The Yin–Yang of cobalamin biochemistry

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The cobalamins are B_{12} cofactors with a reactive cobalt–carbon bond at their core and support the activity of two mammalian enzymes that are both medically important. The reactive organometallic bond of the cofactor can be cleaved either homolytically or heterolytically, but what determines how the enzymes control the fate of this bond?

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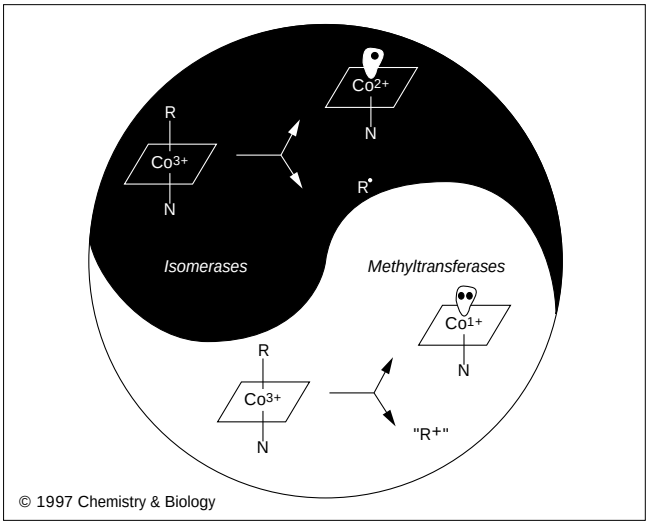
Introduction

The principle of duality or polarity is at the root of Eastern philosophy. In Chinese culture it is called Yin–Yang; in Hindu philosophy it is maya. Cobalamin, appropriately described as nature's most beautiful cofactor [1], represents duality in chemistry. This cofactor has a reactive organometallic bond between cobalt and carbon (the Co–C bond) at its core, which, depending on whether it is in an active site of a methyltransferase or an isomerase, cleaves either heterolytically or homolytically (Fig. 1). This presents an interesting and elusive conundrum: how do these enzymes exert such exquisite control over the destiny of the Co–C bond, selectively permitting one or the other outcome?

Cobalamins contain a central cobalt atom that is tethered equatorially to four nitrogens from the corrin ring, which is believed to be the most ancient of the known tetrapyrrole-derived macrocycles (Fig. 2). Unlike the rings of its perhaps better known structural cousins, the porphyrins and the chlorins, the corrin ring of cobalamin is fairly reduced, affording it a degree of flexibility that is not enjoyed by the other tetrapyrrole cofactors [2]. In the Co^{3+} oxidation state, cobalamins prefer an octahedral geometry. In solution and at physiological pH, the lower axial ligand is also a nitrogen atom, provided by the pendant, intramolecular base, dimethylbenzimidazole (DMB). This leaves only the upper axial position available for a variety of β ligands, including deoxyadenosyl, methyl, water (or hydroxyl), cyano and glutathionyl moieties. The two known cofactor forms are adenosylcobalamin (AdoCbl or coenzyme B_{12}) and methylcobalamin (MeCbl). Cyanocobalamin, or vitamin B_{12}, obtained originally as an artifact of the cofactor isolation procedure [3] is now the form that pervades the food industry.

The determination of the three-dimensional structure of vitamin B_{12}, described in 1956 by Dorothy Hodgkin's laboratory, was a monumental achievement [4] and was followed a few years later by the solution structure of AdoCbl [5]. The latter structure revealed the distinctive Co–C bond, the first organometallic bond to be described in a naturally-occurring compound. Three decades later, X-ray and NMR methods were used to solve the three-dimensional structure of MeCbl [6]. The overall structures of the two alkylcobalamins are very similar despite the difference in the sheer bulk of their upper or β-axial ligands (methyl in MeCbl, deoxyadenosyl in AdoCbl). Salient differences include the extent to which the corrin is puckered, with the 'upward' fold angle (Fig. 2) being 14.8° in MeCbl and 10° in AdoCbl. The Co to axial nitrogen ($Co–N_{axial}$) bond length is 2.19 Å in MeCbl [6], and 2.21 Å in AdoCbl [7].

Figure 1



The Yin–Yang of cobalamin biochemistry. Cobalamin exhibits dual chemical reactivity because the Co–C bond at the core of cobalamin is either cleaved heterolytically when in the active site of a methyltransferase or homolytically when in the active site of an isomerase.

This is consistent with the differences in the pK_a values for protonation of the DMB base in these two cobalamins (2.9 for MeCbl and 3.7 for AdoCbl) [8].

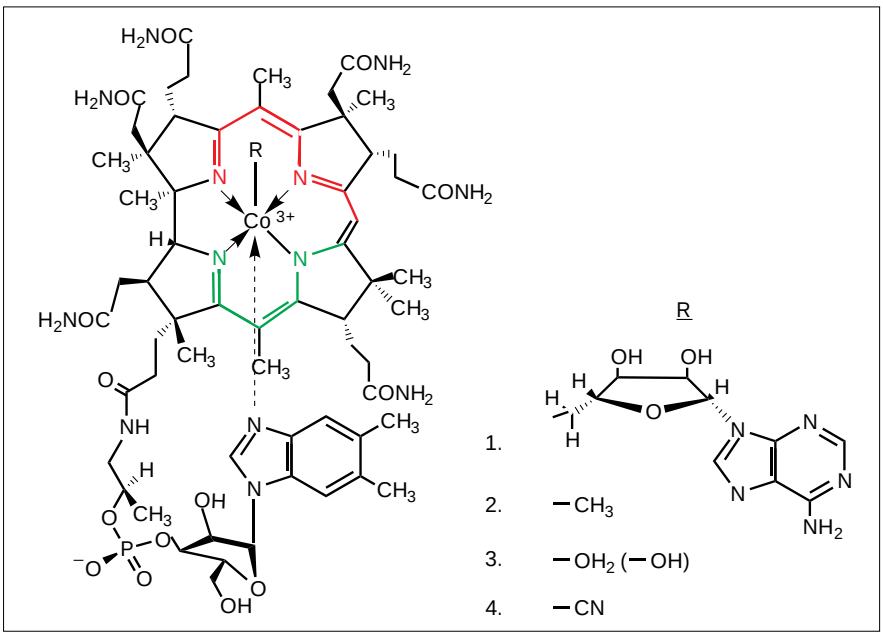
The role of AdoCbl as a cofactor was first described by Barker and coworkers [9] in glutamate mutase, followed a few years later by the discovery of MeCbl in methionine synthase [10]. In nature, this cofactor is associated with two enzyme subfamilies: the isomerases that catalyze 1,2 rearrangement reactions and the methyltransferases that catalyze transmethylation reactions (Table 1). Members of both subfamilies are fairly prevalent in the bacterial world, where the isomerases are involved in fermentative pathways, and the methyltransferases are involved in pathways leading to methionine, acetate, or methane.

