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Radical Peregrinations Catalyzed by Coenzyme B_{12}-Dependent Enzymes†

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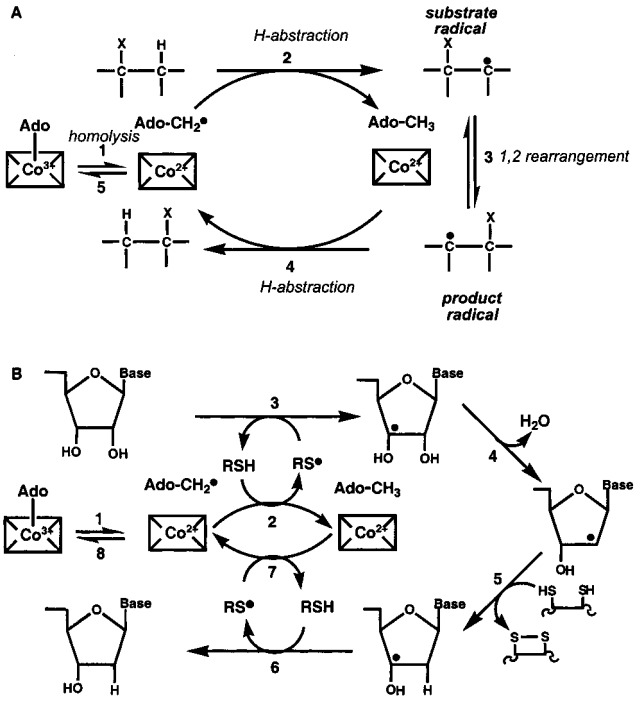
Cobalamins, or derivatives of vitamin B_{12}, are complex organometallic cofactors considered to be the evolutionary matriarch of the family of extant tetrapyrrolic derived macrocyclic cofactors. At their center lies the cobalt atom coordinated equatorially to four pyrrolic nitrogens. In solution and at neutral pH, the lower axial ligand is the intramolecular and bulky base, dimethylbenzimidazole, which extends from ring D via a side chain tether (Figure 1A). Variability is therefore confined to the upper axial position where methyl, deoxyadenosyl, and cyano groups have been described in methylcobalamin (MeCbl),[1] coenzyme B_{12} (or AdoCbl), and vitamin B_{12}, respectively. A distinguishing feature of these cofactors is the presence of a metalloalkyl bond that exhibits dyadic behavior with respect to its mechanism of cleavage. MeCbl-dependent enzymes catalyze transmethylation reactions in which the Co−methyl bond is cleaved heterolytically. In contrast, AdoCbl-dependent enzymes catalyze rearrangement reactions in which the Co−deoxyadenosyl bond fragments homolytically. In Nature, cobalamins serve as cofactors for three different classes of enzymes: isomerases, methyltransferases, and dehalogenases. Of these, the isomerase subfamily appears to be the most diverse and most widely prevalent and is the focus of this article.

The reactions catalyzed by AdoCbl-dependent isomerases involve the 1,2-interchange of a variable group (-OH, $-NH_2$, or a carbon-containing fragment) and a hydrogen atom on vicinal carbons (Scheme 1). An exception to this generalization is encountered in ribonucleotide reductase that catalyzes the formation of deoxyribonucleotides by reduction of the 2′-carbon of ribonucleotides. These chemically difficult reactions are enabled by the deployment of radical chemistry

Scheme 1[a]



[a] (A) General mechanism of rearrangement catalyzed by AdoCbl-dependent enzymes. (B) In class II ribonucleotide reductases, a thiyl radical rather than the initially formed deoxyadenosyl radical abstracts a hydrogen atom from the substrate, and a reduction rather than a rearrangement reaction is catalyzed.

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[1] Abbreviations: MeCbl, methylcobalamin; AdoCbl, 5′-deoxyadenosylcobalamin; AdoCbi, 5′-deoxyadenosylcobinamide; AdoCbi-PMe, 5′-deoxyadenosylcobinamide phosphate methyl ester; AdoCbi-GDP, 5′-deoxyadenosylcobinamide guanosine diphosphate.

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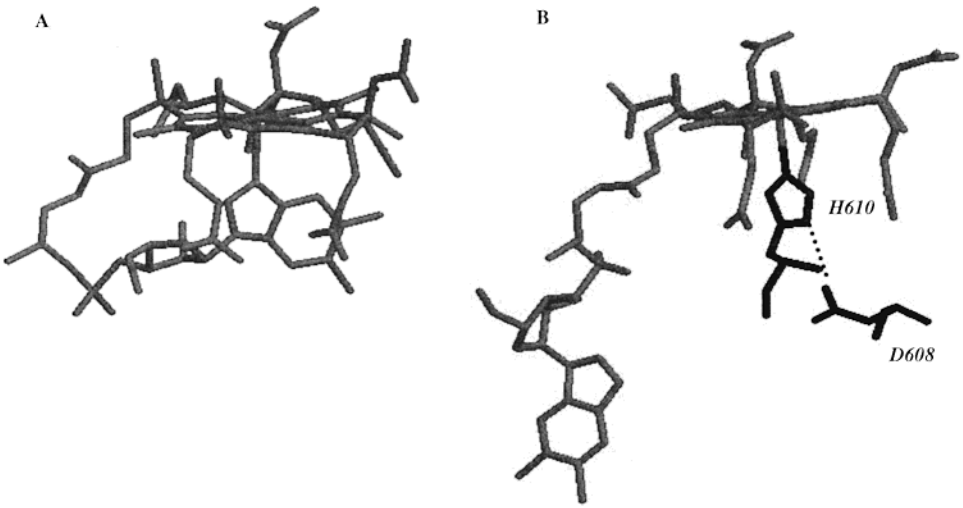


