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Review

Radical catalysis of B_{12} enzymes: structure, mechanism, inactivation, and reactivation of diol and glycerol dehydratases

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Abstract. Enzymatic radical catalysis is defined as a mechanism of catalysis by which enzymes catalyze chemically difficult reactions by utilizing the high reactivity of free radicals. Adenosylcobalamin (coenzyme B_{12}) serves as a cofactor for enzymatic radical reactions. The recent structural analysis of adenosylcobalamin-dependent diol dehydratase revealed that the substrate 1,2-propanediol and an essential potassium ion are located inside a $(\beta/\alpha)_8$ barrel. Two hydroxyl groups of the substrate coordinate directly to the potassium ion which binds to the negatively charged inner part of the cavity. Cobalamin bound in the base-on mode covers the cavity to isolate the active site from solvent. Based on the three-dimensional structure and theoretical calculations, a new mechanism for diol dehydratase is proposed in which the potassium ion plays a direct role in the catalysis. The mechanisms for generation of a catalytic radical by homolysis of the coenzyme Co-C bond and for protection of radical intermediates from undesired side reactions during catalysis are discussed based on the structure. The reactivating factors for diol and glycerol dehydratases have been identified. These factors are a new type of molecular chaperone which participate in reactivation of the inactivated holoenzymes by mediating ATP-dependent exchange of the modified coenzyme for free intact coenzyme.

Key words. Coenzyme B_{12}; adenosycobalamin; diol dehydratase; glycerol dehydratase; enzymatic radical catalysis; enzyme structure and mechanism; mechanism-based inactivation; reactivating factor.

Introduction

Certain enzymes utilize the high reactivity of free radicals to catalyze the reactions. Catalytic radicals originate from either cofactors or protein radicals. In addition to structure and mechanism, there are three important problems for these enzymes: (i) to generate radicals in the active site, (ii) to control highly reactive radical intermediates, and (iii) whether and how to undergo reactivation when inactivated by undesired side reactions. We have attempted to solve these issues with diol dehydratase, an adenosylcobalamin (AdoCbl)-dependent enzyme.

AdoCbl (coenzyme B_{12}) (fig. 1), a naturally occurring organometallic compound, has fascinated chemists and biologists since discovery of a coenzyme form [1] and determination of its structure [2]. It serves as a cofactor for enzymatic radical reactions including carbon skeleton rearrangements, heteroatom eliminations, and intramolecular amino group migrations. An adenosyl radical formed in the active site by homolytic cleavage of the coenzyme Co-C bond triggers the reaction by activating the substrates through abstraction of a hydrogen atom [3–8]. Diol dehydratase catalyzes the dehydration of 1,2-diols to the corresponding aldehydes

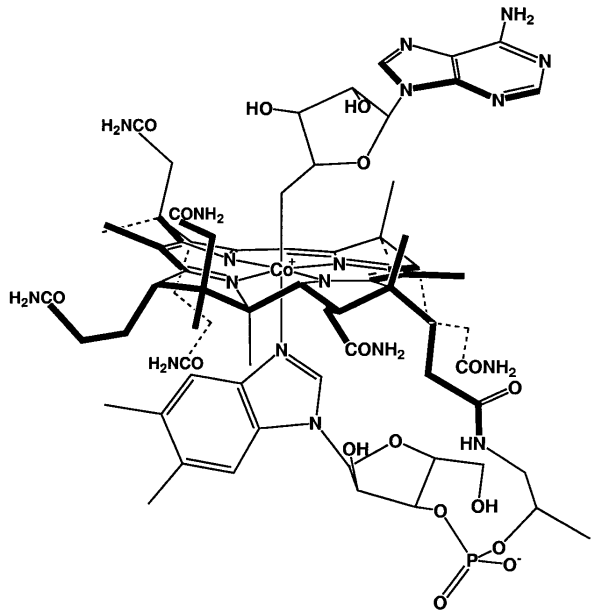


Figure 1. Structure of AdoCbl (coenzyme B_{12}).

(scheme 1) [9, 10]. Because the reaction catalyzed by this enzyme is apparently one of the simplest, the enzyme has been widely used to establish the general mechanism of action of this coenzyme [3, 5]. Extensive structure-function studies of the coenzyme have also been conducted with this enzyme [7, 11, 12]. Glycerol dehydratase is very similar to diol dehydratase in its catalytic properties, with slightly different substrate specificity, as well as in its subunit structure, but is immunochemically distinct from the latter enzyme, playing a different physiological role [7, 12, 13]. The crystal structure of diol dehydratase complexed with cyanocobalamin (CN-Cbl) was determined recently [14]. Reactivating factors for diol and glycerol dehydratases were also recently discovered, and the mechanism of its action established [15, 16].

In this review, I wish to summarize the studies on AdoCbl-dependent diol and glycerol dehydratases from the viewpoint of enzymatic radical catalysis with special emphases on structure, mechanism, inactivation, and reactivation. Structure-function studies of the coenzyme as well as physiological aspects, and gene cloning and expression studies of these enzymes have been reviewed elsewhere [7, 11, 12].

Radical catalysis of B_{12} enzymes

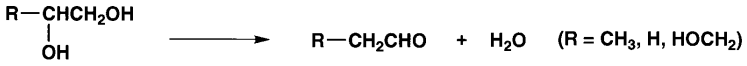
AdoCbl-dependent enzymes

Since Barker and co-workers' [1] discovery of a light-sensitive corrinoid cofactor for the interconversion of glutamate and 3-methylaspartate, many investigators have identified AdoCbl-dependent enzymes and explored their mechanisms of action. AdoCbl participates as a coenzyme for about ten enzymes, which are divided into three classes: (i) those catalyzing carbon skeleton rearrangements (class 1) [8, 17–20], (ii) those catalyzing heteroatom elimination reactions (class 2) [3–7, 12, 21–23], and (iii) those catalyzing intramolecular amino group migrations (class 3) [24] (fig. 2). Although the reactions catalyzed are seemingly quite different, all of them except for ribonucleotide reduction share a common feature—that is, in the AdoCbl-dependent rearrangements, a hydrogen atom migrates from one carbon atom of the substrate to an adjacent carbon atom in exchange for a group X that moves in the opposite direction [3, 4]. In the elimination reactions catalyzed by class 2 enzymes, X is a hydroxyl group on C-2 for diol and glycerol dehydratases and an amino group for ethanolamine ammonia-lyase, and water and ammonia are subsequently eliminated, respectively, from a *gem*-diol and an aminocarbinol formed.

Labeling experiments and stereochemistry

Although the reaction catalyzed by diol dehydratase looks simple, the mechanism of this dehydration is rather complex. Using [1-^{18}O]1,2-propanediol, Rétey et al. [25] demonstrated that the initial migration of a

Scheme 1



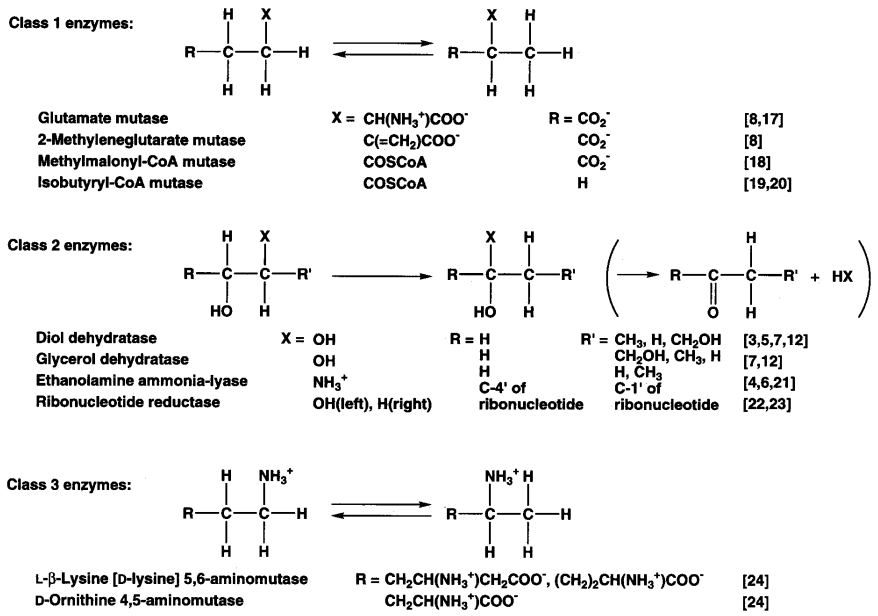


Figure 2. AdoCbl-dependent enzymatic reactions.

hydroxyl group is stereospecific, and that the dehydration of a *gem*-diol undergoes steric control by the enzyme, with only one of the two hydroxyl groups on the prochiral center being eliminated. [^{18}O]- and unlabeled propionaldehydes are formed from (*S*)- and (*R*)-1,2-propanediol, respectively (see scheme 2).

The hydrogen atom moves to the adjacent carbon atom without exchange with solvent protons [26]. Abeles and co-workers [27] demonstrated that the *pro-S* and *pro-R* hydrogen atoms migrate from (*S*)- and (*R*)-1,2-propanediol, respectively. The migrating hydroxyl group is replaced by the hydrogen atom with an accompanying inversion of the configuration of C-2 [27, 28]. Using unlabeled and deuterated substrates, Abeles and co-workers demonstrated that the hydrogen transfer is not necessarily intramolecular, but occurs by an intermolecular process as well [29, 30]. Using a tritiated substrate, they showed that the enzyme-bound coenzyme serves as an intermediate hydrogen carrier, first accepting a hydrogen atom from C-1 of the substrate to C-5′ of the coenzyme and then, in a subsequent step, giving a hydrogen back to C-2 of the product [30–33]. The deuterium isotope effect (k_H/k_D) for the overall reaction is 10 [27], indicating that breaking of the C-H bond is rate determining. The tritium isotope effects (k_H/k_T) for the hydrogen transfer from substrate to coenzyme and from coenzyme to product are 20 and 125, respectively [34]. The reason for the unusually large isotope effect, corresponding to a k_H/k_D of 28, for the latter step remains unclear.

Kinetic experiments performed by Abeles and co-workers indicate that, in the hydrogen transfer process, an enzyme-bound intermediate exists in which the hydrogen abstracted from the substrate and the two hydrogen atoms on C-5′ of the coenzyme all become equivalent [30, 34]. From these results, 5′-deoxyadenosine was postulated to be an intermediate. Its formation was actually demonstrated in inactivation reactions with several substrates [35–37] or coenzyme analogues [38–40]. Its reversible formation was shown with ethanolamine ammonia-lyase [41].