In mammals, cobalamins are only known to be required for the activity of two enzymes, the cytoplasmic methionine synthase, and the mitochondrial methylmalonyl–CoA mutase [11,12]. (Although an AdoCbl-dependent leucine aminomutase has been reported [13] other studies have not confirmed its existence.) Here I review the chemistry, biology, and medical importance of two mammalian B_{12} enzymes.

Methionine synthase: a modular machine

In bacteria, methionine synthase catalyzes the terminal step in the *de novo* biosynthesis of methionine. In mammals, however, methionine is an essential amino acid and so the enzyme has a quite different role. The enzyme functions to recycle homocysteine (forming methionine) and to liberate H_4folate from CH_3–H_4folate, which is the circulating form of the vitamin that is delivered from the bloodstream to the cells. H_4folate is required for purine, pyrimidine and amino-acid biosynthesis. The genes encoding methionine synthase in *Escherichia coli* [14] and more recently, in humans [15,16] have been cloned. The human gene has been mapped to the long arm of chromosome 1 at position 1q43 [15,16]. Despite being widely separated in

Figure 2



Structure of cobalamin. The central cobalt atom has four nitrogen ligands from the corrin ring. R represents a variable axial ligand. The lower axial ligand is a nitrogen atom provided by dimethylbenzimidazole (DMB). There are differences in the extent to which the corrin ring can be puckered, depending on the nature of the lower axial ligand, as defined by the fold angle. This is defined as the angle between the least squares plane through the atoms N21, C4, C5, C6, N22, C9, C10 (red) and C11, N23, C14, C15, C16, N24 (green) [90].

Table 1

Cobalamin-dependent enzymes.

Methyltransferases	Isomerases
Methionine synthase Homocysteine + CH_3–H_4folate→Methionine + H_4folate	*Methylmalonyl-CoA mutase* Methylmalony–CoA→Succinyl–CoA
Corrinoid/Fe protein (i) CH_3–H_4folate + Corrinoid/FeS protein→ H_4folate + CH_3–corrinoid Fe/S protein (ii) CH_3–corrinoid Fe/S protein + CO dehydrogenase→ Corrinoid Fe/S protein + CH_3–CO dehydrogenase	*Glutamate mutase* Glutamate→Methylaspartate
Methyltetrahydromethanopterin:coenzyme M methyltransferase CH_3–H_4MPT + Coenzyme M→H_4MPT + CH_3-coenzyme M	*Methyleneglutarate mutase* 2-Methyleneglutarate→3-methylitaconate
Methanol:2-mercaptoethanesulfonic acid methyltransferase CH_3OH + coenzyme M→CH_3-coenzyme M + H_20	*Ethanoalamine ammonia lyase* Ethanolamine→acetaldehyde + NH_3
	Diol dehydrase Propanediol→propionaldehyde + H_2O
	Isobutyryl-CoA mutase Isobutyryl-CoA→butyryl-CoA
	Glycerol dehydrase Glycerol→β-hydroxypropionaldehyde + H_2O
	D-Ornithine mutase D-Ornithine→2,4-diaminovaleric acid
	L-β-Lysine mutase β-Lysine→L-erythro-3,5-diaminohexanoic acid
	Ribonucleotide reductase NTP→dNTP

evolutionary terms, the primary sequences of the *E. coli* and human proteins are remarkably similar, being 55% identical. The bacterial [17] and mammalian [18] enzymes are large, monomeric proteins with molecular masses of 136 kDa and ~141 kDa respectively.

The bacterial enzyme displays a modular organization (Fig. 3) and separates into component domains following partial tryptic digestion [14,19]. An amino-terminal 61 kDa fragment of the enzyme binds substrates and shows homology to the methyltransferase from *Clostridium thermoaceticum* [20] that binds CH_3–H_4folate, and also to betaine homocysteine methyltransferase [21] which binds homocysteine. The middle 27 kDa domain binds cobalamin [14] and its crystal structure has been determined [22]. The carboxy-terminal 38 kDa domain binds S-adenosylmethionine (AdoMet) [19], and its three-dimensional structure has been determined [23]. Thus, the functions of catalysis and activation appear to be uniquely housed in the amino-terminal and carboxy-terminal domains respectively, while the middle B_{12}-binding domain serves in both cycles.

The most interesting revelation from the crystal structure of the B_{12}-binding domain [22], the first for a protein-bound cobalamin, was that the cofactor is in the DMB 'base-off' conformation, with the lower coordination position taken instead by a histidine, His759, that is donated by the protein (Fig. 4). So, the intramolecular and bulky base that had been viewed with substantial interest by both model chemists and biochemists alike as holding one of the keys to the reactivity of the *trans* Co–C bond, is in fact in an extended conformation with the nitrogen of DMB being ~14 Å away from the cobalt.

