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The structure and the mechanism of action of coenzyme B_{12}-dependent diol dehydratases

Tetsuo Toraya*

Department of Bioscience and Biotechnology, Faculty of Engineering, Okayama University, Tsushima-Naka, Okayama 700-8530, Japan

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Dedicated to the memory of the late Professor Saburo Fukui.

Abstract

Adenosylcobalamin (AdoCbl) (coenzyme B_{12}) serves as a cofactor for enzymatic radical reactions. The recently solved X-ray structure of diol dehydratase in complex with cyanocobalamin (CN-Cbl) revealed the base-on mode of cobalamin binding. The active-site cavity inside the $(\beta/\alpha)_8$ barrel seems to be a common molecular apparatus for coenzyme B_{12}-dependent enzymes to spatially isolate highly reactive radical intermediates. Based on the direct ion–dipolar interactions between two hydroxyl groups of substrate and potassium ion in the active site and theoretical calculations, a new mechanism for diol dehydratase is proposed here in which a potassium ion participates directly in the catalysis. The mechanism of activation of the coenzyme's cobalt–carbon bond by which a catalytic radical is generated in the active site of diol dehydratase is also discussed on the basis of the conformation of the enzyme-bound cobalamin. It was highly suggested that the reactivity of the cobalt atom is controlled by the length of the Co−N bond. Therefore, the role of the 5,6-dimethylbenzimidazole (DBI) moiety of cobalamin coenzyme in the enzyme catalysis is most likely to prevent the enzyme from mechanism-based inactivation by keeping the Co−N bond distance long through steric repulsion between the flattened corrin ring and the base. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Coenzyme B_{12}; Adenosycobalamin; Diol dehydratase; Structure and mechanism

1. Introduction

Adenosylcobalamin (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 beautiful structure [2]. It serves as a cofactor for enzymatic radical reactions including carbon skeleton rearrangements, heteroatom eliminations, and intramolecular amino group migrations. AdoCbl-dependent rearrangements involve migration of a hydrogen atom from one carbon atom of the substrate to an adjacent carbon atom in ex-

Abbreviations: AdoCbl, adenosylcobalamin; AdoH, 5′-deoxyadenosine; CN-Cbl, cyanocobalamin; DBI, 5,6-dimethylbenzimidazole; DFT, density-functional-theory

* Corresponding author. Tel.: +81-86-251-8194; fax: +81-86-251-8264.

E-mail address: toraya@biotech.okayama-u.ac.jp (T. Toraya).

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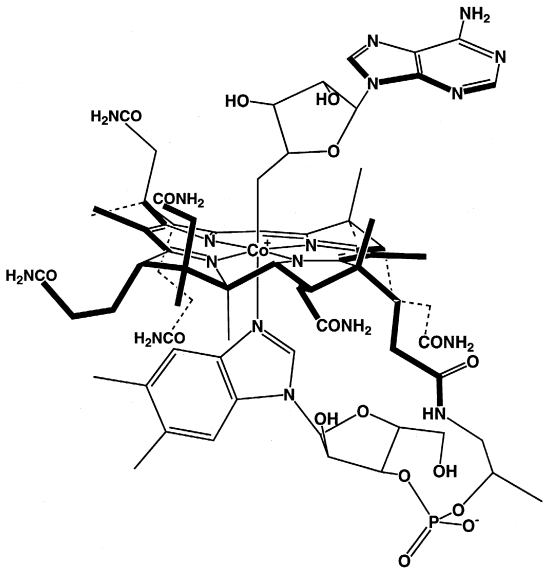
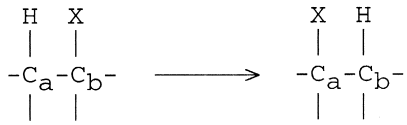


Fig. 1. Structure of AdoCbl (coenzyme B_{12}).

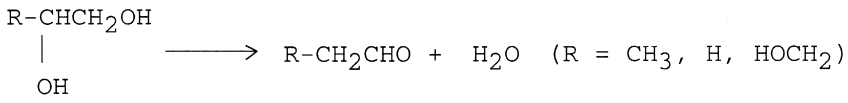
change for group X that moves in the opposite direction [3–8] (Eq. 1). In the rearrangements,



(1)

the adenosyl radical formed in the active site by homolytic cleavage of the coenzyme's Co—C bond triggers the reaction by activating the substrates through abstraction of a hydrogen atom.