FIGURE 1: Conformation of cobalamin bound to the active sites of diol dehydratase (A) and methylmalonyl-CoA mutase (B). H610 coordinates to the cobalt in B and is within hydrogen bonding distance of D608. The deoxyadenosine moiety was not present in either structure.

with the cofactor serving as a latent free radical reservoir. Homolysis of the Co−carbon bond unleashes the radical pair, cob(II)alamin, and the deoxyadenosyl radical. A volley of contained radical peregrination steps follow in which hydrogen atom abstraction by the deoxyadenosyl radical leads to a substrate radical that rearranges to a product radical. Reabstraction of a hydrogen atom from deoxyadenosine yields the product, and recombination of the cofactor radicals completes the catalytic cycle.

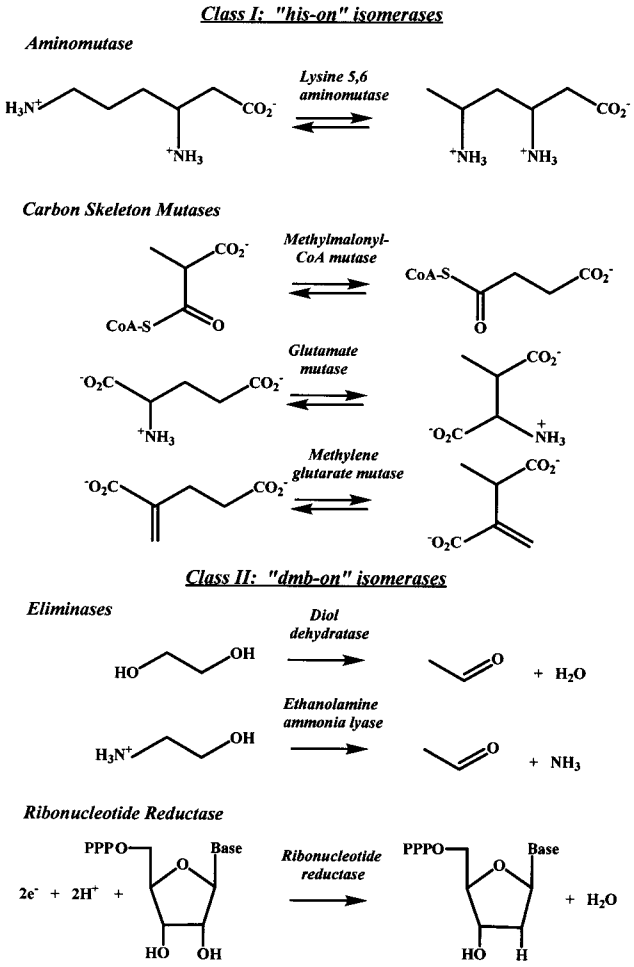
A subject of enduring debate in the field has been the mechanism by which the respective enzymes labilize the Co−C bond. This bond is relatively weak, with a bond dissociation energy of ~30 kcal/mol in aqueous solution, and undergoes homolysis with a rate constant of $\sim 9 \times 10^{-9}$ s^{-1} at 37 °C (*1*−*3*). In contrast, the k_{cat} for some AdoCbl-dependent enzymes is on the order of 10^2 s^{-1}, leading to an estimated trillion-fold rate enhancement that is orchestrated by the enzymes (*4*). In this context, the role of the lower axial ligand, dimethylbenzimidazole, in influencing the strength and therefore the reactivity of the upper axial bond via trans steric and electronic effects has been extensively examined with model compounds. A popular hypothesis invoking the role of conformational distortion of the flexible corrin ring has been widely tested (for example, see refs *5*−*9*). According to the "mechanochemical trigger" hypothesis for labilization of the Co−C bond, an upward flexing of the corrin, induced by the protein or by an inward movement of the lower ligand, would lead to steric crowding on the upper face, thereby weakening the organometallic bond.