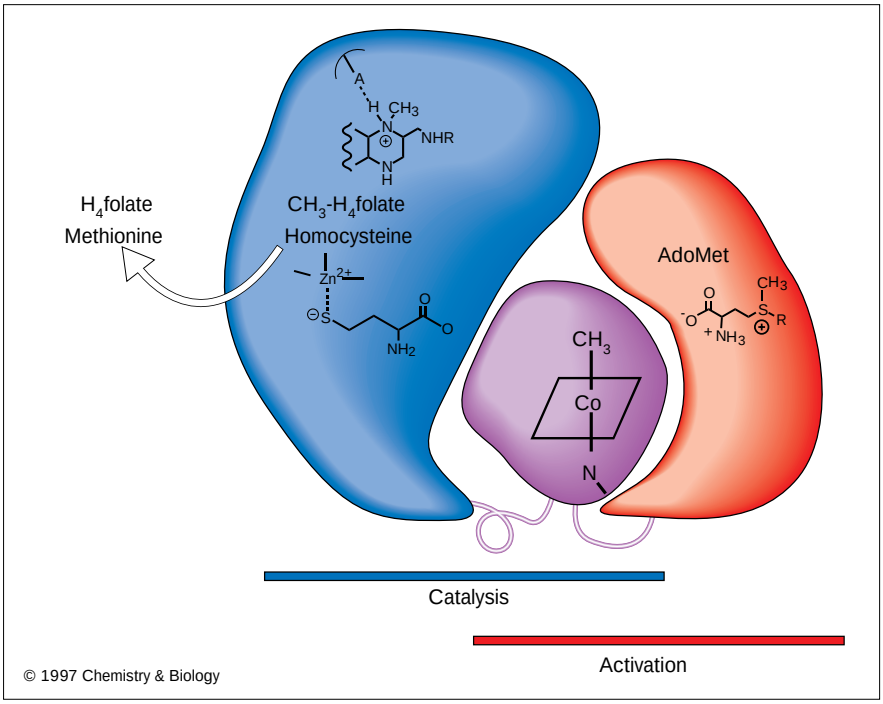
The ligating histidine forms a catalytic triad with Asp757 and Ser810 at the α face of the cobalamin. This triad has been postulated to be involved in shunting protons to and from histidine during catalysis, facilitating coordination-state changes between octahedral MeCbl and square planar cob(I)alamin [22].

Co–C bond heterolysis

Methionine synthase catalyzes successive transmethylation reactions in which a methyl group is transferred from a tertiary amine, CH_3–H_4folate, to cob(I)alamin, and then to the thiolate of homocysteine to generate H_4folate and methionine (Fig. 5). The migrating methyl group is transferred with retention of configuration, a stereochemical outcome which is consistent with the indicated double displacement reaction [24]. Catalysis, however, holds perils for the enzyme, since the intermediate, cob(I)alamin, is supernucleophilic and its remethylation by CH_3–H_4folate competes with adventitious oxidation. The oxidized enzyme is inactive, and can be returned to the catalytic cycle via a reductive activation system that, in addition to reducing equivalents, requires AdoMet as a methyl donor. In bacteria, a well characterized two-protein conduit leads electrons from NADPH to methionine synthase. The two proteins are NADPH–ferredoxin (or flavodoxin) oxidoreductase and flavodoxin [25]. In mammals, a similar arrangement exists, but the identities of the proteins are unknown (Chen and R.B., unpublished observations).

The transfer of a methyl group from CH_3–H_4folate to cob(I)alamin is a challenging and unprecedented reaction in solution and requires displacement of a secondary amine anion. This challenge can be met by activation of

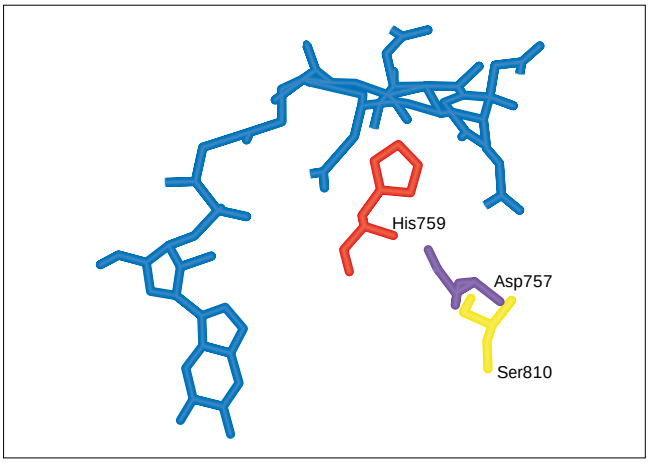
Figure 3



The postulated domain organization of methionine synthase. The amino-terminal domain (blue) binds substrates, the central domain (purple) binds cobalamin, and the carboxy-terminal domain (red) binds AdoMet. The role of an active-site zinc is derived from the published model for cobalamin-independent methionine synthase [33]. The domains involved in catalysis and activation are indicated.

the neutral tertiary amine via oxidation or protonation mechanisms [26]. In the absence of an obvious one-electron or two-electron redox sink, the protonation mechanism is preferred (Fig. 5); displacement of a methyl group from a quarternized amine by Co^+ is chemically precedented [27,28]. If activation does indeed occur via protonation, then the nucleophile, cob(I)alamin, would face a choice between displacing a methyl group and accepting a proton. As cob(I)alamin is highly nucleophilic (n=14.4 on the Pearson scale [29]) but weakly basic (pK_a ~1 [30]), displacement of the methyl group would be predicted [31]. This provides a plausible rationale for why nature uses cob(I)alamins for difficult methyl-group transfers in which protonation may be a common mechanism for the activation of the methyl donor. A B_{12}-independent methionine synthase from *E. coli* catalyzes the same overall reaction, but with a turnover number that is more than 100-fold lower than for the B_{12}-dependent enzyme. The bacterium appears to compensate for this inefficiency by synthesizing the sluggish B_{12}-independent enzyme at 3% of cytoplasmic protein [32].

