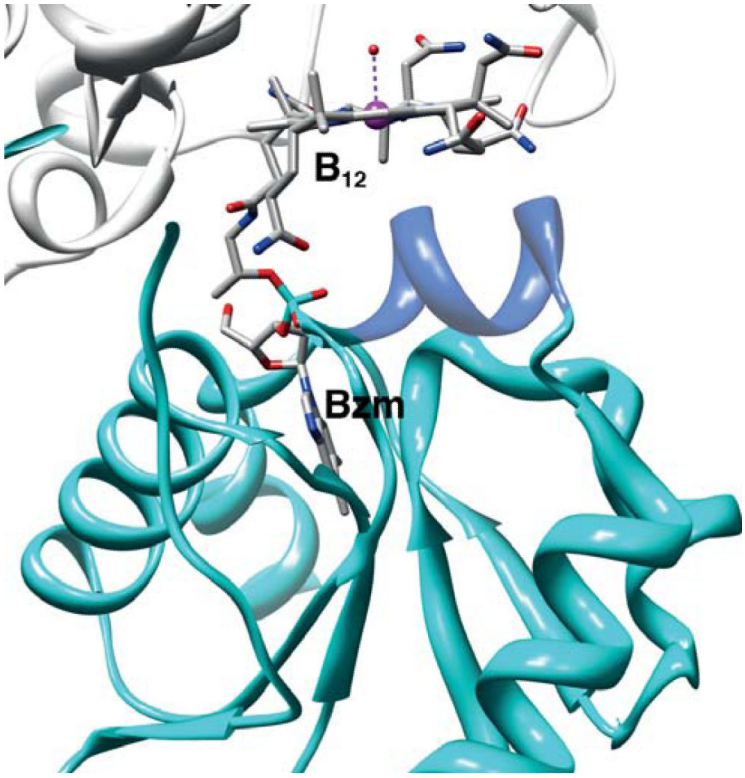

Figure 10.9.
B_{12}-binding site in the *M. thermoacetica* CFeSP (Svetlitchnaia *et al.*, 2006). The region containing the hydrophobic helix (see text) is shown in cornflower blue. Generated using Chimera from PDB ID code 2H9A.

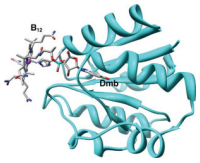

Figure 10.10.
B_{12}-binding site in *M. barkeri* MtaC focusing on the Rossman domain involved in ligating the Dmb moiety, generated from PDB ID code 2I2X, using Chimera.

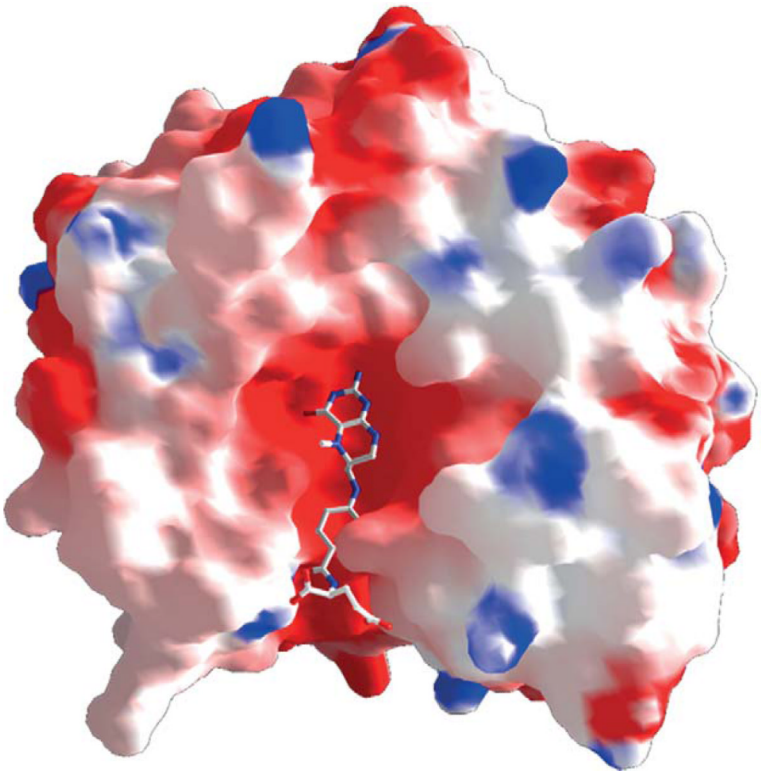

Figure 10.11.
The methyltetrahydrofolate (MTHF)-binding site in the MTHF:CFeSP methyltransferase (AcsE).