However, the determination of the first three-dimensional structures of B_{12} enzymes swept in a paradigm shift within the field with the unexpected revelation that the cobalamin is bound in the active sites of these proteins in a conformation entirely different from that observed in solution (*10*−*12*). The nucleotide tail is held in an extended conformation, and the dimethylbenzimidazole moiety is >10 Å removed from the metal and replaced with a histidine residue embedded in a DX**H**XXG motif in each protein (Figure 1B). This insight into the active site conformation of the cofactor provided by structure determination (*11*, *12*) and by EPR spectroscopy (*13*−*15*) raised a whole new series of questions, including ones about the role, if any, of dimethylbenzimidazole in catalysis and the role of the histidine residue in labilizing the Co−carbon bond. However, further EPR spectroscopic investigations (*16*−*18*) followed by structure elucidations (*19*) led to a new twist, the recognition of a second subclass of AdoCbl-dependent enzymes in which dimethylbenzimidazole is retained as the lower axial ligand. Thus, AdoCbl-dependent enzymes belong to at least two different subfamilies on the basis of their mode of cofactor binding: the "his-on" conformation in methylmalonyl-CoA mutase, glutamate mutase, isobutyryl-CoA mutase, lysine 5,6-aminomutase, and methylene glutarate mutase and the "dmb-on" conformation in dioldehydratase, ethanolamine ammonia lyase, and ribonucleotide reductase (Scheme 2).

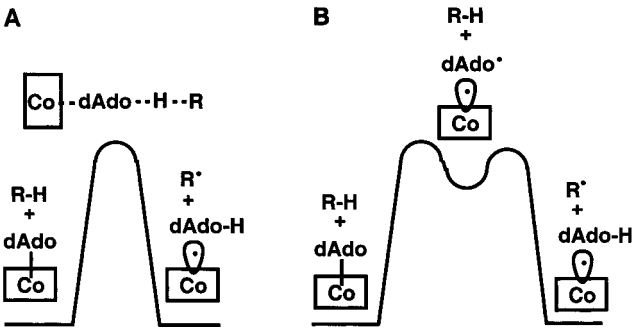
Kinetic Coupling of Homolysis and Substrate (or Protein) Radical Generation Steps

The relative weakness of the Co−carbon bond presents a dilemma for AdoCbl-dependent enzymes, i.e., how to prevent dissipation of the radical potential of the cofactor in the absence of substrate. Recent kinetic investigations of methylmalonyl-CoA mutase, ribonucleotide reductase, and glutamate mutase present novel insights into the strategy that may be employed by these enzymes to control the homolysis reaction. In methylmalonyl-CoA mutase and in glutamate mutase, homolysis of the Co−carbon bond is rapid and displays an unusual sensitivity to isotopic substitution in the substrate. Thus, the homolysis rate is decreased >20-fold when [CD_3]methylmalonyl-CoA (*20*) or [2,4,4-D_3]glutamate (*21*) is utilized instead of the corresponding protiated substrates. These results are interpreted as evidence for kinetic coupling of the homolysis and substrate radical generation steps whereby an unfavorable equilibrium for homolysis is overcome by the favorable equilibrium governing the second (coupling) step (Scheme 3). In glutamate mutase, abstraction of a hydrogen atom from glutamate results in a secondary radical that is expected to be more stable than the primary deoxyadenosyl radical. In methylmalonyl-CoA mutase on the other hand, the coupling step leads to a primary substrate-centered radical that is presumably similar in energy to the deoxadenosyl radical. However, rearrangement leads to a secondary radical that could be further stabilized by the geminal carboxyl substituent. Hence, in methylmalonyl-CoA mutase, the unfavorable equilibrium

Scheme 2: Representative Reactions Catalyzed by Members of the Two Subclasses of AdoCbl-Dependent Enzymes



Scheme 3: Qualitative Free Energy Profiles for Alternative Mechanisms of Kinetic Coupling of Co−C Bond Homolysis to Substrate Radical Generation, (A) Concerted Mechanism and (B) Stepwise Mechanism[a]



[a] RH denotes the substrate.

for the Co−carbon bond homolysis step may be overcome by the eventual formation of the product radical.

In contrast to all other B_{12}-dependent enzymes, the hydrogen atoms at C5′ of AdoCbl undergo solvent exchange in ribonucleotide reductase. This is explained by the presence of a protein-derived thiyl radical (at C408 in the *Lactobacillus caseii* enzyme) that serves as a transit station for the radical between deoxyadenosine and the substrate (*22*, *23*) (Scheme 1B). Mutation of C408 to serine results in the apparent failure of the homolysis step, providing evidence for the coupling of this step to the generation of the more stable thiyl radical (*22*).

These results suggest a mechanism by which the respective enzymes utilize substrate binding energy (or the binding of an allosteric effector in the case of the partial reaction studied in ribonucleotide reductase) to mobilize the radical potential of the cofactor when the enzyme readies to initiate catalysis (Scheme 3). In the absence of substrate, the occurrence of AdoCbl bond cleavage in methylmalonyl-CoA mutase is indicated by stereochemical scrambling of deuterium at C5′, the carbon bonded to the cobalt (*24*). However, recombination of the resulting radicals in the protein cage must be disproportionately favored versus cage escape to explain the observed absence of radical products in the absence of substrate (*25*). This strategy of kinetic coupling may be more generally exploited by enzymes that utilize cofactors as free radical reservoirs, such as in the family of *S*-adenosylmethionine-dependent enzymes where the formation of a high-energy deoxyadenosyl radical is postulated (*26*).