Figure 4



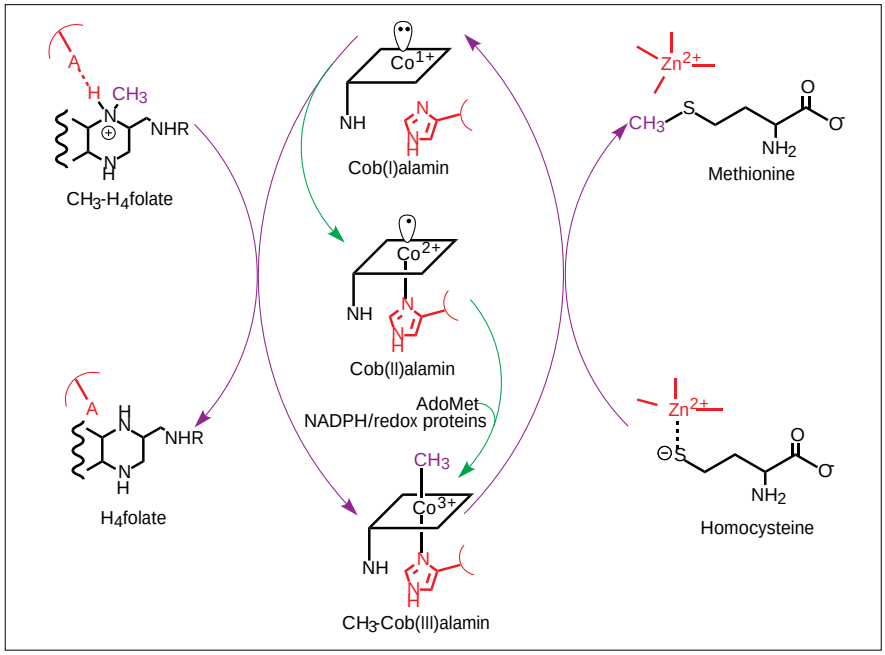
The conformation of MeCbl bound to the cobalamin domain of methionine synthase [22]. The amino acid residues of the catalytic triad are indicated.

Methyl transfer from MeCbl to the thiolate of homocysteine is precedented in a nonenzymic reaction [10]. Presumably the nucleophilicity of homocysteine is enhanced in the active site by deprotonation. In the B_{12}-independent enzyme, Zn^{2+} serves as a Lewis acid and activates homocysteine via coordination [33]. This may represent a mechanism that is general to other methyltransferases that employ a thiolate substrate [33].

The heterolytic Co–C bond cleavage of MeCbl is enhanced 10^5-fold by methionine synthase [34]. Several experiments have attempted to uncover the role of the lower axial base in labilizing the upper axial Co–C bond. Equilibration experiments using MeCbl and cob(I)inamide (cob(I)alamin minus the DMB base), have attributed an

Figure 5

The postulated reaction catalyzed by methionine synthase. A methyl group (purple) is transferred in the reaction from CH_3–H_4folate to cob(I)alamin and then to the thiolate of homocysteine to produce H_4folate and methionine. Hypothetical contributions (except for the histidine ligand) from the protein to the reaction mechanism are indicated in red. The role of an active-site zinc is derived from the published model for the cobalamin-independent methionine synthase [33]. The arrows in green represent the competing oxidation and activation steps.



~4.2 kcal mol^{-1} increase in Co–C bond strength to nucleotide coordination by the lower base [35]. Recent resonance Raman [36] and near IR FT-Raman [37] studies reveal the absence of *trans* effects on the strength of the Co–C bond in the ground state. The Co–C stretching frequencies in a series of alkylcobalamins in the base-on and base-off conformations have been assigned [36]. While the trend in stretching frequencies parallels the trend in the Co–C bond dissociation energy (BDE), the presence or absence of the lower base has no effect on the Co–C stretching vibration. Ionization of the α imidazole ligand to methylcob(III)inamide (MeCbi) to imidazolate similarly has no effect on the Co–C stretching frequency [37]. These results indicate that the catalytic contribution of the histidine ligand is likely to be expressed later along the reaction coordinate rather than in the ground state.

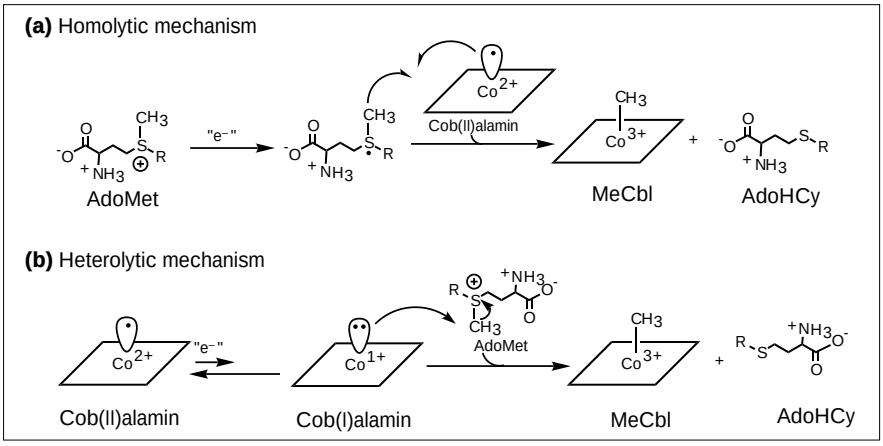
The Co-CH_3 stretching frequency of MeCbl bound to methionine synthase has not been determined, so ground state destabilization of the organometallic bond in the enzyme active site cannot be evaluated. The Co–CH_3 stretching frequency in the corrinoid/FeS protein from *C. thermoaceticum* is observed at 429 cm^{-1} [38]. This is significantly lower than that of free MeCbl (n = 505 cm^{-1}, Co–C_{BDE} = 37 kcal mol^{-1} [39]) and similar to that of AdoCbl (n = 429 cm^{-1} and 443 cm^{-1}, Co–C_{BDE} = 31.5 kcal mol^{-1} [40]), suggesting that the Co–C bond may be weakened in the active site.

Replacement of His759 by glycine in methionine synthase compromises catalytic turnover, and decreases the rate of demethylation by a factor of 10^5 [34]. Replacement of the Asp757 and Ser810 residues of the catalytic triad have modest effects on enzyme activity. Substitutions at all three positions diminish the stability of enzyme-bound MeCbl to light, and enhance AdoMet-dependent reactivation. These results have been interpreted as evidence that the catalytic triad contributes to Co–C bond strength [34]. It is attractive to consider a common methyl-transfer mechanism for all B_{12}-dependent enzymes. From this perspective, the lower ligand would appear to be inessential for demethylation, because the cofactor is in the 'base-off' conformation in the corrinoid/FeS protein in all three oxidation states [41]. The stability of cob(I)alamin to oxidation and of the Co–C bond to photolysis are both dictated by the surrounding protein architecture [42]. The possibility of distal conformational changes in the methionine synthase mutants cannot be ruled out because they were apparently not examined. These studies do, however, suggest that deprotonation or reprotonation of the catalytic triad is of minor importance in stabilizing the transition state in catalytic turnover [34], and leave open its role in this cycle.