Radical intermediates and a minimal mechanism

The formation of Cbl^{II} during catalysis was observed by optical [36, 42–44] and electron paramagnetic resonance (EPR) [43, 45–48] spectroscopy. The high-field doublet and the low-field broad signals of EPR spectra were assigned to an organic radical and low-spin Co(II) of Cbl^{II}, respectively [49, 50]. The doublet signal was interpreted to arise from a weak exchange interaction of the organic radical with Co(II). An interaction of the free radical with the Co(II) center at least 6 Å away was suggested from a model proposed for the system [49], in reasonable agreement with the distances between Co and C-1 and C-2 of 1,2-propanediol of 8.37 and 9.03 Å, respectively [14]. The distance of closest approach of water to Co(II) was estimated to be 10.2 Å [46]. The generation of an organic radical intermediate at a kinet-

ically competent rate in the enzymatic reaction was also demonstrated by EPR spectroscopy [48]. Thus, it was established that the radical species formed by homolysis of the Co-C bond of AdoCbl is involved in catalysis, and that hydrogen migrates as a hydrogen atom.

A minimal mechanism proposed by Abeles and co-workers for the rearrangements catalyzed by diol dehydratase and other AdoCbl-dependent enzymes is illustrated in figure 3 [3–5]. The interaction between apoenzyme and coenzyme leads to the activation of the Co-C bond of the coenzyme for homolytic cleavage forming Cbl^{II} and the adenosyl radical. In the absence of substrate, only a small fraction of the coenzyme is present in the dissociated form. The addition of substrate to the complex shifts the equilibrium so that a major fraction of the coenzyme is converted to the dissociated form. The adenosyl radical that is formed then abstracts a hydrogen atom from the substrate, producing a substrate-derived radical and 5′-deoxyadenosine. The substrate radical rearranges to the product radical, which then abstracts a hydrogen atom from 5′-deoxyadenosine. This leads to the formation of the final product and regeneration of the coenzyme.

Concept of enzymatic radical catalysis

Ionic mechanisms are favorable for reactions which take place in a polar solvent like water, because polar transition states are stabilized by solvation. It is true that most enzymatic reactions are catalyzed by ionic mechanisms, but it should be noted that radical mechanisms can also be possible for certain enzymes, because X-ray structures of enzymes have revealed that the active sites are rather hydrophobic. Since the detection of radical intermediates by EPR in the reactions catalyzed by AdoCbl-dependent enzymes [45, 46, 51, 52] as well as non-B_{12} ribonucleotide reductase [53] in 1972–1973, many enzymes have been established to catalyze by a radical mechanism and have been attracting general interest in this rapidly expanding field [54–65].

These enzymes utilize the high reactivity of free radicals to catalyze the reactions. Such radicals originate from either cofactors or protein radicals. AdoCbl is a biochemical radical generator which forms an adenosyl radical only when necessary [55]. In addition, *S*-adenosylmethionine was also shown to serve as a donor for an adenosyl radical [56–59]. The involvement of protein radicals, stable and transient, in enzyme catalysis was established and extensively reviewed [60–65]. I will limit the scope of this review to enzymatic radical catalysis of AdoCbl-dependent enzymes.

AdoCbl-dependent carbon skeleton rearrangements catalyzed by class 1 enzymes (fig. 2) involve activation of the neighboring C-C bond by H· abstraction. The reactions accompanying the C-C bond cleavage become possible if a hydrogen atom is abstracted by an adenosyl radical from a neighboring carbon atom of the substrate. AdoCbl-dependent heteroatom eliminations and intramolecular amino group migrations catalyzed by class 2 and class 3 enzymes, respectively, involve activation of the neighboring C-O or C-N bond by H· abstraction. For cleavage of bonds between a carbon atom and poor leaving group X, such as OH^- and NH_3, activation of substrates by the removal of a hydrogen atom from the neighboring carbon atom is required. If the C-H bond of substrates is not activated by an acid-base mechanism, such activation is achieved only by abstraction of a hydrogen atom using the high reactivity of radicals or a metal-containing cofactor [56]. The validity of such speculation may be supported by the discovery of the AdoCbl-independent counterpart of diol dehydratase from *Clostridium glycolicum* [66, 67]. This enzyme is supposed to be an iron-containing radical enzyme, and its mechanism of action may be related to that of AdoCbl-dependent enzymes. Stubbe and co-workers demonstrated that AdoCbl-dependent and -independent ribonucleotide reductases catalyze by essentially an identical radical mechanism, although they differ in the ways they generate the thiyl radical, a transient protein radical [60–62]. Frey and co-workers reported that lysine 2,3-aminomutase, an AdoCbl-independent aminomutase, uses the adenosyl radical derived from *S*-adenosylmethionine [56, 57]. From these findings, it seems likely that these reactions take place only by radical mechanisms.

Radical-catalyzed reactions have a common feature—substrates of these enzymes are not activated by an acid-base mechanism. It is worth noting that all of these reactions are chemically difficult under mild, physiolog-

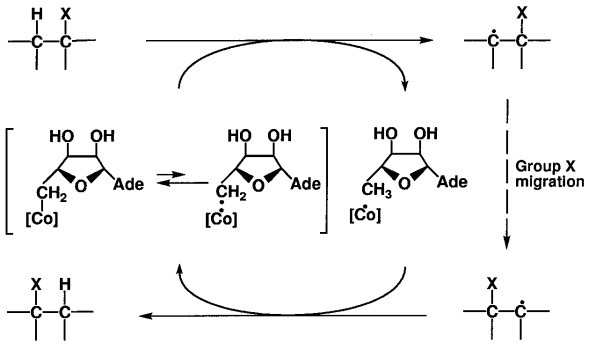


Figure 3. The minimal mechanism for AdoCbl-dependent rearrangements. Homolytic cleavage of the Co-C bond of enzyme-bound AdoCbl and adenosyl-radical-catalyzed enzymatic rearrangements. [Co], cobalamin; Ade, 9-adeninyl; X, a generic migrating group.

ical conditions without enzymes. The substrates of these enzymes become activated by abstraction of a hydrogen atom from non-activated C-H bonds with a radical species in the active site, forming a substrate-derived radical. Cleavage of a C-C, C-O, or C-N bond after the adjacent bond to a radical now takes place easily. Enzymatic radical catalysis can therefore be defined as a mechanism of catalysis by which enzymes catalyze chemically difficult reactions by utilizing the high reactivity of free radicals.

The concept of enzymatic radical catalysis can be expressed by a simplified potential energy diagram shown in figure 4. A common feature of this type of reaction is that the conversion of a substrate to a product is thermodynamically possible but kinetically very difficult because of the extremely high energy of a transition state (fig. 4A). If a catalytic free radical (R·) is introduced into the active site, it becomes stabilized by abstracting a hydrogen atom from a substrate, forming RH and a substrate-derived radical (S·) (fig. 4B). By coupling with this hydrogen abstraction, the barrier height for the transition state decreases markedly. After conversion of S· to a product-derived radical (P·), P· abstracts a hydrogen atom back from RH to form a product (PH) and regenerate the catalytic radical (R·). That is to say, the transition state ($S^{\ddagger} + RH$) becomes relatively more stabilized to the ground state ($SH + R\cdot$) by coupling with the hydrogen abstraction. As a result, the activation energy ($\Delta G_e^{\ddagger}$) is decreased markedly by the enzyme, which results in a large rate enhancement of thermodynamically possible but kinetically difficult reactions.

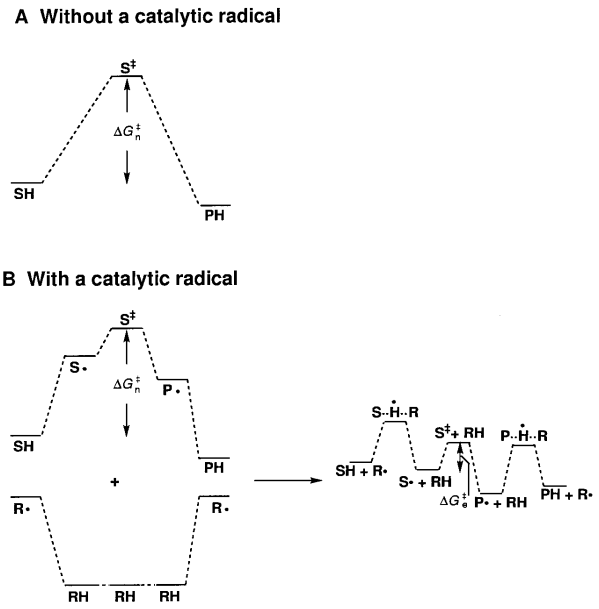


Figure 4. The concept of enzymatic radical catalysis expressed by a potential energy diagram. SH, substrate(s); PH, product(s); $S^{\ddagger}$, transition state; R·, catalytic radical. Relative barrier heights are arbitrary. With (*B*) or without (*A*) a catalytic radical.

Three-dimensional structure of diol dehydratase

Overall and subunit structures

The crystal structure of diol dehydratase complexed with cyanocobalamin (CN-Cbl) and substrate has recently been solved and refined at 2.2-Å resolution [14]. Diol dehydratase exists as a dimer of a heterotrimer, $(\alpha\beta\gamma)_2$, consistent with the subunit structure deduced from molecular-weight determination [68]. Figure 5A shows the mix of wire and schematic models of the dimeric form viewed along a non-crystallographic two-fold axis. The interaction between two α subunits contributes exclusively to dimerization of the heterotrimer, and two β and two γ subunits are separately bound by dimer α_2. Diol dehydratase dissociates into two dissimilar protein components, designated F and S, upon DEAE-cellulose column chromatography in the absence of substrate [72, 73]. Components F and S were identified as the monomeric β subunit and the dimeric $\alpha_2\gamma_2$ complex, respectively [68].

Each heterotrimer ($\alpha\beta\gamma$) binds one molecule of CN-Cbl (fig. 5B). The cobalamin molecule is located between the α and β subunits, an upper (Coβ) ligand being oriented to the direction of the α subunit, although the cyano group could not be located in the electron density map.

The α subunit includes a $(\beta/\alpha)_8$ barrel structure in the central part (fig. 6A). This structure, the so-called TIM (triose-phosphate isomerase) barrel, was found in methylmalonyl-CoA mutase [74] and recently in glutamate mutase [75] as well, although the reactions catalyzed are quite different. CN-Cbl is accommodated in the area composed of the C-terminal side of each β strand constituting the TIM barrel, while the substrate 1,2-propanediol and K^+, an essential cofactor [9, 76], are buried deep in the barrel (fig. 6A, B). The N-terminal and C-terminal regions of the α subunit consisting of many α helices and a few β strands, as well as the γ subunit consisting mainly of α helices surround the outer parts of the barrel and seem to support the α subunit to maintain the overall structure.