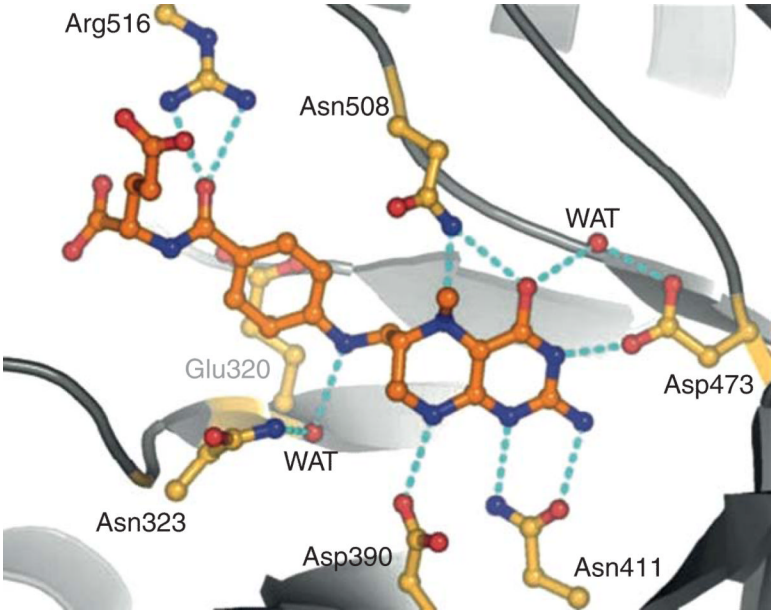

Figure 10.12.
Methyltetrahydrofolate (MTHF)-binding site in MetH. From Evans *et al.* (2004).

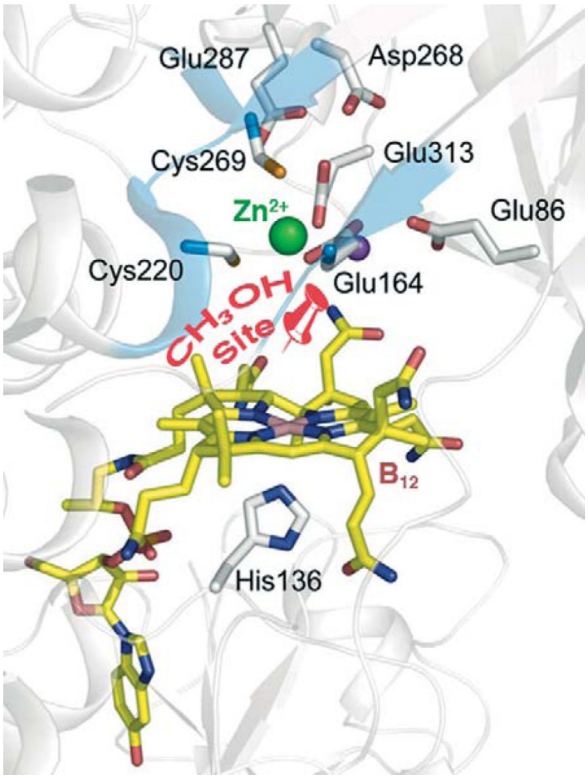

Figure 10.13.
Proposed methanol-binding site between the Co and Zn ions (pinpoint) in MtaBC. Modified from Hagemeier *et al.* (2006). Generated from PDB ID code 2I2X using Chimera.