Diol dehydratase catalyzes the AdoCbl-dependent conversion of 1,2-diols to the corresponding aldehydes (Eq. 2) [9,10].



(2)

Because the reaction catalyzed by this enzyme is apparently simple, the enzyme has been widely used to study the general mechanism of action of this coenzyme [3,5]. The structure–function studies of the coenzyme has also been conducted most extensively with this enzyme [7,11,12]. Glycerol dehydratase is an enzyme isofunctional to diol dehydratase with slightly different substrate specificity, but immunochemically distinct from the diol enzyme, bearing a different physiological role [7,13]. It is believed that both enzymes catalyze reactions by essentially the same mechanism, because these enzymes show high sequence homologies [14–16] and similar cofactor requirements [7,12]. Recently, the crystal structure of diol dehydratase in complex with cyanocobalamin (CN-Cbl) has been solved [17].

In this paper, I review the current status of our studies on the structure and the mechanism of action of diol and glycerol dehydratases with special emphasis on the role of a monovalent cation in the enzyme catalysis. Distributions and physiological roles of the enzymes as well as gene cloning and expression studies are also briefly summarized here. General aspects of diol and glycerol dehydratases [7,12] as well as structure–function studies of AdoCbl in diol dehydratase [7,11,12] have recently been reviewed elsewhere.

2. Distributions, physiological roles, gene cloning, and expression

2.1. Distributions

Both AdoCbl-dependent diol dehydratase and glycerol dehydratase are produced by some genera of Enterobacteriaceae, e.g. *Klebsiella* and *Citrobacter*, when the bacteria grow anaerobically in complex media containing 1,2-propanediol and glycerol, respectively [13,18,19]. *K. oxytoca* ATCC 8724 [18–20] and *Salmonella*

typhimurium [21] produce only diol dehydratase. *Propionibacterium* [18,22], *Flavobacterium* [23], *Nocardia* [24], and *Lactobacillus* [25] species produce diol dehydratase, whereas other *Lactobacillus* [26,27] and *Clostridium* [28] species produce glycerol dehydratase.

AdoCbl-independent diol dehydratase of *Clostridium glycolicum* was reported to be a membrane-associating radical enzyme [29–31].

2.2. Physiological roles

1,2-Propanediol, 1,2-ethanediol, and glycerol are more reduced substrates than carbohydrates and fermented by only a limited number of bacteria under anaerobic conditions without external electron acceptors. NADH formed by the oxidation of 1,2-diols or glycerol must be reoxidized to NAD^+ for internal redox balance, which is important for this type of fermentation to proceed [18,32]. The abilities of bacteria to grow anaerobically on 1,2-diols or glycerol without exogenous electron acceptors were found to be closely related with those of the bacteria to produce specific dehydratases [18].