The β subunit has a Rossmann-fold-like structure in the central part which may play an important role in the contact with the lower ligand of cobalamin. The remaining parts consisting mainly of many α helices and a few anti-parallel β strands surround the Rossmann-fold structure. Surface representations of the α and β subunits viewed from the β and α subunits, respectively, are shown in figure 5C, D.

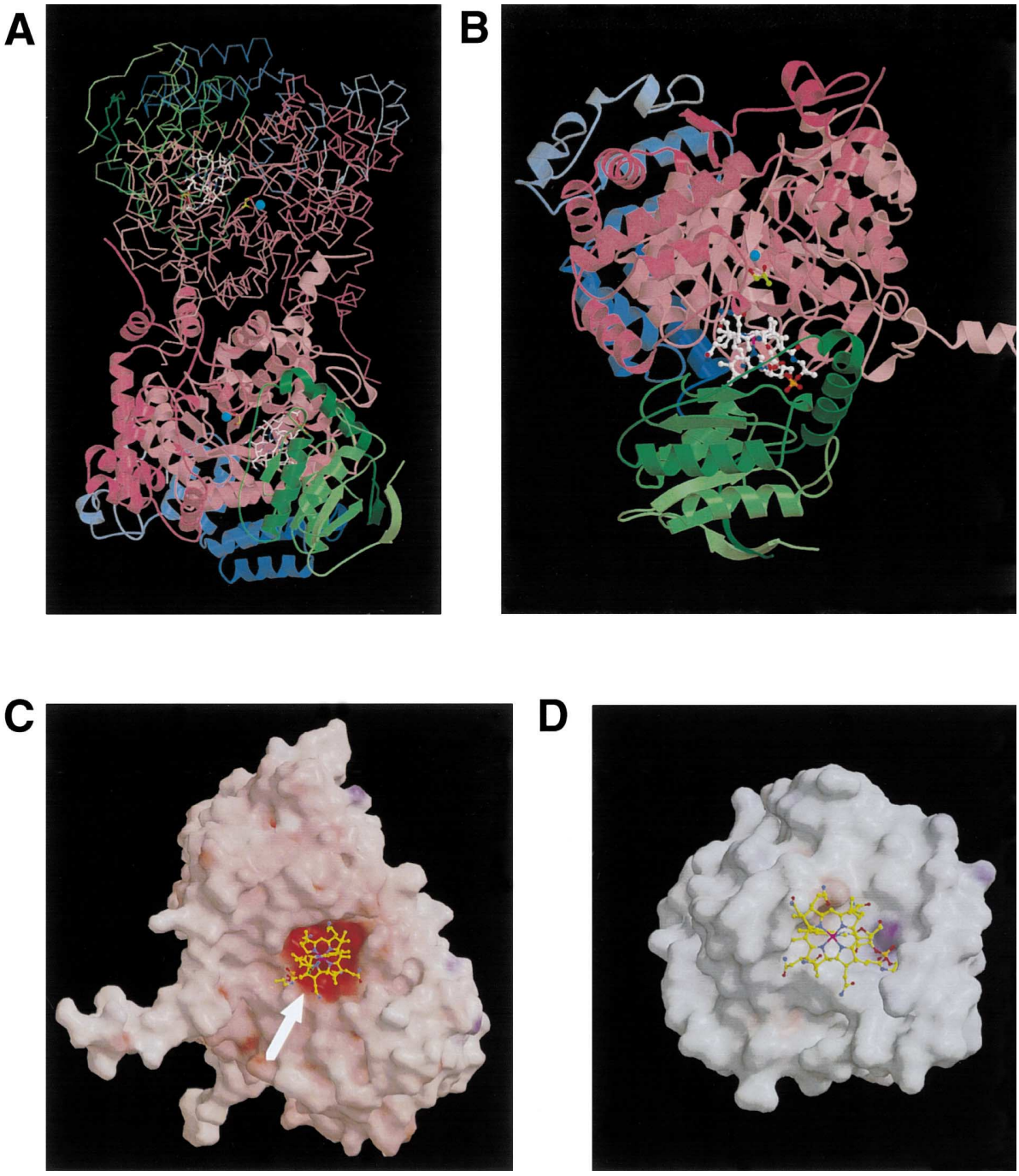


Figure 5. Structure of the diol dehydratase·CN-Cbl complex. (*A*) $(\alpha\beta\gamma)_2$: overall structure. (*B*) $\alpha\beta\gamma$ heterotrimer unit. Drawn with MOLSCRIPT [69] and RASTER3D [70] using the coordinates obtained by Yasuoka and co-workers [14]. Pink, green, and blue colors are used for the α, β, and γ subunits, respectively, darkening continuously from the N-terminal to the C-terminal sides. (*C*, *D*) α subunit viewed from the β subunit (*C*) and β subunit viewed from the α subunit (*D*). Surface representations with electrostatic potential distributions were drawn with GRASP [71] and RASTER3D [70] using the same coordinates. Red and blue areas show regions with negative and positive charges, respectively. Cobalamin and 1,2-propanediol are shown as ball-and-stick models whose carbon atoms and bonds are colored in yellow and green, respectively.

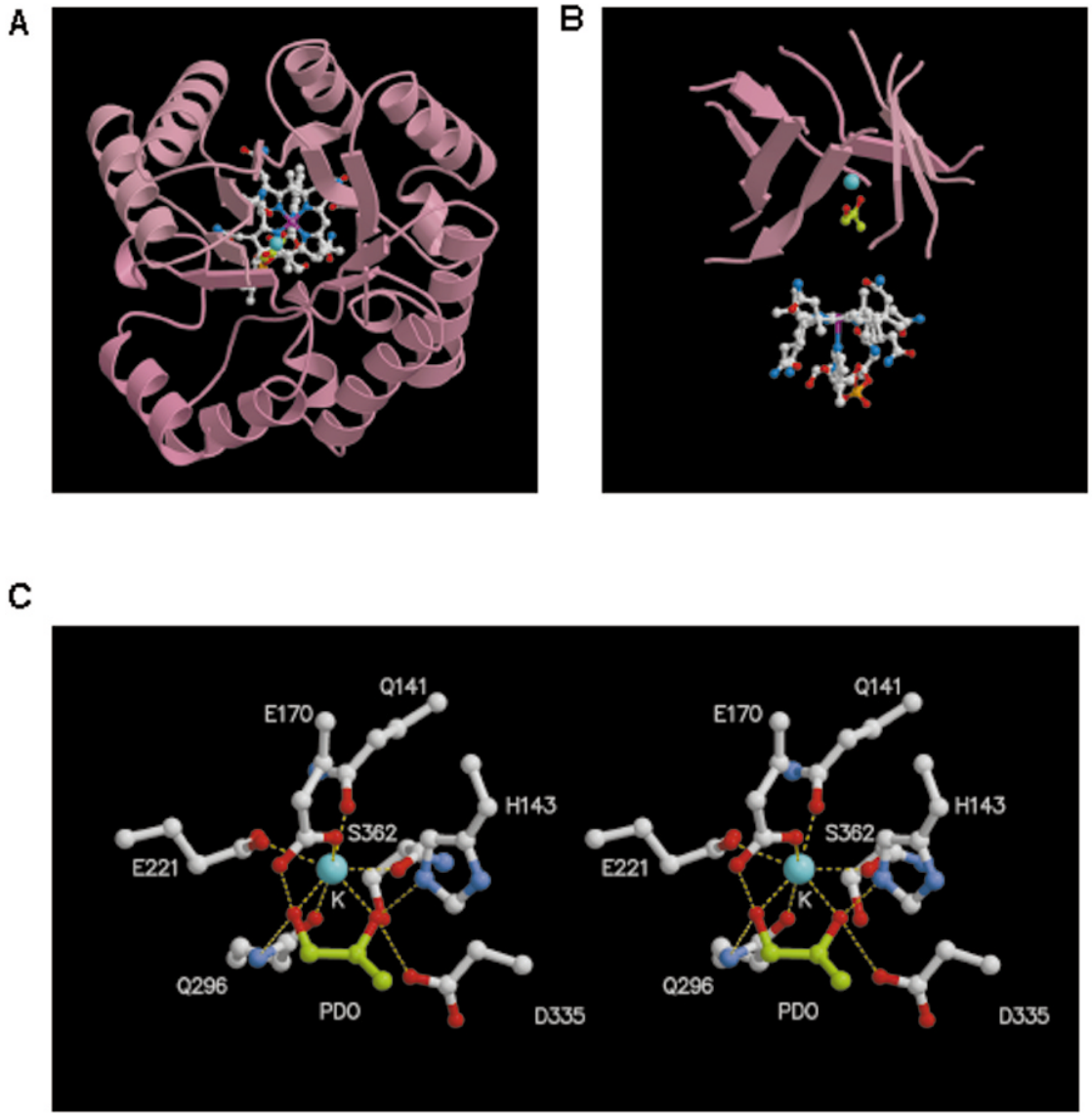


Figure 6. Structure of the active site of diol dehydratase. Drawn with MOLSCRIPT [69] and RASTER3D [70] using the coordinates obtained by Yasuoka and co-workers [14]. The eight β strands composing the barrel are colored in pink, darkening continuously from the N-terminal to the C-terminal sides. Carbon atoms and bonds in the substrate and K^+ are shown in green-yellow and cyan, respectively. (*A*, *B*) Views from the directions perpendicular (*A*) and parallel (*B*) to the plane of the corrin ring of cobalamin. (*C*) A stereoview of the active-site residues interacting with substrate and K^+.

Cobalamin binding

One of the striking features in the structure of diol dehydratase is that the 5,6-dimethylbenzimidazole (DBI) nucleotide moiety is coordinated to the cobalt atom in the corrin ring [14]. This was the first crystallographic indication of the so-called 'base-on' mode of cobalamin binding to proteins, although this mode of cobalamin binding was indicated by EPR studies with ^{15}N-labeled coenzyme or its analogue [77, 78]. In contrast, the X-ray structures of the methionine synthase cobalamin-binding domain [79], methylmalonyl-CoA mutase [74], and glutamate mutase [75], together with EPR studies on these enzymes [79–81], show that the DBI ligand is displaced from the cobalt atom and, instead, the imidazole group of a certain histidine residue in the cobalamin-binding motif part (DxHxxG) of proteins is ligated to the cobalt atom in these enzymes. This motif is conserved in all the enzymes cata-

lyzing carbon skeleton rearrangements [82–85], methionine synthase [86], and L-β-lysine (D-lysine) 5,6-aminomutase [87], but not in the enzymes catalyzing heteroatom elimination reactions [88–92]. Recently, the base-on mode of cobalamin binding was shown by EPR with ribonucleotide reductase of *Lactobacillus leichmannii*, another cobalamin enzyme catalyzing a heteroatom elimination [93]. It was thus concluded that there are at least two types of B_{12} proteins with different ancestors, as suggested by motif analysis.