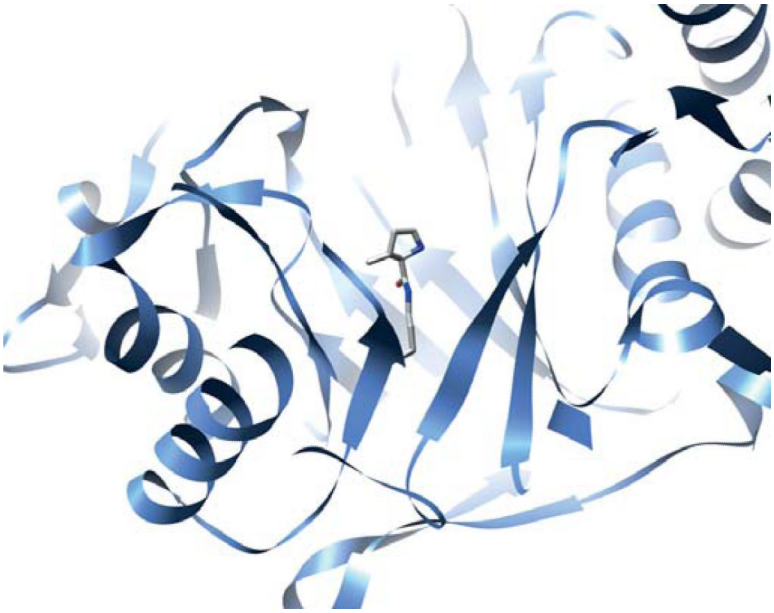

Figure 10.14.
Pyrrolysine (stick diagram) in the channel formed by the triosephosphate isomerase (TIM) barrel of MtmB. Generated from 1NTH (Hao *et al.*, 2002) using Chimera.

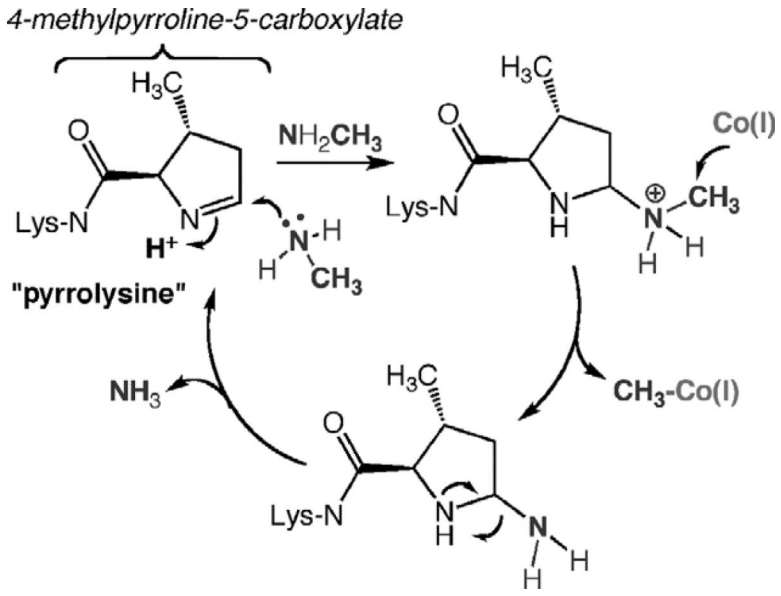

Figure 10.15.
Proposed role of pyrrolysine in activating methylamines. Modified from Hao *et al.* (2002).

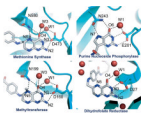

Figure 10.16.
Proton transfer networks without an obvious proton donor. From Doukov *et al.* (2007).

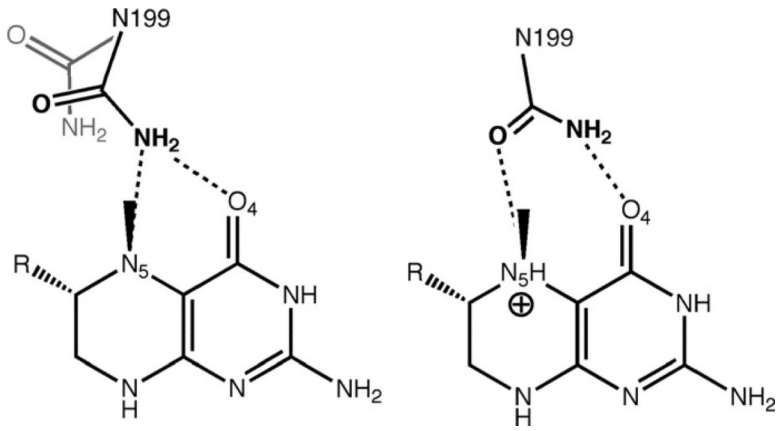

Figure 10.17.
Left: Asn199 in the apo and MeTr-bound states. Right: Proposed transition state for proton transfer. Modified from Doukov *et al.* (2007).