Are methyl transfer reactions conformationally gated?

The in-line transfer of a methyl group to the same cobalamin acceptor from two distinct methyl donors, CH_3–H_4folate and AdoMet, bound to two domains, suggests a molecular ping-pong play in which rather substantial domain movements may be necessary. Although rapid-reaction kinetic studies provided evidence for a heterolytic transfer of the methyl group from CH_3–H_4folate

Figure 6



Alternative mechanistic proposals for reductive methylation of methionine synthase. (a) In the homolytic mechanism, a single electron transfer to AdoMet is followed by the recombination of a transient methyl radical with cob(II)alamin. (b) In the heterolytic mechanism, the thermodynamically unfavorable reduction of cob(II)alamin to cob(I)alamin is driven by coupling to an exergonic AdoMet dependent methylation reaction.

to homocysteine via cob(I)alamin [43], the mechanism of methyl transfer from the second donor, AdoMet, is unclear. Both homolytic and heterolytic mechanisms have been considered (Fig. 6). In the heterolytic mechanism, the thermodynamically unfavorable reduction of cob(II)alamin to cob(I)alamin ($E^o = -526$ mV [44]) is driven by coupling to an exergonic AdoMet-dependent methylation reaction [25]. In the homolytic mechanism, a single electron transfer to AdoMet precedes recombination of a transient methyl radical with cob(II)alamin [34]. However, unlike other AdoMet-dependent enzymes such as the anaerobic ribonucleotide reductase and lysine 2,3 amino mutase for which radical-based mechanisms are postulated, methionine synthase does not have a redox-active metal cluster that is believed to be important in the generation of the adenosyl (not methyl) radical.

If both methyl-transfer reactions use the same intermediate, cob(I)alamin, how does the enzyme recognize that it is in the catalytic mode and select CH_3–H_4folate, or in the activation mode and select AdoMet? Do domain conformations gate access of the methyl donor as they gate electron-transfer reactions in some systems [45]? For instance, the oxidized enzyme might favor a conformation in which the B_{12}-binding domain and AdoMet-binding domain are aligned for methyl-group transfer. There is some evidence for this in the bacterial methionine synthase. The proximal redox partner, flavodoxin, binds preferentially to the form of the enzyme that contains cob(II)alamin instead of MeCbl [46]. Oxidization state driven conformational changes are implicated in methyltetrahydromethanopterin:coenzyme M methyltransferase in which sodium ion translocation is coupled to the B_{12}-dependent methyltransferase activity [47].

Methionine synthase and medicine

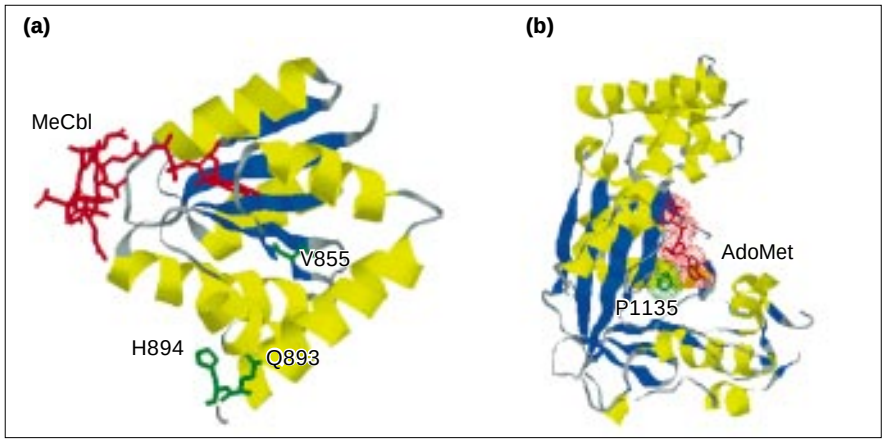
Studies spanning the last decade have shown that elevated homocysteine is an independent risk factor for coronary artery diseases [48] and neural tube defects [49]. It is estimated that the concentration of homocysteine is raised in 15–40% of patients with coronary, cerebral, or peripheral arterial diseases [48]. Severe hyperhomocysteinemia is inherited as an inborn error of metabolism, an autosomal recessive condition that is accompanied by megaloblastic anemia and neurological disorders. The only known pathways of homocysteine metabolism in humans, with the exception of those in kidney and liver cells, are via transmethylation catalyzed by methionine synthase and transsulfuration catalyzed by cystathionine β-synthase. The recent cloning of the human gene for methionine synthase led to mutation detection studies in patient populations [15,16]. The first mutations (Pro1173Leu, ΔIle881 and His920Asp) and two polymorphisms (Arg61Lys and Asp919Gly) have been detected in cblG (methylcobalamin deficient and decreased methionine synthase activity) patients who have severe hyperhomocysteinemia [16,50]. Interestingly, these are clustered in the B_{12}-binding and AdoMet-binding domains of the protein (Fig. 7).

Methylmalonyl-CoA mutase: a radical rearranger

In mammals, methylmalonyl-CoA mutase is a mitochondrial matrix enzyme that converts methylmalonyl-CoA to succinyl-CoA in catabolic pathways leading from branched-chain amino acids, odd-chain fatty acids, and cholesterol. In some bacteria such as *Propionibacterium shermanii*, the mutase is important in the reverse metabolic direction, linking production of propionate to succinate. The human [51] and *P. shermanii* [52] genes encoding methylmalonyl-CoA mutase have been cloned. The human gene has been mapped to chromosome 6p12–21.2 [53]. The human (α2) and bacterial (αβ heterodimer) enzymes have native molecular masses of ~150 kDa.