For accommodating cobalamin, the side chains of a group of amino acid residues provide a space which is mainly surrounded by hydrophilic groups [14]. Five amide groups of the six peripheral side chains of the corrin ring form hydrogen bonds with amino acid residues in the α and β subunit. The ribose and phosphate groups of the nucleotide ligand are mainly surrounded by side chains of hydrophilic residues in the β subunit. Hydrogen bonds between phosphate oxygen and a few hydrophilic residues, a salt bridge between phosphate oxygen and the ε-NH_3^+ group of Lysβ135, and loose hydrophobic contacts between the DBI moiety and some hydrophobic residues are involved in the enzyme-coenzyme interactions. There are no significant interactions between the apoenzyme and the functional groups of the α-ribose moiety except for the hydrogen bonding between Proβ155 and 2′-OH.

The bond distance between Co and N-3 of DBI is 2.50 Å. Such an elongated Co-N bond was reported with methylmalonyl-CoA mutase [74] and recently with glutamate mutase [75] as well, in clear contrast to the normal Co-N bond length in free cobalamins [94–97] and in methionine synthase-bound methylcobalamin [98]. These results suggest that homolysis of the Co-C bond is favored rather than its heterolysis by lengthening the Co-N bond. The corrin ring is almost planar with a dihedral angle of the northern and southern least square planes of 3° [14]. This value is close to that reported for methylmalonyl-CoA mutase-bound cobalamin [74], in clear contrast to 17.9° for free CN-Cbl [94, 95]. Because no strong interactions between amino acid residues of diol dehydratase and the DBI moiety are found, it is likely that the hydrogen-bonding interactions between enzyme and coenzyme amide side chains make the corrin ring almost planar. It seems reasonable that the Co-N bond is lengthened by steric repulsion between the flattened corrin ring and the bulky DBI moiety [14].

Active-site cavity in a TIM barrel

The substrate 1,2-propanediol is bound inside the TIM barrel, near K^+ rather than the cobalt atom of cobalamin (fig. 6B) [14]. Active-site residues interacting with substrate and K^+ are shown in figure 6C. Besides coordination to K^+, the O(2) atom of 1,2-propanediol forms hydrogen bonds with Aspα335 and Hisα143, and the O(1) with Gluα170 and Glnα296. The methyl group and other carbon atoms of 1,2-propanediol have some hydrophobic contacts with nearby amino acid residues. The corrin ring of cobalamin is located in the 'bottom' of the barrel, which is defined here as the C-terminal side of the barrel (fig. 6A, B). Although the position of the adenosyl group of the coenzyme has not yet been determined, there is enough room to accommodate the adenosyl group above the corrin ring. Therefore, the bottom part of this barrel above the corrin ring must be the active site of this enzyme. A similar TIM barrel structure was reported with methylmalonyl-CoA mutase [74] and glutamate mutase [75]. Because highly reactive radical intermediates are involved in the reactions catalyzed by AdoCbl-dependent enzymes, these species must be protected by the enzymes from undesired side reactions, as stressed by Rétey in his concept of 'negative catalysis' [99], or escape out of the active site. Such protection can be achieved by their spatial isolation inside the barrel. Although these two enzymes and diol dehydratase bind cobalamin in quite different manners, all three bind substrates inside the barrel. Thus, this architecture may be considered as a common molecular apparatus for radical reactions catalyzed by AdoCbl-dependent enzymes.

In the surface representations of the α and β subunits shown in fig. 5C, D, the active site of the enzyme inside the TIM barrel is located in the cavity in the central region of the α subunit [14]. K^+ binds to the negatively charged inner part of the cavity, whereas cobalamin covers the cavity to isolate the active site from solvent molecules. The center of the barrel behind K^+ is filled with large side chains of amino acid residues. In contrast, Mancia et al. [74] reported that the substrate is bound to methylmalonyl-CoA mutase, penetrating the TIM barrel.

There are two cracks in the β_8 barrel between the third and fourth and between the seventh and the eighth β strands. The latter crack is much closer to the solvent [14]. A neutral, hydrophilic region around a loop between the eighth β strand and the α helix following it may provide a substrate-uptake path to the active site near the pyrrole ring D of cobalamin. The postulated entrance area of the path is shown with the arrow in the surface representation of the α subunit (fig. 5C). Unlike this enzyme and glutamate mutase [75], the TIM barrel of substrate-free methylmalonyl-CoA mutase splits apart to bind succinyl-CoA or methylmalonyl-CoA [100].

K^+ in the active site

Essential K^+ exists in the TIM barrel and is coordinated by five oxygen atoms originated from Glnα141,

Gluα170, Gluα221, Glnα296, and Serα362 (fig. 6C) [14]. The sixth and seventh coordination positions are occupied by O(1) and O(2) of substrate 1,2-propanediol. The K-Co distance is 11.7 Å. Monovalent cations such as K^+ were reported to be essential cofactors for diol dehydratase [9, 76]. It is evident that K^+ bound in the active site in the presence of substrate is no longer exchangeable with NH_4^+ under the crystallization conditions [101], although NH_4^+ shows higher affinity for diol dehydratase than K^+ [76]. This suggests that the K^+-binding site in the complex is not accessible to the solvent. K^+ is required for tight binding of cobalamins to apoenzyme [76, 102], suggesting that K^+ bound in the barrel participates in the binding of cobalamin to apoenzyme by affecting the barrel structure in the α subunit. The TIM barrel may undergo deformation in the absence of K^+, which results in weakening or destruction of the enzyme-coenzyme interactions.

K^+ is bound to the enzyme as a hepta-coordinated complex in the presence of substrate. Although the X-ray structure of substrate-free diol dehydratase has not yet been determined, it seems reasonable to assume that a water molecule is ligated to K^+, forming an octahedral, hexa-coordinated complex in the absence of substrate. It is likely that the amide oxygen (main chain) of Serα362 changes its position upon complexation with the substrate. This would cause a conformational change in the enzyme which is important for switching the substrate-free form to the substrate-bound form.

Detailed mechanism of action of diol dehydratase

Possible pathways of the hydroxyl group migration

In the above-mentioned descriptions of the minimal mechanism for diol dehydratase, the pathway of the hydroxyl group migration remained almost obscure, although several proposals were made on the bases of non-enzymatic model reactions and theoretical calculations [103–106]. The long distance between Co and substrate revealed by the X-ray analysis [14] excludes a possibility that Co(II) of Cbl^{II} is directly involved in the rearrangement of a substrate-derived radical to a product-derived one by transalkylation through organocobalamin intermediates [107]. Co(II) is considered as a 'spectator' in the 1,2-shift of the hydroxyl group in the diol dehydratase reaction. Instead, two pathways seem to be consistent with the X-ray structure of diol dehydratase.

In one pathway, the hydroxyl group migration from C-2 to C-1 is postulated to take place via a cyclic transition state. Based on ab initio calculations as well as model reactions, Golding and co-workers proposed that protonation of the migrating hydroxyl group facilitates the rearrangement [103, 104]. They pointed out that protonation or even partial protonation with NH_4^+ reduces the barrier height for the transition state in the hydroxyl group migration [105].

An alternative pathway with a radical anion intermediate would also be consistent with the X-ray structure. The pK_a values of the hydroxyl groups attached to a carbon radical were reported to be about 5 pH units lower than those of the corresponding alcohols [108]. Formation of such a radical anion intermediate may be favored by K^+ through electrostatic stabilization. This could ensure deprotonation of the hydroxyl group on C-1 by COO^- of Gluα170 for *S*-isomer. The abstraction of the hydroxyl group from C-2 may be facilitated by the resulting oxyanion on C-1 through σ-π overlapping as well as by its coordination to K^+. The carbon atom of C=O which is polarized by interaction with general acid COOH of Gluα170 could undergo suprafacial nucleophilic attack by the abstracted hydroxide ion. This alternative pathway, essentially similar to the hypothetical one proposed by Buckel and co-workers [106], however, may be incompatible with the constant activity of diol dehydratase at pH 6.0–10.0 [9].

Direct participation of K^+ in the enzyme catalysis

Density-functional theory (DFT) computations were carried out to determine whether K^+ can assist the 1,2-shift of the hydroxyl group in the substrate-derived radical [109]. Two pathways of hydroxyl group migration were considered. The first is a stepwise abstraction/readdition reaction that can proceed via a direct hydroxide abstraction by K^+, and the second is a concerted hydroxyl group migration assisted by K^+. From computations, only a transition state for the latter concerted mechanism was found. The migration of the hydroxyl group requires 86.2 kJ/mol in the absence of K^+. As shown in the potential energy diagram (fig. 7), the barrier height for the transition state from the complexed radical decreases only by 9.7 kJ/mol upon coordination of the migrating hydroxyl group to K^+, which corresponds to a 42-fold rate acceleration at 37 °C. The hydroxyl group migration still requires a high activation energy, but this might not be unreasonable, because the deuterium isotope effect ($k_H/k_D = 10$) indicates that the hydroxyl group migration is not rate determining [27]. The net binding energy upon replacement of the K^+-bound H_2O for substrate is 44.7 kJ/mol. Such a large binding energy would be at least partly utilized to facilitate the Co-C bond homolysis through the substrate-induced conformational change of the enzyme [109].

Proposed mechanism

Figure 8 illustrates a new overall mechanism for diol dehydratase which we propose based on the computational results [109] as well as the three-dimensional structure of the enzyme [14]. Although the X-ray structure of the substrate-free enzyme (1) has not yet been determined, it is likely that the binding of 1,2-propanediol to the active site converts a hexa-coordinated complex of K^+ into the hepta-coordinated one (2) through the coordination of its two hydroxyl groups to K^+, displacing sixth ligand H_2O [14]. This could change the position of the Serα362 ligand of the eighth β strand of the $(\beta/\alpha)_8$ barrel and thus induce a conformational change in the protein which triggers homolytic cleavage of the Co-C bond of AdoCbl, forming an adenosyl radical and Cbl^{II}.

The specific hydrogen atom of the substrate (*proS* and *proR* hydrogen atoms of *S*- and *R*-enantiomers, respectively) [27] is abstracted by the adenosyl radical, producing a substrate-derived radical and 5′-deoxyadenosine (3).