Co−carbon bond homolysis is rapid and not rate-determining in all AdoCbl-dependent enzymes where it has been monitored by stopped-flow spectroscopy (*20*, *21*, *27*, *28*). A detailed characterization of the kinetic and thermodynamic parameters describing Co−carbon bond homolysis has so far been described for ribonucleotide reductase (*29*, *30*) and methylmalonyl-CoA mutase (*31*). In solution, thermolysis of AdoCbl is characterized by an unfavorable equilibrium and a $\Delta G^{\ddagger}$ of 30 kcal/mol at 37 °C. In contrast, the coupled homolysis steps catalyzed by ribonucleotide reductase and methylmalonyl-CoA mutase are characterized by equilibrium constants close to unity and a sizable lowering of $\Delta G^{\ddagger}$ of ∼15−17 kcal/mol that corresponds to a rate acceleration of $\sim 10^{12}$-fold (*29*−*31*).

A corollary of the kinetic coupling hypothesis for Co−carbon bond cleavage is that the Co−carbon bond is not substantially destabilized in the ground state, i.e., in the resting holoenzyme. This expectation is supported by experimental data for at least two enzymes. First, comparison of the resonance Raman spectra of AdoCbl free in solution and bound to methylmalonyl-CoA mutase reveals that the ν(Co−C) stretching vibration is minimally perturbed in the bound state (*32*, *33*). It is downshifted by 6 cm^{-1}, corresponding to a destabilization of ∼0.5 kcal/mol. Second, the Co−carbon bond dissociation enthalpy for AdoCbl bound to ribonucleotide reductase is estimated to be similar to the value for the free cofactor (*30*). In contrast, in dioldehydratase, studies with cofactor analogues have led to the conclusion that approximately half of the 10^{12}-fold destabilization is expressed in the ground state with the remainder being realized following substrate binding (*34*). Significant weakening of the Co−carbon bond in the absence of substrate is expected to lead to enzyme inactivation. Nature appears to compensate for this profligacy by taking recourse in an ATP-dependent repair system that catalyzes the release of the inactive cofactor (*35*).

Despite the differences in their quaternary structures, and the lack of overall primary sequence homology, the structures of methylmalonyl-CoA mutase (*11*) and glutamate mutase (*12*) reveal strikingly similar architectures. Furthermore, despite the difference in the cofactor conformation between these two enzymes and diol dehydratase (*19*), they share similarities in the folds used for cofactor binding. Thus, the

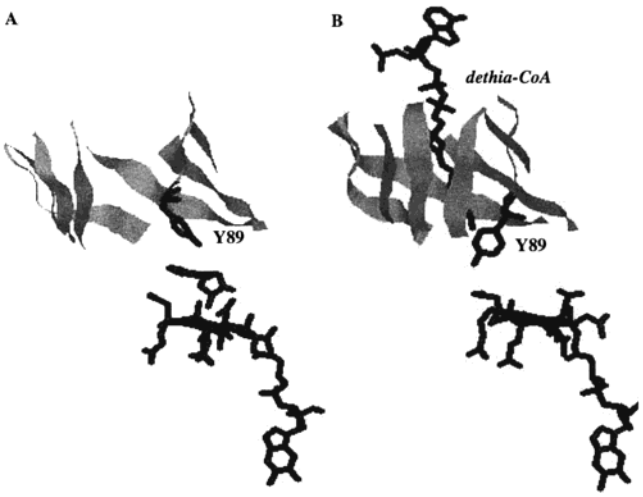


FIGURE 2: Conformational change that accompanies substrate binding in methylmalonyl-CoA mutase. (A) Structure of holoenzyme in the absence of substrate. Only the β-sheets that form part of the TIM barrel on the upper face of the cofactor are shown. Active site residue Y89 is located above the adenine ring of the cofactor. (B) The substrate analogue, dethia-CoA, pierces through the TIM barrel, and the β-sheets move in around it. This results in Y89 moving in closer to the corrin ring (*37*). Density for the deoxyadenosine moiety was not present in this structure.

lower or α-face of the cofactor is docked in a Rossman fold-like structure, a motif widely utilized for nucleotide binding. The upper or β-face is constructed from another common fold, the TIM barrel, and is deeply buried. In methylmalonyl-CoA mutase, the substrate pierces through the entire length of the TIM barrel in a fashion that is apparently unique and, in so doing, accomplishes the task of sealing off the active site from the solvent (*36*, *37*). In the absence of substrate, the adenine ring of the cofactor is sandwiched between Y89 and the corrin ring (Figure 2). Substrate binding induces a substantial conformational change as the barrel snaps around the pantethiene tail and pushes Y89 toward the corrin ring into the area previously occupied by the deoxadenosine moiety. Thus, substrate binding energy appears to be harnessed by methylmalonyl-CoA mutase to mobilize Y89 as a molecular wedge that pries apart the Co−carbon bond (*37*).