Although the methyltransferases and isomerases that have been sequenced so far do not share any overall

Figure 7

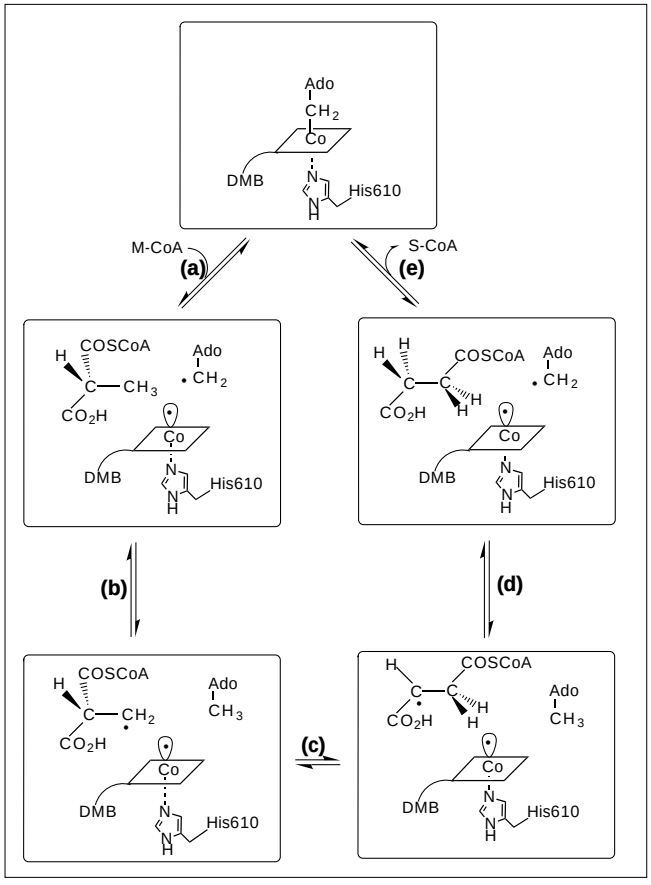


The localization of mutations (dark green) found in human methionine synthase in the homologous *E. coli* enzyme. (a) Portion of the B_{12}-binding domain [22] in which homologous residues at which mutations have been found are shown in green. Bacterial Val855, Gln893 and His894 correspond to human Ile881, Asp919 and His920 respectively. (b) AdoMet binding domain of *E. coli* methionine synthase [23] in which the bacterial residue Pro1135 (corresponding to human Pro1173) is shown to be in contact with the bound AdoMet.

sequence homology, there is a region including and surrounding the coordinating histidine residue that is conserved [54]. UV, visible and electron paramagnetic resonance (EPR) spectroscopic studies had indicated that cofactor binding to methylmalonyl-CoA mutase is accompanied by a ligand replacement reaction in which a histidine in the active site replaces DMB [55]. The crystal structure of the bacterial methylmalonyl-CoA mutase reveals that the α ligand is His610 [56] and that it is coordinated to cobalt via an unusually long bond (2.45 Å; the distance observed for the free cofactor is 2.21 Å) [7]). The crystal structure of the mutase has the cofactor in a mixture of inactive forms (cob(II)alamin and hydroxycobalamin (OHCbl)) with a substrate fragment, dethia CoA, bound to it. Simulations of extended X-ray fine structure (EXAFS) spectroscopic data of AdoCbl bound to the mutase active site provide two possible minima for the Co–N_{axial} distance at 2.13 Å and 2.45 Å (E. Scheuring, R. Padmakumar, R.B. and M.R. Chance, unpublished results).Two of the three catalytic triad residues (His759 and Asp757) found in methionine synthase have homologs (His610 and Asp608) in the mutase structure. The third, Ser810, is replaced by Lys604 in the mutase; but the two residues do not have the same sidechain orientation (with respect to their hydrogen-bonding Asp partners) and have different positions in the primary sequences relative to the coordinating histidine. The role, if any, of these residues in the isomerization reaction is unknown.

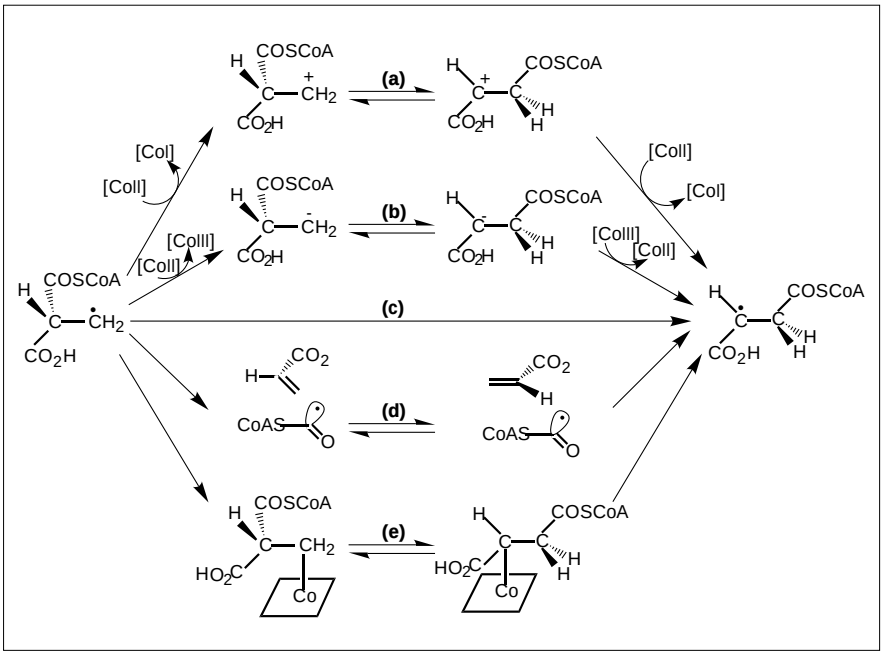
The first step in the mutase-catalyzed reaction is postulated to be the homolytic fission of the Co–C bond of the cofactor to generate a carbon-centered deoxyadenosyl radical and a metal-centered cob(II)alamin radical (Fig. 8a). Homolysis of the reactive Co–C bond is accelerated by a factor of ~$10^{12\pm1}$ by the enzyme [57]. In the next step, the adenosyl radical is believed to initiate the rearrangement reaction by abstracting a hydrogen atom from the methyl

Figure 8



Postulated reaction mechanism for methylmalonyl-CoA mutase. M-CoA and S-CoA refer to methylmalonyl-CoA and succinyl-CoA respectively. (a) The homolytic fission of the cofactor generates a deoxyadenosyl radical and a cob(II)alamin radical. (b) A reactive primary radical is formed on the substrate. (c) The primary radical rearranges to form a secondary radical on the substrate. (d) The product is formed on reabstraction of a hydrogen atom. (e) The deoxyadenosyl and cobalamins recombine to complete the cycle.