Computational results indicate that the 1,2-shift of the hydroxyl group in the substrate radical takes place through the cyclic transition state [103–105, 109]. In this mechanism, the activation energy for the transition state from the complexed substrate radical decreases only partially upon coordination of the migrating hydroxyl group to K^+ and still remains high [109]. Smith et al. [105] indicated by ab initio calculations that the barrier height for the transition state in the hydroxyl group migration can be lowered by partial protonation with NH_4^+. The X-ray structure of the active site revealed that the hydroxyl group on C-2 is hydrogen bonded to Hisα143 and Aspα335 (fig. 6C). However, a possibility that, in the diol dehydratase reaction, the interaction of the migrating hydroxyl group with the imidazolium ion of Hisα143 is important for further stabilization of the transition state might be less likely, because the rate of the diol dehydratase reaction is constant in the pH range from 6.0 to 10.0 [9]. The hydroxyl group seems to migrate from C-2 to C-1 with hydrogen bonding to these residues and with the C-2-C-

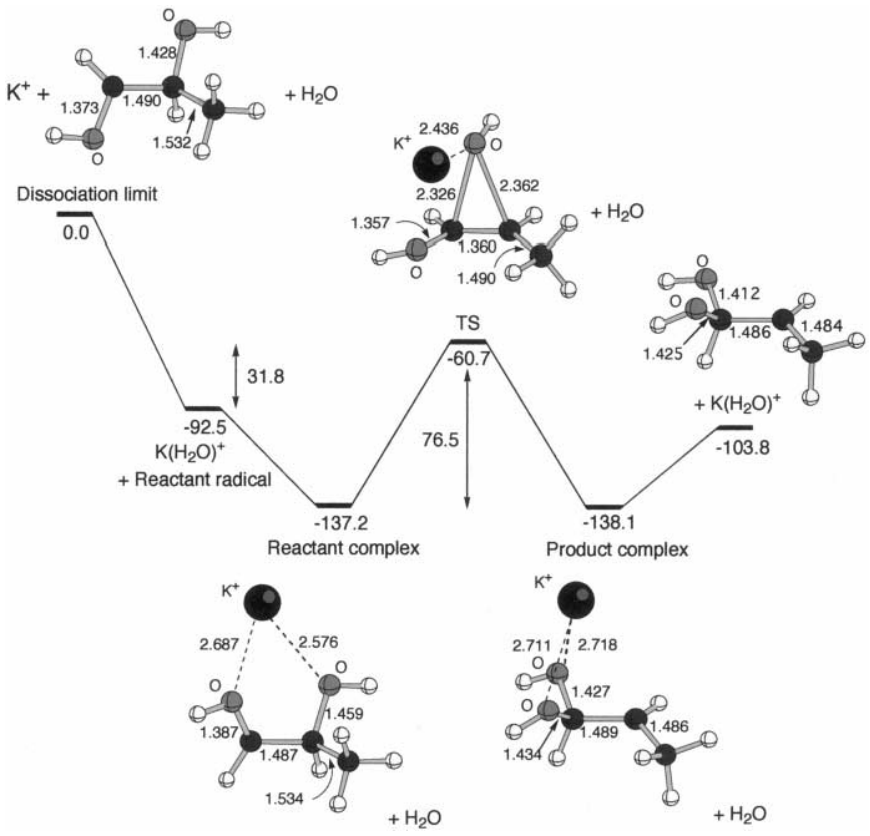


Figure 7. A potential energy diagram for the migration of the hydroxyl group from C-2 to C-1 in the 1,2-dihydroxypropyl radical in the presence of K^+ [109]. Bond length in Å and energies in kJ/mol.

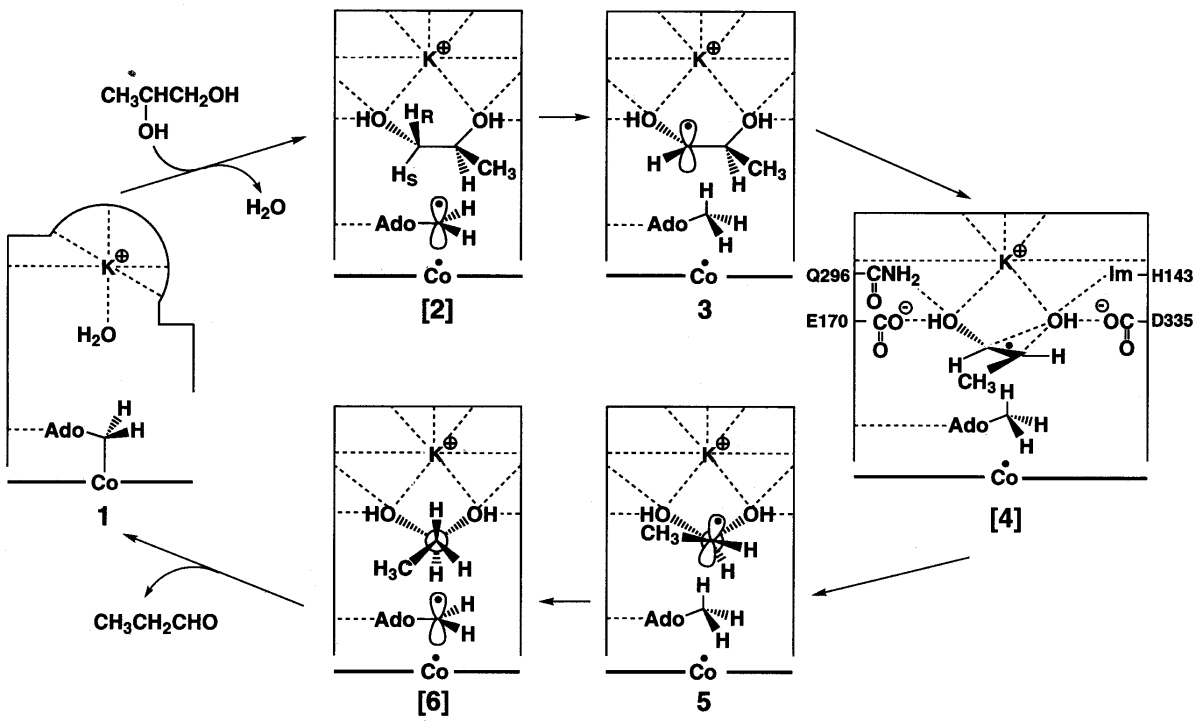


Figure 8. The proposed mechanism for diol dehydratase. The reaction with the (*S*)-1,2-propanediol is illustrated. -Co-, cobalamin; $AdoCH_2$, adenosyl group; Im, imidazole group of Hisα143. The complexes with a number in parentheses show transient species.

1 bond turning around through the transition state (4) illustrated in figure 8. It seems reasonable to assume that the enzyme-bound substrate changes its orientation during the reaction, because the two hydroxyl groups are much more strongly bound to the active site through ion-dipolar interactions as well as hydrogen bonding than the C-C-C backbone of the substrate is bound through hydrophobic or van der Waals interactions.

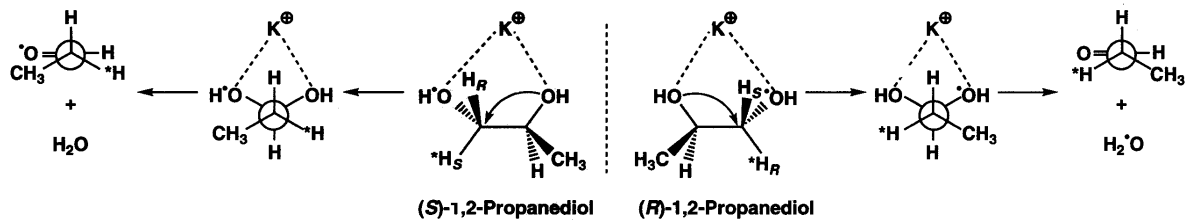
The *gem*-diol radical (5) formed abstracts a hydrogen atom back from 5′-deoxyadenosine, producing *gem*-diol and the adenosyl radical (6). As a result, the configuration of C-2 would be inverted in the reaction. The *gem*-diol is then dehydrated, producing propionaldehyde and H_2O. Removal of the migrated hydroxyl group would also be facilitated by its coordination to K^+. The product aldehyde loses its affinity for K^+ because of the loss of the hydroxyl groups and is thus released from the active site. A decrease in the coordination number of K^+ would bring about the conformational change back to the substrate-free form (1), which accompanies recombination of the adenosyl radical with Co(II) of Cbl^{II} to regenerate the coenzyme. A bond energy released upon reformation of the Co-C bond would ensure the release of the product from the active site and the conformational change of the enzyme to the substrate-free form.

The mechanism proposed here can explain all the biochemical results reported so far [5, 12]. It was shown that the monovalent cation is not a spectator but one of the 'conductors' in this enzyme catalysis.

Stereochemical course of the reaction

Figure 8 illustrates the reaction with (*S*)-1,2-propanediol. When each enantiomer of 1,2-propanediol is run independently, the rate with the *R*-isomer is 1.7–1.8 times higher than that with the *S*-isomer [27, 110, 111]. But, when racemic 1,2-propanediol is used as substrate, the *S*-isomer reacts at a faster rate than the *R*-isomer [112]. Jensen and Neese [112] indicated that this reversal is due to the ratio of K_m values ($K_{m(R)}/K_{m(S)} = 3.1–3.2$). This suggests that the modes of binding of the *R*- and *S*-isomers are different. From the fact that, among the diastereomers of 2,3-butanediol, only the *meso*-isomer serves as a substrate [113], it would be reasonable to assume that (*R*)- and (*S*)-1,2-propanediol are bound to the active site in the opposite orientation as the mirror image (scheme 2). Since the distances of C-1 and C-2 to the cobalt atom are almost equal [14], hydrogen ab-

Scheme 2



straction would occur with both enantiomers. In the cases of *S*- and *R*-isomers, the *proS* and *proR* hydrogens would be abstracted by the adenosyl radical, respectively. Gluα170 and Aspα335 might at least partially share a common catalytic function. If Hisα143, Aspα335, and K^+ are assumed to constitute the only site for dehydration, the oxygen atom derived from C-1 in the *S*- and *R*-isomers would be retained in the product and lost into the solvent, respectively. Hence, the pseudo symmetry of the substrate binding site of diol dehydratase accounts for the unusual stereospecificity [27, 110–112], stereochemical course [25, 27, 28], and substrate specificity [10, 113].