Radical Flights: Bridging the Distance between the Cofactor and Substrate

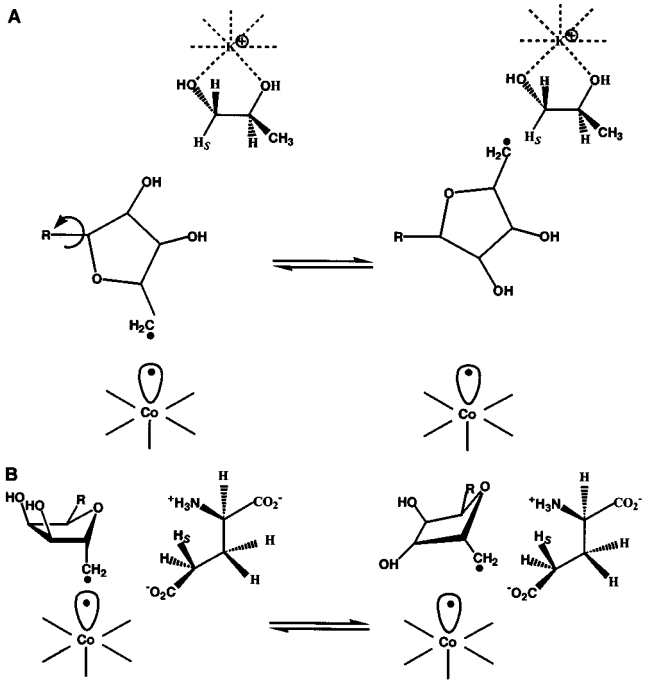
The general mechanism depicted for AdoCbl-dependent enzymes predicts the presence of radical pair intermediates in which one of the radicals is metallic and the other organic. This prediction is borne out by the observation of unique EPR spectra that fall into two classes. The first class, exemplified by diol dehydratase and ethanolamine ammonia lyase, is characterized by the presence of an axial spectrum with g values typical of low-spin cob(II)alamin and an organic radical signal centered at $g = 2$ that is split into a doublet due to hyperfine interaction with the unpaired electron on the cobalt (*38*−*40*). Simulation of the ethanolamine ammonia lyase spectrum yielded an interradical distance estimate of ∼11 Å (*41*). In contrast, in glutamate mutase (*42*), ribonucleotide reductase (*43*), and methylmalonyl-CoA mutase (*25*, *44*), a novel axial EPR spectrum is observed with a g value of ∼2.1. Simulations of the spectra in ribonucleotide reductase (*45*) and in glutamate mutase (*42*) provide an interradical distance estimate of ∼7 Å. With the exception of ribonucleotide reductase, where a cysteinyl radical is coupled to cob(II)alamin (*23*), the second radical in the other enzymes is organic in nature and appears to correspond to the most stable radical that is predicted on the basis of chemical grounds. Isotopic labeling studies have failed to provide evidence for the accumulation of the deoxyadenosyl radical in the EPR spectra, consistent with the proposition that it is a high-energy intermediate and likely to be present only at very low concentrations.

Thus, the EPR spectra demand that the radical on deoxyadenosine, which is initially spin correlated and proximal to cob(II)alamin, is propagated over a significant distance during generation of the substrate-derived radicals that accumulate under steady-state turnover conditions. There are at least two mechanisms by which the radical can journey between the cofactor and substrate. The first is an indirect route in which a protein radical serves as an intermediate between deoxyadenosine and the substrate, and the second is a direct route interconverting the deoxyadenosine and substrate radicals. With the exception of ribonucleotide reductase, where there is unequivocal evidence for the role of a protein radical (*23*), there is no evidence for the involvement of a protein radical in any other AdoCbl-dependent enzyme, and kinetic evidence against one in cases where this issue has been examined (*46*−*48*).

The forward propagation of the deoxyadenosyl radical must then be triggered by conformational changes that result in either a small or large separation from the cobalt. Recently elucidated structural evidence from two enzymes, diol dehydratase (*49*) and glutamate mutase (*50*), provides fascinating insights into how this may be achieved (Scheme 4). Thus, in diol dehydratase, where a larger distance must be traversed (the distance between substrate C1 and cobalt in the crystal structure is 8.4 Å), rotation around the *N*-glycosidic bond allows the deoxyadenosyl radical to swing between the cofactor and substrate, providing a 6.6 Å reach. In contrast, in glutamate mutase where the distance that needs to be bridged is shorter, a pseudorotation of the ribose ring can change the orientation of the C5′ radical from pointing toward the cobalt to pointing toward the substrate carbon. A prediction of the "conformational toggle mechanism" for radical propagation is that C5′ of deoxyadenosine must be in close contact with the substrate carbon to permit hydrogen atom abstraction. A recent ENDOR study on ethanolamine ammonia lyase reveals that the deoxyadenosine moiety swings ∼7 Å from its original position in AdoCbl to within van der Waals contact distance with the substrate C1 which is ∼12 Å away from the cobalt, and provides strong evidence in support of this mechanism (*51*).