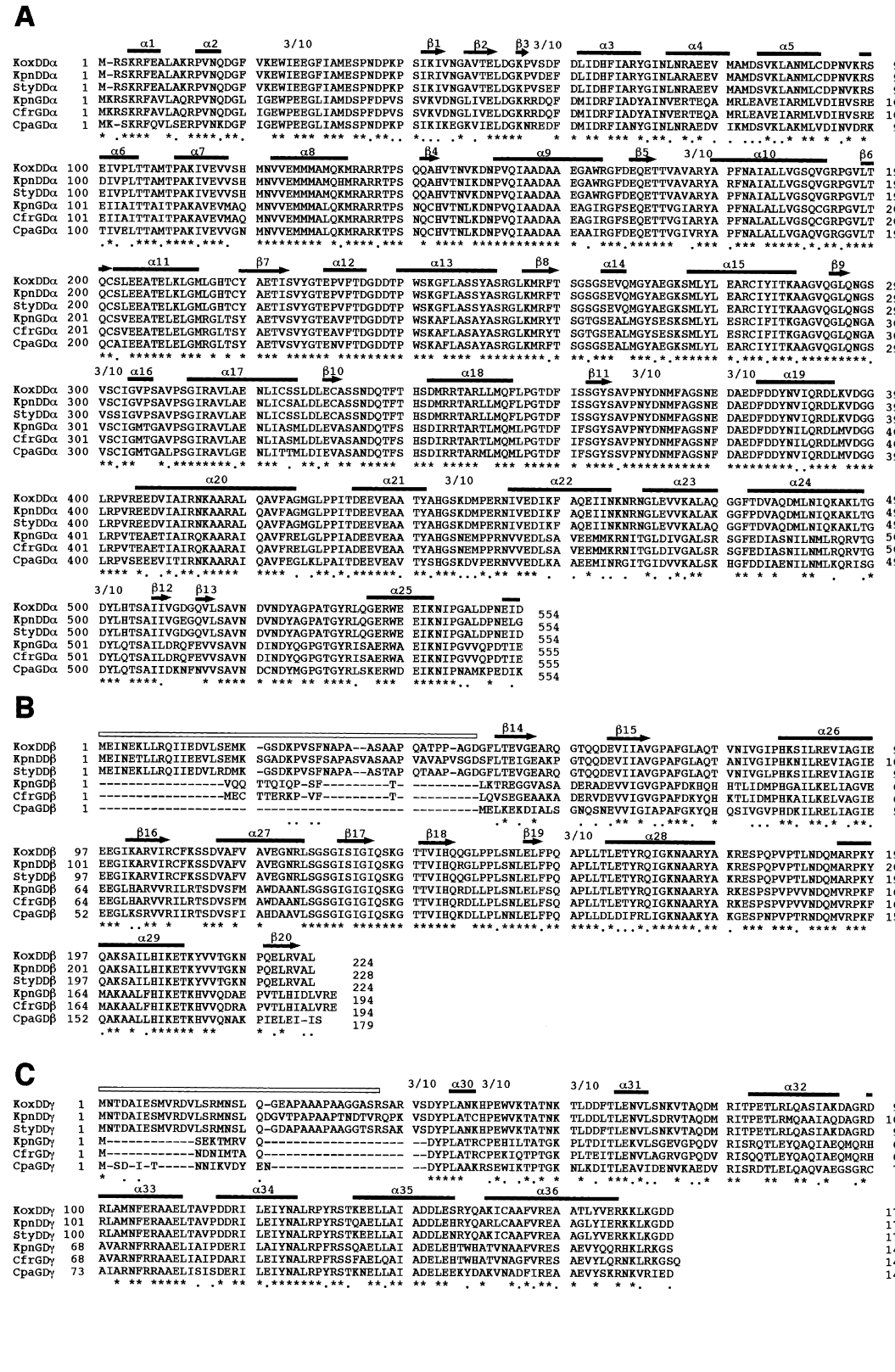
1,2-Diols are fermented to the corresponding acids and alcohols [22,33]. Diol dehydratase, alcohol dehydrogenase, CoA-dependent aldehyde dehydrogenase, phosphotransacetylase, and acetate kinase activities were shown to be present in cell-free extracts of 1,2-diol-grown cells. From these facts, it was indicated that 1,2-diols are fermented to the acids and alcohols through aldehydes, acyl-CoA, and acyl phosphates [21,22,29,33,34]. The physiological role of diol dehydratase was therefore concluded to be to produce an aldehyde, which serves as both an electron acceptor and a metabolic intermediate in the fermentation of 1,2-diols [7,12].

Upon anaerobic dissimilation of glycerol, 1,3-propanediol is accumulated in the growth medium. Among the four enzymes encoded by the *dha* regulon, i.e., glycerol dehydrogenase, dihydroxyacetone kinase, glycerol dehydratase, and 1,3-propanediol dehydrogenase, the first two enzymes participate in the oxidation and subsequent phosphorylation of glycerol, whereas the latter two enzymes constitute a branch for reoxidation of reducing equivalents (NADH) [35,36]. The physiological role of glycerol dehydratase is therefore to produce an electron acceptor, β-hydroxypropionaldehyde, in the glycerol fermentation [7,12]. In some bacteria, glycerol dehydratase can be substituted by isofunctional diol dehydratase, which is induced in a low level by glycerol [18,20,35].