Formation of a catalytic radical by homolysis of the Co-C bond of the coenzyme

Coenzyme analogues modified in the adenosyl group or the peripheral side chains of the corrin ring

Analogues of AdoCbl in which the adenine or β-D-ribose moiety of the adenosyl group or the peripheral side chains of the corrin ring are modified have been synthesized and examined for coenzymic activity with diol dehydratase [for reviews see refs 7, 11, 12 and references therein]. The importance of the nitrogen atoms of the adenine moiety for manifestation of catalytic function and for activation (labilization) of the Co-C bond decreases in the order N-7 > 6-NH_2 > N-3 > N-1 [42, 114]. The β-D-ribofuranose ring itself, possibly its rigid structure, is essential for activity [115]. The importance of the functional groups of the ribose moiety decreases in the order β-D-ribofuranose ring $\gg$ 3′-OH > 2′-OH > -O- [115]. Formation of Cbl^{II} during catalysis was spectroscopically observed with active analogues, but not with inactive ones.

The binding site for the adenosyl group of AdoCbl is present in diol dehydratase which is spatially limited and highly specific for adenine nucleosides [116]. $\Delta G°$ upon binding of the adenosyl group and the adenine moiety of the coenzyme to diol dehydratase was estimated to be approximately -27.6 and -25 kJ/mol at 37 °C from the K_D values for 5′-deoxyadenosine and adenine, respectively [114].

Isomers of AdoCbl analogues in which one of the three amide groups of the propionamide side chain of the corrin ring is converted to -COO^-, -$COOCH_3$, or -$CONHCH_3$ vary considerably in coenzymic activity for diol dehydratase [43], suggesting the importance of hydrogen bond donation from coenzyme to apoenzyme. The X-ray structure determined recently shows that the peripheral amide side chains form hydrogen bonds with hydrophilic side chains of nearby amino acid residues [14]. It was concluded that steric distortion of the corrin ring induced by the specific tight interactions of the coenzyme amide side chains with apoenzyme are essential for catalysis by facilitating homolytic cleavage of the Co-C bond and by stabilizing radical intermediates.

Cleavage of the Co-C bond of sterically hindered alkylcobalamins bearing neither adenine nor functional groups is accelerated at least ten times upon binding to apoenzyme [117]. Such labilization of the Co-C bond is considered to be caused by steric distortion of the corrin ring.

Coenzyme analogues modified in the nucleotide moiety

Among analogues in which the α-D-ribose of the nucleotide moiety is substituted by an oligomethylene group, 5,6-dimethylbenzimidazolyl trimethylene and tetramethylene analogues are active coenzymes for diol dehydratase, indicating that neither the α-D-ribofuranose ring nor functional groups of the ribose are essential for catalysis [39]. The loop size corresponding to ribose is optimal for catalysis and for tight binding to apoenzyme. Therefore, the D-ribose moiety seems important as a spacer to keep base DBI in the proper position.

Benzimidazolyl, pyridyl and imidazolyl trimethylene analogues are active as coenzymes for diol dehydratase [11, 39, 118], but the analogues with less bulkier bases tend to undergo mechanism-based inactivation during catalysis (table 1). It was suggested that the nucleotide loop structure is indispensable for tight binding to this enzyme [119, 120].

Adenosylcobinamide methyl phosphate (AdoCbi-PMe) is a totally inactive coenzyme but behaved as a compe-

titive inhibitor [40]. Because adenosylcobinamide (AdoCbi) itself neither functions as a coenzyme nor binds tightly to apoenzyme [119], it is clear that the phosphodiester group is essential for tight binding to the apoenzyme. AdoCbi-PMe undergoes homolytic cleavage of the Co-C bond upon binding to apoenzyme in the presence of substrate, although it is inactive as coenzyme [40]. It can therefore be concluded that, in diol dehydratase, the presence of a base moiety is not absolutely required for activation of the Co-C bond by apoenzyme but is essential for catalysis.

Mechanism of activation of the coenzyme Co-C bond

How do the enzymes generate catalytic radicals in the active site? This is a generally important issue for the radical-catalyzed reactions. A catalytic radical in the AdoCbl-dependent rearrangements is the adenosyl radical which is formed in the active site by homolytic cleavage of the Co-C bond of the coenzyme. Since the Co-C bond of AdoCbl is stable in the dark but sufficiently weak (BDE = about 109 [121], about 126 [122] kJ/mol), only a modest labilization by interaction with apoprotein would be required for homolysis. The X-ray structure of diol dehydratase as well as the structure-function studies of AdoCbl revealed that the cobalamin moiety of AdoCbl is tightly bound to apoenzyme by specific interactions through peripheral amide side chains of the corrin ring [43] and the phosphate group of the nucleotide moiety [40]. As a result of enzyme-coenzyme interaction, the corrin ring becomes almost planar and the Co-N bond is unusually lengthened by steric repulsion between the flattened corrin ring and DBI [14]. Enzyme-induced distortion of the corrin ring has been proposed to sterically labilize the Co-C bond [43]. The axial base coordination to the cobalt atom is not absolutely required for labilization of the Co-C bond [40], suggesting that a mechanochemical activation is less likely. Although the extent of acceleration of the Co-C bond cleavage by tight interactions at the cobalamin moiety is rather small without the adenosyl group [117], this could lead to labilization of the Co-C bond of AdoCbl to homolysis rather than to heterolysis. It has been pointed out from the non-enzymic model studies that a long Co-N bond would preferentially destabilize Co^{III} relative to Co^{II} [123] or inhibit Co-C heterolysis as well as accelerate Co-C homolysis [124]. In addition to the interactions at the cobalamin moiety, the interactions with apoenzyme at the adenine moiety of the adenosyl group are also essential for cleavage of the Co-C bond of the coenzyme [114]. There is biochemical evidence for the presence of the adenosyl-group-binding site in diol dehydratase [116], although its position has not yet been crystallographically determined. The strong attraction of the coenzyme adenosyl group to its binding site in the apoenzyme could produce a kind of angular strain or tensile force which leads to labilization of the Co-C bond [125].

In the absence of substrate, only a small fraction of the coenzyme is in the dissociated form [42, 45, 46]. When the substrate is added to the system, most of the coenzyme undergoes homolysis of its Co-C bond at a kinetically competent rate [48]. This indicates that a substrate-induced conformational change of the enzyme brings about further strain which triggers homolytic cleavage of the Co-C bond. Resonance raman spectra showed that AdoCbl binding to methylmalonyl-CoA mutase in the absence of substrate changes the corrin ring conformation but leaves the Co-C bond unaffected [126]. Acceleration of Co-C bond homolysis upon binding to apoenzyme in the presence of substrate was explained by Pratt [127] in terms of steric crowding at the upperface of AdoCbl. From the comparison of X-ray structures of substrate-free and substrate-bound methylmalonyl-CoA mutase, Evans and co-workers [74, 100] indicated that substrate binding induces a conformational change which was suggested to destroy the binding site for the adenosyl group, displacing it from the cobalt atom and forming the radical species. The rate acceleration of the Co-C bond cleavage with diol

Table 1. Correlation between k_{cat}/k_{inact} and bulkiness or basicity of the base.

Analogue	Relative activity (%)	K_m or K_I (μM)	$k_{cat}/k_{inact} \times 10^{-4}$	Base moiety			
				structure	bulkiness	basicity (pK_a)	reference
AdoCbl	(100)	0.80	144	DBI	very large	6.09	43
AdoCbiP-DBIPr	59	0.82	70	DBI	very large	6.09	39
AdoCbiP-BIPr	40	2.7	9.3	benzimidazole	large	5.48	11
AdoCbiP-PyPr	25[a]	0.29	1.4	pyridine	medium	5.19	118
AdoCbiP-ImPr	8[a]	0.99	0.33	imidazole	small	6.95	39
AdoCbiP-Me	0	2.5	~0	none	no	–	40

[a] Calculated from the initial velocity.

dehydratase in the presence and absence of substrate was calculated to be 10^{11}- to 10^{12}-fold [48, 128] and 10^6- to 10^7-fold (M. Yamanishi and T. Toraya, unpublished data), respectively. It is therefore evident that the Co-C bond homolysis of the coenzyme is facilitated in two steps—rate acceleration upon binding of the coenzyme to apoenzyme by a factor of 10^6–10^7 and further rate enhancement upon binding of substrate to the holoenzyme by a factor of 10^4–10^6. These accelerations corresponding to $\Delta\Delta G^{\ddagger}$ of 33–42 and 25–38 kJ/mol, respectively, at 37 °C would be achieved by utilizing the binding energies released upon binding of the coenzyme and the substrate, respectively, to the enzyme. These values of $\Delta\Delta G^{\ddagger}$ seem reasonable, because there are twelve hydrogen bonds and a salt bridge between the apoenzyme and cobalamin and four hydrogen bonds and two ion-dipolar interactions between the enzyme (K^+) and 1,2-propanediol [14]. In contrast to diol dehydratase, methylmalonyl-CoA mutase [74] and glutamate mutase [75] do not possess a monovalent cation in the active sites, although the substrate-binding energy is considered to be utilized to facilitate the homolysis of the coenzyme Co-C bond in these enzymes as well. A possible reason for this difference may be that substrates of diol dehydratase are too small to obtain such a large binding energy needed for facilitation of the Co-C bond homolysis. Thus, the coordination of substrates to K^+ in the active site is important for increasing the substrate binding energy in diol dehydratase.

Control of radical intermediates and mechanism-based inactivation

Inactivation by glycerol, other substrates, and O_2

Although the initial velocity with glycerol as substrate is the highest among substrates for both diol and glycerol dehydratases, the enzymes undergo concomitant irreversible inactivation by glycerol during catalysis [10, 111, 129, 130]. This mechanism-based inactivation involves irreversible cleavage of the Co-C bond, forming 5′-deoxyadenosine and an unknown cobalamin species showing an alkylcobalamin-like spectrum. The unknown cobalamin species is converted very slowly to OH-Cbl. The cobalamin species that is formed remains tightly bound to the enzyme, inactivating the enzyme irreversibly. Such inactivation forming 5′-deoxyadenosine and Cbl^{II} or OH-Cbl is caused by 1,2-ethanediol and other substrates as well [3, 77, 111, 131]. It can therefore be concluded that these enzymes tend to undergo mechanism-based inactivation, probably because they catalyze reactions by a radical mechanism. With diol dehydratase, a substrate which generates a smaller magnitude of exchange interaction between low-spin Co(II) and an organic radical intermediate in EPR spectra is a more efficient mechanism-based inactivator [131]. This result suggests that a radical-intermediate-stabilizing effect of the holoenzyme during catalysis decreases with distance between the radical pair.

The holoenzyme of diol dehydratase is also inactivated by glycolaldehyde [35] or chloroacetaldehyde [36], forming Cbl^{II} and 5′-deoxyadenosine. Holoenzymes of both diol and glycerol dehydratases undergo inactivation by O_2 in the absence of substrate [9, 35, 130, 132]. The formation of OH-Cbl upon inactivation suggests that inactivation is caused by reaction of the activated Co-C bond of the coenzyme with O_2 [35].