It is thus tempting to speculate that in the subclass of enzymes where the interradical distance is small, such as in methylmalonyl-CoA mutase, pseudorotation of the ribose ring could result in radical propagation from the cofactor to substrate. In contrast, where the distance is large, as in ethanolamine ammonia lyase, a different means, namely, glycosidic bond rotation, would accomplish the same end. Importantly, this mechanism for radical propagation argues strongly against a concerted mechanism for the coupled homolysis−substrate radical generation step (Scheme 3A). A discrete deoxyadenosyl radical must exist, albeit at very

Scheme 4: Schematic Representation of Conformational Toggles for Radical Translation from the Cofactor to Substrate[a]



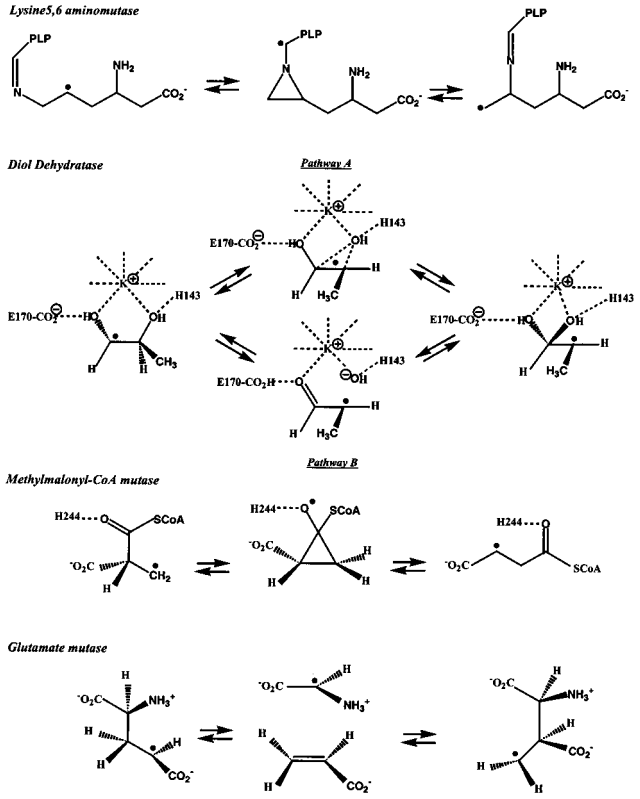
[a] (A) In diol dehydratase, rotation of the ribose ring around the *N*-glycosidic bond moves the radical at C5′ ~6.6 Å away from the cobalt. (B) In glutamate mutase, pseudorotation of the ribose ring moves the radical at C5′ ~3 Å away from the cobalt.

low concentrations, and undergo a conformational change to partake in the next step.

Quantum Mechanical Tunneling during Hydrogen Atom Transfer from the Substrate to Cofactor

With the exception of the reaction catalyzed by ribonucleotide reductase, a minimum of two hydrogen atom transfers are predicted to occur in reactions catalyzed by all AdoCbl-dependent enzymes (Scheme 1). The overall kinetic isotope effects associated with these reactions are normal, i.e., lie within the range predicted for semiclassical, behavior. However, exalted kinetic isotope effects have been reported for the component hydrogen atom transfer steps from the substrate to the deoxyadenosyl radical [in methylmalonyl-CoA mutase (*20*, *52*) and glutamate mutase (*21*)] and from deoxyadenosine to the product radical [in diol dehydratase (*53*) and ethanolamine ammonia lyase (*54*)]. The anomalously large isotope effects suggest the involvement of nuclear tunneling whereby the hydrogen atom traverses through (rather than over) the energy barrier as it crosses from the reactant to the product well. In the methylmalonyl-CoA mutase-catalyzed reaction, an Arrhenius analysis of the temperature dependence of the isotope effect provides strong evidence for tunneling behavior (*52*). Thus, large deviations in the values of A_H/A_D (0.078 ± 0.009) and $E_{a_D} - E_{a_H}$ (3.41 ± 0.07 kcal/mol) are observed that dwell outside the range predicted for semiclassical behavior ($A_H/A_D = 0.7-1$ and $E_{a_D} - E_{a_H} = 1.15$ kcal/mol). Interestingly, the difference in the activation enthalpy for deuterium versus hydrogen transfer is non-zero ($\Delta H^{\ddagger}_D - \Delta H^{\ddagger}_H = 5.9$ kcal/mol), suggesting a role for protein dynamics in promoting nuclear tunneling. It is likely that tunneling will emerge as a common catalytic strategy for hydrogen atom transfers in other AdoCbl-dependent enzymatic reactions.