Figure 9

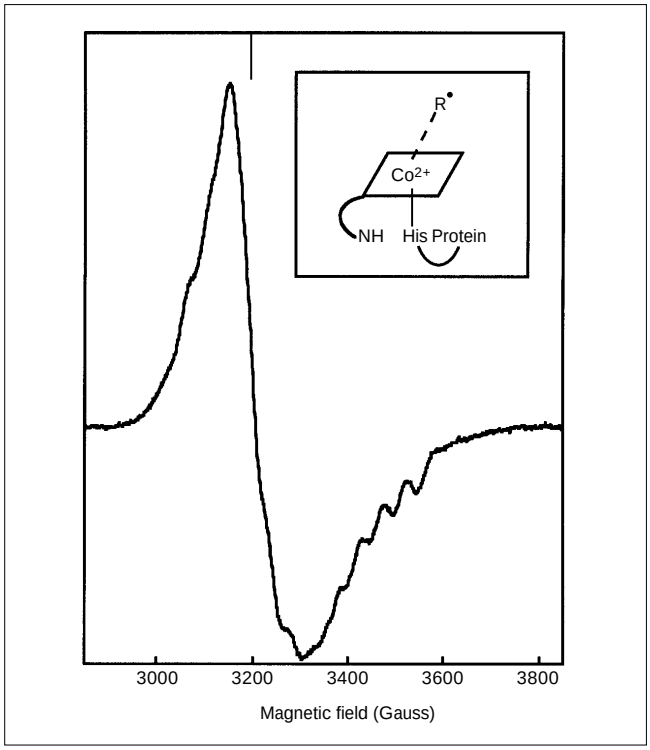


Alternative mechanisms for rearrangement of methylmalonyl-CoA to succinyl-CoA. Routes (a) to (e) represent rearrangements via the following intermediates: (a) carbonium ion, (b) carbanion, (c) direct radical rearrangement pathway, (d) fragmentation pathway and (e) organocobalt adduct. The ionic intermediates could be formed via reversible oxidation/reduction reactions of the cobalamin, denoted as [Co] here.

group of methylmalonyl-CoA to generate a reactive primary radical on the substrate (Fig. 8b). The primary radical then rearranges to form a secondary radical on the product (Fig. 8c). This substitution at adjacent carbon atoms occurs with retention of configuration [58]. The nature of the rearrangement step itself (i.e., whether it occurs via a free radical, a carbonium ion, a carbanion, an organocobalt intermediate or via a fragmentation pathway [59]; Fig. 9) is not known, and a number of studies have attempted to model this step [60–66]. Reabstraction of a hydrogen atom (Fig. 8d) to give the product, and recombination of the deoxyadenosyl and cobalamin radicals (Fig. 8e) complete the catalytic cycle.

This mechanism predicts that radical-pair intermediates are formed during catalysis. This has been tested using EPR spectroscopy of the mutase trapped in steady-state catalytic turnover in the presence of either substrate [67,68] or substrate analogs [69,70]. In all cases, a broad axial spectrum with $g_{xy} = 2.11$ and $g_z = 2.0$ has been observed (Fig. 10). The contribution of cob(II)alamin to this species can be readily discerned on the basis of the observed cobalt hyperfine splitting pattern. Selective isotope substitutions in the substrate indicate that the other member of the radical pair is organic and derived from the substrate. Thus, the paramagnetic intermediate observed by EPR spectroscopy is cob(II)alamin spin coupled to a substrate-derived radical (Fig. 10, inset). Other AdoCbl-dependent enzymes have also been shown to catalyze formation of a $g_{xy} \approx 2.11$ signal in the presence of their respective substrates [71–73]. In glutamate mutase, isotope substitutions

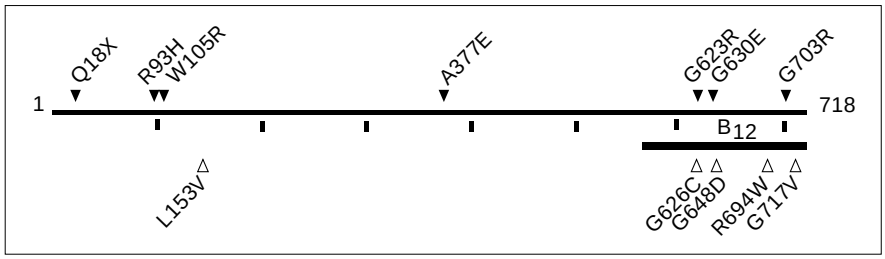
Figure 10



EPR spectrum of the radical-pair intermediate on methylmalonyl-CoA mutase. The line marker is at g = 2.11. The inset shows the postulated structure of the radical-pair intermediate, in which cob(II)alamin is spin coupled to a substrate-derived radical.

Figure 11

Map of mutations isolated from patients with methylmalonic acidemia belonging to the mut^0 class (▼), and to the mut$^-$ class (△). The predicted site for cobalamin binding is indicated.



and use of substrate analogs indicate that the 'other' radical is substrate-derived [72]. In ribonucleotide reductase from *Lactobacillus casei*, the 'other' member of the radical-pair intermediate is a protein-derived thiyl radical [73].