Inactivation by coenzyme analogues

Time courses of the diol dehydratase reaction with certain analogues of AdoCbl as coenzymes are not linear due to concomitant inactivation during catalysis [38, 39, 42, 115, 118]. This tendency is most marked with the imidazolyl and pyridyl trimethylene analogues with which the reaction ceases almost completely within about 2 and 8 min, respectively [39, 118]. Upon incubation of the imidazolyl or pyridyl analogue with apoenzyme in the presence of substrate, a Cbl^{II}-like species irreversibly accumulates as inactivation progresses, while EPR signals due to an organic radical quickly disappear [39, 118, 133]. When the adenosyl form of [$^{14}N_2$]- and [$^{15}N_2$]imidazolyl analogues of the coenzyme are used with unlabeled apoenzyme, the octet in the EPR spectra of the inactivated enzyme shows superhyperfine splitting into triplets and doublets, respectively [77, 133]. It was therefore concluded that cobalamin is bound to this enzyme with DBI coordinating to the cobalt atom. The same conclusion was reached by Rétey and coworkers with AdoCbl containing [$^{15}N_1$]DBI [78].

AdoCbi-PMe, an inactive coenzyme, brings about irreversible inactivation of the enzyme in the presence of substrate [40]. It is converted to cob(II)inamide methyl phosphate upon inactivation, whereas no signals due to an organic radical are observed [40, 133]. These results demonstrate that the extinction of organic radical intermediates results in inactivation of the enzyme by these coenzyme analogues.

The mechanism of inactivation by coenzyme analogues

Upon inactivation with the imidazolyl analogue and AdoCbi-PMe, the adenosyl group of the analogues is converted to 5′-deoxyadenosine [39, 40]. The apoenzyme resolved from the inactivated complexes by acid ammonium sulfate fractionation is reconstitutable into catalytically active holoenzyme with AdoCbl, indicating that the apoenzyme itself is not inactivated during the process. The inactivation by these analogues takes place

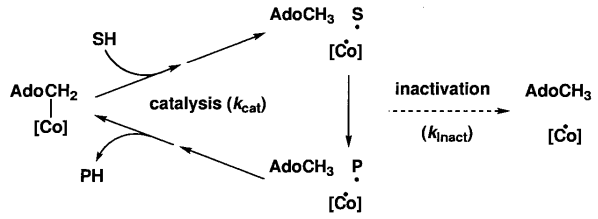


Figure 9. Mechanism-based inactivation of diol dehydratase by coenzyme analogues [39, 40, 135]. RCH_3, 5′-deoxyadenosine; [Co], cobalamin; SH, substrate; PH, product.

only in the presence of substrate. From these results, the inactivation by the coenzyme analogues seems mechanistically similar and can be considered as a sort of mechanism-based inactivation (fig. 9) [39, 40, 133]. That is, a certain radical intermediate common to the normal catalytic cycle is quenched by undesired side reactions or escape out of the active site, which makes it impossible to regenerate the coenzyme. This leads to inactivation, because the modified coenzyme remains tightly bound to the apoenzyme and is not exchangeable with free intact AdoCbl.

As judged from k_{cat}/k_{inact} values (table 1), the imidazolyl and pyridyl analogues cause inactivation at much higher probability than the 5,6-dimethylbenzimidazolyl analogue or AdoCbl itself. Therefore, they are considered 'suicide coenzymes' [39, 118]. In the case of inactivation with AdoCbi-PMe, the k_{cat}/k_{inact} value is almost zero [40]. Because this is an extreme case of suicide inactivation, we propose calling such an analogue a 'pseudocoenzyme'.

Role of DBI in B_{12} catalysis

How do the enzymes control highly reactive radical intermediates? This is a generally important issue for continuous progress of the radical-catalyzed reactions. In diol dehydratase [14] as well as methylmalonyl-CoA mutase [74] and glutamate mutase [75], radical species could be protected from solvent molecules by spatial isolation inside the TIM barrel. In addition, another mechanism controlling the reactivity of radical intermediates seems to exist.

With coenzyme analogues, in diol dehydratase, coordination of a nitrogenous base of the nucleotide moiety plays a pivotal role in stabilizing the highly reactive radical intermediates and is therefore obligatory for catalytic turnovers [39, 40, 118]. In this sense, Cbl^{II} is not simply a spectator but a 'stabilizer' for organic radical intermediates. What property of the base is related to its radical-stabilizing effect? The apparent radical-stabilizing effect of the base correlates well with its bulkiness rather than its basicity (table 1) [11]. Therefore, the steric effect of the base seems important for stabilizing radical intermediates during catalysis.

Diol dehydratase binds cobalamin in the base-on mode. It is thus likely that a bulky base exerts its effect by a so-called 'trans' effect. The X-ray structure revealed that the corrin ring of cobalamin is almost flattened upon binding to diol dehydratase and the Co-N bond is lengthened. These are also the cases in methylmalonyl-CoA mutase [74]. The Co-N bond is lengthened in glutamate mutase [75] as well, but not in methionine synthase [98], although an imidazole group of a certain histidine residue is ligated to the cobalt atom in these enzymes. It is therefore likely that the homolysis of the Co-C bond is much more favored than its heterolysis by lengthening the Co-N bond, which enables the continuous progress of radical reactions. It was suggested strongly that the reactivity of the cobalt atom towards a product radical via the adenosyl radical for reformation of the Co-C bond is controlled by the Co-N bond distance. A longer Co-N bond would be expected with a bulkier base like DBI, because of the steric repulsion between the flattened corrin ring and the base moiety [14]. Thus, the role of the DBI moiety in enzyme catalysis is most likely to prevent a B_{12} enzyme from mechanism-based inactivation [11]. This must be at least one of the reasons why most of the naturally occurring corrinoids contain a unique, bulky base DBI.

Regeneration of a catalytic radical by a reactivating factor

In situ reactivation

Because radical enzymes tend to undergo inactivation by extinction of radical intermediates [62], another important issue for radical-catalyzed reactions is whether and how the inactivated enzymes are reactivated. Diol and glycerol dehydratases both undergo mechanism-based inactivation by glycerol during catalysis [10, 111, 129, 130], although their intrinsic physiological roles are different [7, 12]. The physiological role of glycerol dehydratase is to produce an electron acceptor, β-hydroxypropionaldehyde, in the fermentation of glycerol [134–137]. In some bacteria, glycerol dehydratase is substituted by isofunctional diol dehydratase for its essential role in the glycerol fermentation, because the diol enzyme is induced to a low level by glycerol [135, 136, 138]. Mechanism-based inactivation of glycerol and diol dehydratases by glycerol is therefore enigmatic, since glycerol is a growth substrate for the bacteria which produce these enzymes. This apparent inconsistency was solved by our finding that the glycerol-inactivated as well as O_2-inactivated holoenzymes of these

enzymes in permeabilized cells (in situ) of *Klebsiella pneumoniae* and *K. oxytoca* undergo rapid reactivation in the presence of AdoCbl, ATP, and Mg^{2+} [139, 140]. The glycerol-inactivated holoenzyme of diol dehydratase in permeabilized cells (in situ) of *Escherichia coli* harboring a plasmid containing the diol dehydratase genes (*pddABC*) and their flanking regions is also rapidly reactivated in the presence of free AdoCbl, ATP, and Mg^{2+} [141]. The in situ reactivation was also observed when the enzyme was inactivated by an imidazolyl coenzyme analogue during dehydration of 1,2-propanediol [141]. The experiments with recombinant *E. coli* carrying deletion mutant plasmids demonstrated that certain protein(s) encoded by the 3′-flanking region of the diol dehydratase genes are required for the in situ reactivation of the inactivated enzyme. Inactive complexes of the glycerol and diol enzymes with CN-Cbl, aquacobalamin, and pentylcobalamin are activated in situ in the presence of AdoCbl, ATP, and Mg^{2+}, but the complexes with adeninylbutylcobalamin and adeninylpentylcobalamin (AdePeCbl) are not [140, 141]. These results suggest that the inactivated holoenzymes are reactivated in situ in the presence of ATP and Mg^{2+} by exchange of the modified coenzyme for free intact AdoCbl.

Genes for a reactivating factor

Permeabilized *E. coli* cells coexpressing the diol dehydratase genes with the two open reading frames in the 3′-flanking region are able to reactivate glycerol-inactivated diol dehydratase as well as activate the enzyme · CN-Cbl complex in situ in the presence of free AdoCbl, ATP, and Mg^{2+} [142]. Thus, these open reading frames were identified as the genes encoding a putative reactivating factor for diol dehydratase and designated *ddrA* and *ddrB* genes after it. The genes encode polypeptides consisting of 610 and 125 amino acid residues with predicted molecular weights of 64,266 and 13,620, respectively. We proposed calling a complex of these proteins 'diol dehydratase-reactivating factor'. Sequence homologies indicate that the *ddrA* and *ddrB* genes of *K. oxytoca* corresponds to the *pduG* and *pduH* genes, respectively, in the *pdu* operon of *Salmonella typhimurium* [143] with unknown functions [142] (fig. 10A).

Recently, we identified two open reading frames of *K. pneumoniae* with unknown functions in the *dha* regulon as the genes for a reactivating factor for glycerol dehydratase and designated them *gdrA* and *gdrB* [144]. The reactivation of the inactivated glycerol dehydratase by the gene products was confirmed in permeabilized recombinant *E. coli* coexpressing GdrA and GdrB proteins with glycerol dehydratase. The DdrA and DdrB proteins are highly homologous to the products of *gdrA* (*dhaB4*) and *gdrB* (*orf2b*) of *K. pneumoniae* [145] and *orfZ* and *orfX* of *Citrobacter freundii* [90] and *Clostridium pasteurianum* [146] (fig. 10B), respectively. The DdrB protein shows substantial homology to the β subunits of diol dehydratase [88] and glycerol dehydratase [89] as well.