Scheme 5: Alternative Pathways for the Rearrangement Reactions in Representative Members of AdoCbl-Dependent Isomerases



A factor that is deemed to be important for promoting tunneling is the width of the reaction barrier, i.e., the distance between the donor and acceptor sites over which the atom is transferred (*55*). In this light, recent spectroscopic and structural data provide provocative evidence that the 5′-carbon of deoxyadenosine is in van der Waals contact with the substrate carbon from which hydrogen atom transfer occurs in diol dehydratase (*49*) and in ethanolamine ammonia lyase (*51*). Experimental evaluation of this model can be sought by studying the dependence of the kinetic isotope effect on modulation of the donor−acceptor distance either via mutagenesis of key active site residues or by use of substrate analogues, and complemented by high-resolution crystal structures that monitor the effects of these changes.

Rearrangement Step: Anions, Cations, Fragments, or Cyclic Intermediates?

The mechanism of the rearrangement step represents the most poorly understood aspect of AdoCbl-dependent reactions. While the hydrogen atom is transferred intermolecularly, from the substrate to deoxyadenosine (or to the active site cysteine in ribonucleotide reductase) before being returned to the product, the variable group migrates intramolecularly. The differences in the chemical reactivity of the groups that are rearranged by these enzymes necessitate different strategies for facilitating this step (Scheme 5). Thus, in lysine 5,6-aminomutase, the chemical problem of amino group migration is apparently solved by the formation of a Schiff base with pyridoxal phosphate that renders the nitrogen

sp^2-hybridized and serves to stabilize the radical via delocalization (*56*). Computational studies indicate protonation of the PLP ring would further enhance the reaction via captodative stabilization of the cyclic intermediate (*57*). In contrast, in diol dehydratase, a hydroxyl group shifts between adjacent carbon atoms and at least two plausible mechanisms can be considered (Scheme 5). The first is a direct rearrangement via a cyclic transition state (pathway A) that could be facilitated by protonation of the hydroxyl group as suggested by ab initio molecular orbital theory-based calculations (*34*). The structure of diol dehydratase reveals an active site K^+ that coordinates both hydroxyl groups of the substrate and H143 that is within hydrogen bonding distance of the C2 OH group (*19*). Both the potassium ion and H143 could play a role in stabilizing the transition state in this pathway. The second pathway involves the formation of a radical anion intermediate and is predicated by the reported lowering of the pK_a value of a hydroxyl group on a carbon radical by 5 units versus the value for the corresponding alcohol (*58*). In the active site of diol dehydratase, E170 could function as a general base to deprotonate the C1 OH group and K^+ could assist in electrostatic stabilization of the resulting radical anion intermediate and in elimination of the C2 OH group which would subsequently add to C1 to form a gem-diol intermediate (pathway B). Recent density functional theory-based computations aimed at evaluating the contribution of the K^+ ion in the diol dehydratase mechanism predict a modest stabilization by K^+ coordination for the pathway involving a cyclic transition state but failed to find a transition state solution for the elimination−addition pathway (*34*).

The general properties of ethanolamine ammonia lyase in which an amino group migrates appear to be more similar to those of diol dehydratase rather than to those of lysine 5,6-aminomutase. However, significant gaps exist in our understanding of the details by which the reaction proceeds. Thus, it is unclear if the reaction involves an elimination−addition step (as in pathway 2 for dioldehydratase) to give an amino alcohol intermediate or if the amino group is directly expelled as ammonia. In contrast, elegant isotope labeling studies with diol dehydratase have established the intermediacy of a gem-diol species that suffers enzymatic dehydration to yield the aldehyde product (*59*).

Glutamate mutase is apparently singular in its ability to effect rearrangement of an sp^3-hybridized carbon in the absence of additional cofactors, a situation that precludes a cyclic transition state. A plausible mechanism for this rearrangement is the fragmentation of the substrate radical to a glycyl radical and acrylate followed by addition to C2 of acrylate to form product (*60*). Recent freeze-quench studies provide evidence for this mechanism by demonstrating the formation of very low concentrations of acrylate but at a kinetically competent rate (*61*).

In the remaining carbon-skeleton mutases, methylmalonyl-CoA mutase, isobutyryl-CoA, and methyleneglutarate mutase, the migrating carbon is sp^2-hybridized, and a cyclopropyl transition state is the simplest mechanism that can be considered. Computational studies predict that the barrier for direct radical rearrangement can be lowered substantially by partial proton transfer, while the fragmentation−recombination mechanism as proposed for glutamate mutase is predicted to have a significantly higher barrier (*62*). The structure of methylmalonyl-CoA mutase reveals the presence of H244 within hydrogen bonding distance of the carbonyl group of CoA. Mutagenesis of this residue to alanine, glutamine, or asparagine results in a modest lowering of the catalytic efficiency, and the loss of one of the two kinetic pK_a's in the pH versus activity profile of the H244A mutant compared to the wild-type enzyme (*63*). Importantly, the structure of one of these mutants has been determined and confirms the absence of structural alterations other than at the site of mutagenesis (*64*). Previous kinetic studies had revealed that the barrier to the interconversion between the substrate and product radical is low; i.e., the rearrangement step is not rate-determining in the reaction catalyzed by methylmalonyl-CoA mutase (*47*). Hence, although the catalytic penalty resulting from mutation of His244 is modest, the results are consistent with a role for this residue in proton donation.