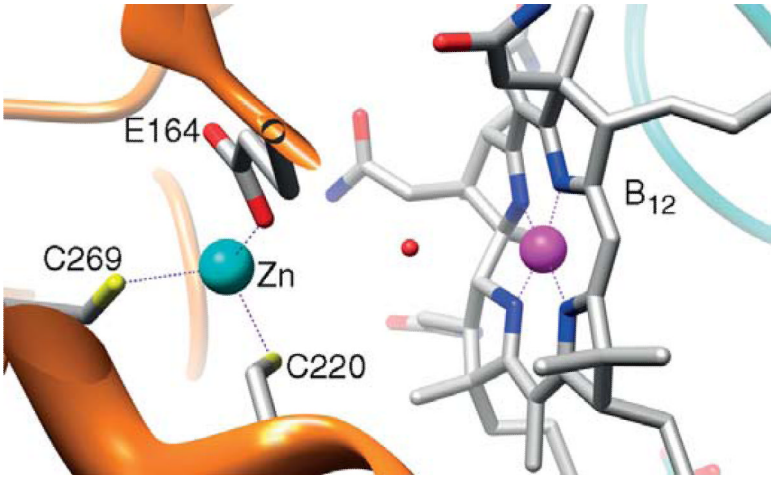

Figure 10.18.
Zn site in *M. barkeri* MtaB (gold) where methanol is proposed to be activated (Hagemeier *et al.*, 2006). The MtaC subunit is shown in cyan. Generated using Chimera from PDB# 2I2X.

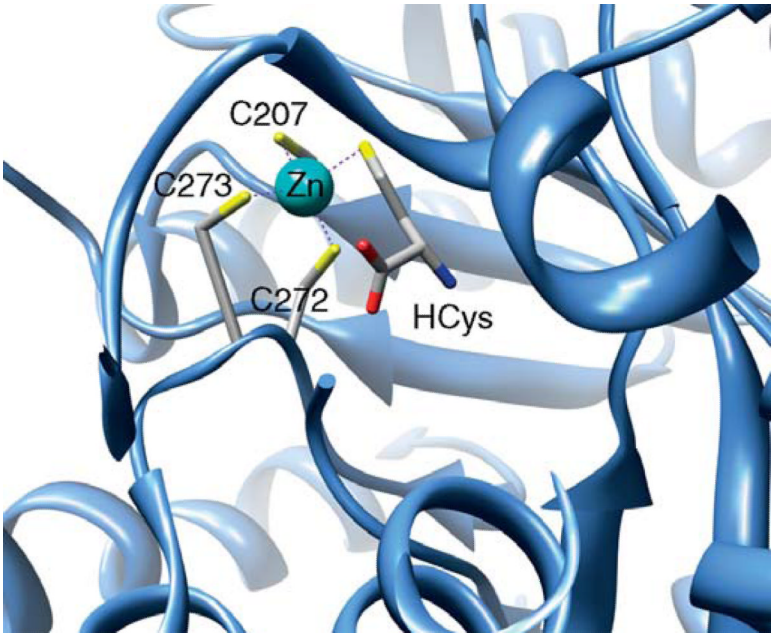

Figure 10.19.
Homocysteine ligated to Zn within the triosephosphate isomerase (TIM) barrel of the N-terminal domain of MetH. Generated from PDB ID# 1Q8A using Chimera.

Table 10.1

B_{12}-dependent methyltransferases

Methyl donor	Methyltransferase components	Biological system	Ultimate methyl acceptor	Reference
MTHF	MetH	Methionine Synthesis	Homocysteine	(Matthews, 2001)
CH_3NH	MtmABC	Methanogenesis	Coenzyme M	(Krzycki, 2004)
$(CH_3)_2N$	MtbABC	Methanogenesis	Coenzyme M	(Soares *et al.*, 2005)
$(CH_3)_3N$	MttABC	Methanogenesis	Coenzyme M	(Soares *et al.*, 2005)
CH_3SH	MtsAB	Methanogenesis	Coenzyme M	(Tallant *et al.*, 2001)
MTHMPT	MtrA-H	Methanogenesis	CoM	(Gottschalk and Thauer, 2001)
CH_3OH	MtaABC	Methanogenesis	Coenzyme M	(Hagemeier *et al.*, 2006)
MTHF	AcsABCDE	Acetogenesis	THF, CODH/ACS	(Doukov *et al.*, 2007)
MTHF	MtaABC	Acetogenesis	THF	(Das *et al.*, 2007)
CH_3OAr	MtvABC	Acetogenesis	THF	(Naidu and Ragsdale, 2001)
CH_3Cl	CmuAB	Dehalorespiration	THF	(Studer *et al.*, 2001)