In vitro reactivation by the reactivating factor

Upon mechanism-based inactivation of diol dehydratase by glycerol, the coenzyme is modified through irreversible cleavage of its Co-C bond, resulting in inactivation of the enzyme by tight binding of the modified coenzyme to the active site. Recombinant DdrA and DdrB proteins of *K. oxytoca* copurified to homogeneity from overexpressing *E. coli* exist as a tight complex with an apparent molecular weight of 150,000, indicating that the subunit structure of the reactivating factor is most likely A_2B_2 [15]. The factor not only reactivates glycerol-inactivated and O_2-inactivated holoenzymes but also activates the enzyme·CN-Cbl complex in vitro in the presence of free AdoCbl, ATP, and Mg^{2+} [15]. The reactivating factor mediates ATP-dependent exchange of the enzyme-bound, adenine-lacking cobalamins, for free AdoCbl, an adenine-containing cobalamin through intermediary formation of apoenzyme (fig. 11) in the presence of ATP and Mg^{2+}. The reverse is not the case. The modified coenzyme released from the enzyme is back converted to AdoCbl by reductive adenosylation in the presence of reducing agent(s) and ATP. This conversion is catalyzed by cobalamin reductases and Cbl^{I}:ATP adenosyltransferase.

Molecular chaperone-like action of the reactivating factor

The mechanism of reactivation of diol dehydratase by its reactivating factor was investigated in vitro using the enzyme·CN-Cbl complex as a model for inactivated holoenzyme [16]. The factor shows extremely low but distinct ATP-hydrolyzing activity. It forms a tight complex with apoenzyme in the presence of ADP, but not at all in the presence of ATP, indicating that ADP and ATP forms of reactivating factor are high- and low-affinity forms for diol dehydratase, respectively. Incubation of the enzyme·CN-Cbl complex with the reactivating factor in the presence of ADP brings about release of the enzyme-bound cobalamin, leaving the tight apoenzyme·reactivating factor complex (fig. 12A). Although the resulting complex is inactive even in the presence of added AdoCbl, it dissociates by incubation with ATP, forming the apoenzyme which is reconstitutable into active holoenzyme with added coenzyme. It was thus established that the reactivation of the inactivated holoenzyme by the factor in the presence of ATP and Mg^{2+} takes place in two steps: (i) ADP-dependent

cobalamin release, and (ii) ATP-dependent dissociation of the apoenzyme·reactivating factor complex. ATP plays dual roles as a precursor of ADP in the first step and as an effector to change the factor into the low-affinity form for diol dehydratase. The enzyme-bound AdoCbl is also susceptible to exchange with free AdoPeCbl, although to a much lesser degree. The mechanism for discrimination of adenine-containing cobalamins from adenine-lacking cobalamins can be explained by the difference in formation equilibrium

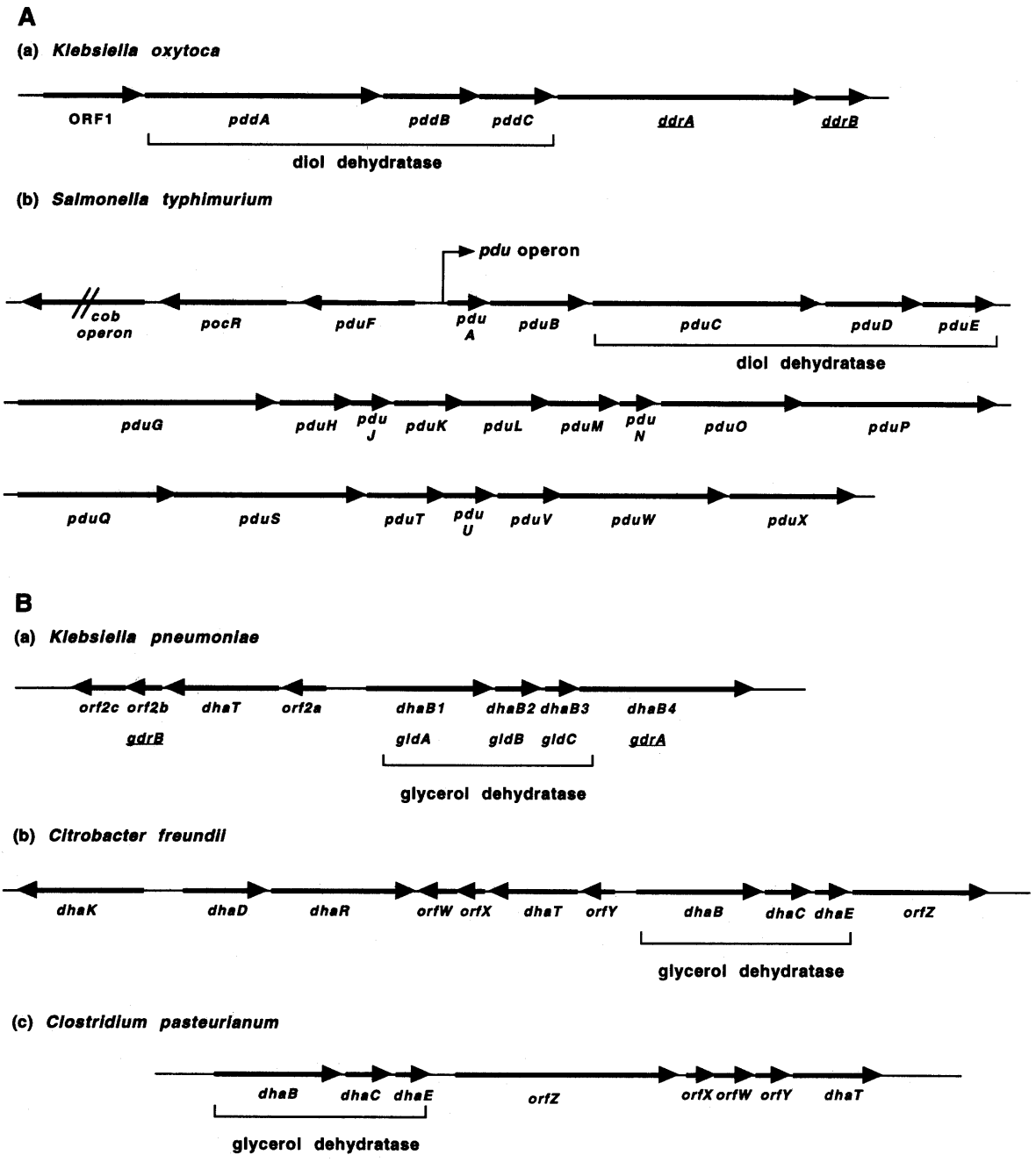


Figure 10. Gene organizations of *pdu*/*cob* regulons [88, 142, 143] (*A*) and *dha* regulons [89, 90, 145, 146] (*B*). The underlined genes encode reactivating factors for diol dehydratase (*A*) and glycerol dehydratase (*B*), respectively.

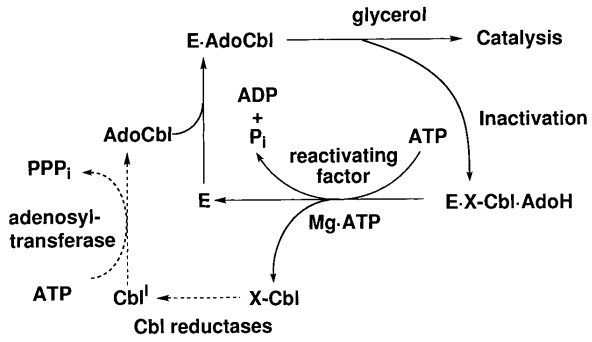


Figure 11. Reactivation of glycerol-inactivated holodiol dehydratase by the cobalamin exchange mechanism [15]. E, apodiol dehydratase; AdoH, 5′-deoxyadenosine; Cbl^I, cob(I)alamin; X-Cbl, unidentified cobalamin formed upon mechanism-based inactivation by glycerol. Dashed lines represent the conversion of free X-Cbl to AdoCbl.

constants (K_{eq}) of the cobalamin·enzyme·reactivating factor ternary complexes (fig. 12B). From the K_D value for adenine, the presence of adenine in the upper axial ligand is expected to decrease K_{eq} by a factor of 1.7×10^4. We concluded that the reactivating factor is a new type of molecular chaperone that participates in reactivation of the inactivated enzymes [16].

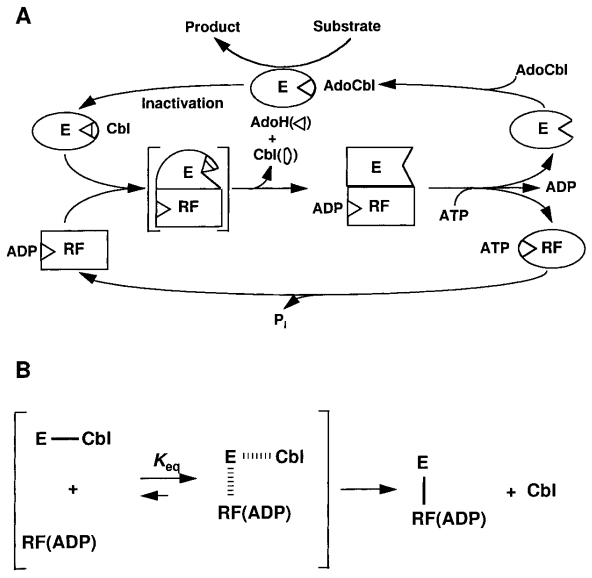


Figure 12. A proposed mechanism for reactivation of inactivated holodiol dehydratase by the reactivating factor (*A*) and a possible mechanism of discrimination of the enzyme-bound adenine-lacking cobalamins from adenine-containing cobalamins (*B*) [16]. E, apodiol dehydratase; RF, reactivating factor; AdoH, 5′-deoxyadenosine; Cbl, cobalamin.

Concluding remarks

Enzymatic radical catalysis is one of the most important mechanisms for a group of enzymes which catalyze by a non-polar mechanism. Our understanding of this concept is rapidly growing but still not deep enough. It was less than 30 years ago that AdoCbl-dependent enzymes were first demonstrated to catalyze by a radical mechanism. Recent structural analyses of diol dehydratase and another two AdoCbl-dependent enzymes revealed a common molecular architecture which ensures radical catalysis of these B_{12} enzymes. The direct involvement of K^+ in the catalysis of diol dehydratase would add a new page to the biochemistry of monovalent cations. The presumption and confirmation of the functional roles of the active-site residues by site-directed mutagenesis as well as theoretical calculations would lead us to a fine mechanism of action of diol dehydratase in the near future. Important clues have been obtained so far to understand how the enzyme protects reactive radical intermediates from undesired side reactions. However, further understanding of the mechanism of activation of the coenzyme Co-C bond must await solution of the X-ray structure of the adenosyl-group-binding site as well as the structure of the substrate-free form of the enzyme. The discovery of a reactivating factor for diol and glycerol dehydratases and the elucidation of its molecular chaperone-like action would certainly be a milestone in investigating how B_{12} enzymes regenerate radicals when inactivated by extinction of radical intermediates. The generality of these findings obtained with diol dehydratase must be pursued in the future for a better understanding of the enzymatic radical catalysis of B_{12} enzymes.

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