The class II ribonucleotide reductases exploit AdoCbl as a radical generator to form the working thiyl radical that initiates the reaction by abstraction of a hydrogen atom from C3′. The overall reaction involves elimination of the C2′ OH group and reduction of C2′ by electrons that are ultimately derived from NADPH. An elimination mechanism akin to pathway 2 for dioldehydratase (Scheme 5) can also be considered for ribonucleotide reductase. Elimination of the C2′ OH group would lead to a ketyl radical intermediate that would be subsequently reduced by electron transfer from a pair of active site cysteines.

The crystal structures of AdoCbl-dependent enzymes now reveal the identities of active site residues that may play a role in facilitating the rearrangement reaction. Mutagenesis studies will be a valuable approach for evaluating their roles and revealing the mechanistic strategies that are employed.

Tail Service: The Role of the Nucleotide Tail and the Lower Ligand

The nucleotide tail in cobalamins is the largest peripheral ornamentation found in any tetrapyrrolic cofactor. Its role in AdoCbl-dependent enzymes is unclear, particularly in light of its displacement to a location distal to the active site in the class I or his-on subgroup of enzymes. In diol dehydratase, extensive structure−function analyses with cofactor derivatives modified in the nucleoside moiety have revealed that substitution of dimethylbenzimidazole with the sequentially smaller bases, benzimidazole, pyridine, and imidazole, is paralleled by a decrease in catalytic activity and an increased susceptibility to inactivation (*65*). These results suggest that the steric bulk of the trans axial base may indeed play a role in controlling reactivity in this and perhaps other members of the dmb-on subfamily. Excision of the nucleoside moiety yields the cofactor analogue, AdoCbi-PMe, which retains tight binding and undergoes substrate-induced Co−C bond homolysis but does not support catalysis (*66*). Thus, a critical role for the base in diol dehydratase appears to be in facilitating turnover by minimizing unproductive escape of the deoxyadenosyl radical during catalysis.

In methylmalonyl-CoA mutase, elimination of the nucleotide tail has a modest effect on the cofactor binding and contributes ~1 kcal/mol (*67*). In contrast, the nucleotide tail has a profound influence on organizing the active site for catalysis, as evidenced by the failure of H610 to engage as

the lower axial ligand in the presence of truncated cofactor analogues and by their inability to support catalysis. This is rationalized by a comparison of the structures of the B_{12}-binding domains of apoglutamate mutase (*68*) and the corresponding domain in holomethylmalonyl-CoA mutase (*11*), which reveals that the region is largely preorganized. However, a major difference is in a segment of the protein that leads away from the coordinating histidine residue that transitions from a disordered loop to an α-helix in the presence of the cofactor. In contrast, the cofactor analogue, AdoCbi-GDP, which has a bulky but different nucleotide, is competent in catalysis (*69*). These observations suggest that occupancy of the binding pocket rather than the identity of the base itself is important for promoting catalysis in this enzyme.

The rationale for the ligand switch orchestrated by the class I his-on enzymes is unknown. Is it a vestige of evolution, inherited from the presumably more ancient MeCbl-dependent transmethylases, where it provides the enzymes with a mechanism for allowing multiple conformational changes that signal the active versus the inactive state (*70*)? Or is it an evolutionary booty selected for an advantage that it conferred on fine-tuning the catalytic potential of these enzymes? Dual mutagenesis approaches of the cofactor and of active site residues have begun to illuminate this question. In glutamate mutase, disruption of the coordinating histidine (or the aspartate residue to which it is hydrogen bonded) results in poor binding of the cofactor and is accompanied by an $\sim 10^3$-fold diminution in enzyme activity (*71*). Similar results are obtained with mutagenesis of the corresponding histidine residue in methylmalonyl-CoA mutase (M. Vlasie and R. Banerjee, unpublished results). However, the cofactor analogue, AdoCbi-GDP, binds to methylmalonyl-CoA mutase in a his-off state and incurs a modest catalytic penalty (~4-fold on k_{cat} and ~100-fold on k_{cat}/K_m) (*69*). While these results suggest a minor role for the histidine residue in catalysis, they do not rigorously exclude the possibility that with AdoCbi-GDP the histidine becomes transiently engaged as a ligand to cobalt during catalytic turnover. Together, the observations with glutamate mutase and methylmalonyl-CoA mutase suggest that loss of the histidine impacts cofactor binding and suggest that the importance of the histidine and aspartate residues may be structural and that they function in organizing a high-affinity cofactor binding site. Model studies are consistent with a modest effect (870-fold) of imidazole ligation in the Co−C bond cleavage step (*72*), especially from the perspective of the overall trillion-fold rate enhancement achieved by these enzymes.

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