Co–C bond homolysis: a trillion-fold acceleration

The aspect of B_{12}-dependent isomerases that has received the most attention has been their estimated $10^{12\pm1}$-fold rate enhancement of Co–C bond homolysis [57]. The Co–C bond is fairly weak, but on the enzyme and in the presence of substrate a mammoth labilization occurs corresponding to a $\Delta\Delta G^{\ddagger}$ of 16 kcal mol^{-1} [74]. In the debate over the factors that govern this weakening, significant emphasis has been placed on *trans* steric [75–78] and *trans* electronic [2,79] effects. The bulk of the lower ligand controls the extent of corrin-ring pucker — the upward fold angle decreases from 18°–11.3° when DMB is replaced by imidazole in solution [80]. Cob(II)alamin in the mutase active site appears to be relatively flat, however, and the geometry of enzyme-bound AdoCbl is unknown [55]. Resonance Raman studies on AdoCbl reveal that the lower axial base has no effect on the Co–C bond strength in the ground state [36]. Kinetic studies indicate that coordination by DMB favors Co–C bond dissociation by 10^2 [57] while equilibrium measurements indicate that the contribution of the lower base to homolytic cleavage is negligible [35]. Taken together, these results suggest that the modest *trans* effect observed in the kinetic studies is expressed in the transition state, and that significant contribution to Co–C bond weakening must be derived from factors other than *trans* ligand effects.

Although the discussion of destabilization mechanisms has tended to concentrate on ground-state steric distortion methods, the role of the substrate in labilizing the Co–C bond has also been considered. Of the two alternatives, the use of the intrinsic substrate binding energy, is a more attractive mechanism, providing the enzyme with a *modus operandi* to control cofactor reactivity. Significant ground-state destabilization of the Co–C bond in the active site would promote homolysis and, possibly, escape of the adenosyl radical leading to inactive enzyme. With methylmalonyl-CoA mutase, inactivation in the absence of substrate is not observed.

The rearrangement of methylmalonyl-CoA to succinyl-CoA minimally involves two hydrogen-transfer steps, as indicated in Figure 8. Kinetic isotope effects for the human enzyme [81] and bacterial enzyme [82,83] have been measured. These studies indicate that hydrogen-atom transfer is only partially rate limiting [83]. Stopped-flow spectrophotometric studies have revealed that Co–C bond cleavage is very rapid (the observed rate constant, $k_{obsd} > 600\,s^{-1}$ at 25°C), and therefore not rate limiting in catalytic turnover ($k_{cat} \approx 60\,s^{-1}$ at 25°C). Surprisingly, in the presence of [CD_3]-methylmalonyl-CoA, the Co–C bond homolysis rate is reduced ~20-fold. These results have been interpreted as evidence that cleavage of the organometallic bond (Fig. 8a) and H-atom abstraction from substrate (Fig. 8b) are coupled and that the intrinsic substrate binding energy contributes to labilization of the Co–C bond [84]. (A substrate isotope effect on Co–C bond cleavage has also been observed in glutamate mutase; E.N.G. Marsh, personal communication.)

Tritium from 5′-[CT_3] AdoCbl is partitioned in a ratio of 3:1 (succinyl-CoA : methylmalonyl-CoA) regardless of whether the reaction proceeds with substrate or product [84]. This indicates that the barrier to the interconversion of substrate and product is low, and, together with the stopped-flow studies, suggests that another step such as product release is rate limiting. None of the tritium is released to solvent or remains bound to the enzyme, arguing against the intermediacy of a protein radical [83].

Identification of the intrinsic binding energy as contributing to Co–C bond labilization does not, however, furnish information on the mechanism by which this is achieved. Protein sidechains interacting with the cofactor may function like forklifts to labilize the Co–C bond by steric distortion following substrate binding. Both the cofactor and substrate provide surfaces rich in the potential for interactions with the protein, and a number of these have been identified in the mutase crystal structure [56]. It is interesting in this regard, that substitution of

AdoCbl by 2′5′ dideoxy AdoCbl (in which the 2′OH of the ribose ring is replaced by hydrogen), results in an inactive enzyme [85].

A number of cofactor and substrate analogs have been used to elucidate the minimal structural determinants of reactivity. With the availability of the three-dimensional structure of the bacterial mutase, a similar approach will be employed to evaluate the roles of individual active-site residues. In an interesting study, a series of cofactor analogs with variable alkyl spacers between the cobalt and the 4′ carbon of deoxyadenosine, designed to mimic the 'posthomolysis intermediate' of AdoCbl, was employed [86]. The analog with a C6 spacer had the lowest inhibition constant, suggesting that the adenosine moiety may move some 10 Å away from the cobalt following homolysis.

The role, if any, of the displaced DMB in catalysis is presently unknown. In the extended conformation, it provides extensive surfaces for binding to the active sites of both methionine synthase and methylmalonyl-CoA mutase. But both methionine synthase [87] and methylmalonyl-CoA mutase [55] can bind cofactor analogs with truncated nucleotide tails. Although these analogs were reported to be active with methionine synthase [87], it is very uncertain whether these compounds were free of contaminating cobalamin. Neither AdoCbi nor AdoCbiPMe are active with the mutase although they bind fairly tightly to the enzyme ([55]; R.B., unpublished data).

Methylmalonyl-CoA mutase and medicine

Impaired functioning of methylmalonyl-CoA mutase either caused by genetic defects or induced by cobalamin deficiency leads to methylmalonic acidemia in which precursors and abnormal metabolites of methylmalonyl-CoA accumulate [88]. Depending on the severity of the condition, the clinical consequences range from benign to neonatal death. Patients with inborn errors in methylmalonyl-CoA mutase metabolism are classified as being either mut^- (enzyme activity is detectable), or mut^o (enzyme activity is not detectable). Over 20 mutations have been reported in the human gene (reviewed in [89]). A number of these are clustered in the carboxy-terminal B_{12}-binding domain (Fig. 11). These variants represent a road map, provided by nature, of catalytically important residues; however the penalties associated with individual mutations remain to be explored.

Conclusions

The dual reactivity of the Co–C bond of alkylcobalamins associated with the isomerase and methyltransferase subfamily is enigmatic. The factors modulating the reactivity of the organometallic bond to selectively permit homolytic (in isomerases) or heterolytic (in methyltransferases) cleavage remain unknown. The crystal structures of methionine synthase and methylmalonyl-CoA mutase will guide future experiments designed to explore this and other mechanistic issues. Finally, mutation analysis studies will determine whether population polymorphisms in the human methionine synthase gene are correlated with risk for neural tube defects and/or cardiovascular diseases.

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