2.3. Gene cloning, sequence homologies, and heterologous expression

The genes of *K. oxytoca* encoding diol dehydratase were cloned by using a synthetic oligonucleotide as a hybridization probe [14]. The three sequential genes *pddABC* encode the α, β, and γ subunits of the enzyme, respectively. Recently, the sequences of the genes for diol dehydratases in *S. typhimurium* [37] and *K. pneumoniae* [38] have also been reported. The genes for glycerol dehydratases in *K. pneumoniae* and *Citrobacter freundii* were cloned by us [15] and by Seyfried et al. [16], respectively. The clostridial genes have also been cloned recently [39]. The α, β, and γ subunits of this enzyme are also encoded by the three sequential genes.

Alignment of the amino acid sequences of the α, β, and γ subunits of diol and glycerol dehydratases [14–16,37–39] indicates high homologies between these dehydratases (Fig. 2). The organizations of the genes for diol and glycerol dehydratases are very similar. High sequence homologies suggest that the dehydratases are evolutionarily related [38]. Because several bacteria of Enterobacteriaceae produce both enzymes in response to the growth substrates, it is likely that both enzymes were formed from a common ancestor enzyme by gene redundancy and evolved to the present ones adapted to their respective metabolic roles. The consensus sequence DxHxxG, which forms a part of the cobalamin-binding motif [40–42] was not found in either enzyme. This motif is


A
B
C

present in all the enzymes catalyzing carbon skeleton rearrangements [43–46] as well as methionine synthase [47] and D-lysine 5,6-aminomutase [48], but not in either enzymes catalyzing heteroatom elimination reactions including ribonucleotide reductase [14–16,49,50].

High-level expression of the genes for diol dehydratase [14] and glycerol dehydratase [15,16] in *Escherichia coli* was achieved. It is now possible to purify a large quantity of the recombinant enzymes from overexpressing *E. coli* strains ([51], Tobimatsu et al., to be published). When the genes encoding the subunits of diol dehydratase were overexpressed individually or in various combination in *E. coli* [51], the α and γ subunits mutually required each other for correct folding, forming a soluble, active $\alpha_2\gamma_2$ complex (component S). Expression of the β subunit in a soluble, active form (component F) was promoted by coexpression with both α and γ subunits, probably by coexistence with component S. It is thus evident that each subunit mutually effects the correct folding of the others in this heterooligomer enzyme.

3. Three-dimensional structure

3.1. Overall structure

The crystal structure of diol dehydratase in complex with CN-Cbl and substrate 1,2-propanediol has recently been determined and refined at 2.2-Å resolution [17]. The enzyme exists as a dimer of heterotrimer $(\alpha\beta\gamma)_2$, which is consistent with the subunit structure deduced from molecular weight determination [51]. Fig. 3A 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 exclusively contributes 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 [54,55]. Components F and S were identified as the monomeric β subunit and the dimeric $(\alpha\gamma)_2$ complex, respectively [51].

Each heterotrimer $(\alpha\beta\gamma)$ binds one molecule of CN-Cbl (Fig. 3B). The cobalamin molecule is located between the α and β subunits, an upper axial ligand being oriented to the direction of the α subunit. The cyano group was unable to be located in the electron density map.

3.2. Structure of each subunit

The secondary structure of diol dehydratase is also shown in Fig. 2. The α subunit contains a $(\beta/\alpha)_8$ barrel structure in the central part (Fig. 4A). This structure, so-called triose-phosphate isomerase (TIM) barrel, has been found in methylmalonyl-CoA mutase [41] and recently in glutamate mutase [42] as well, although the reactions catalyzed are quite different. The cobalamin molecule is accommodated in the area which is 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 [9,56], are buried deeply in the barrel (Figs. 3B and 4B). The N-terminal and the C-terminal regions of the α subunit consist of many α helices and a few β strands and surround the outer parts of the barrel. The γ subunit consisting mainly of α helices is located far from the coenzyme and is in full contact with the α subunit. The roles of the γ subunit and the N-terminal and the C-terminal parts of the α subunit might be to support the barrel structure of the α subunit to maintain the overall structure.

Fig. 2. Alignment of amino acid sequences of the subunits of diol dehydratases (DD) and glycerol dehydratases (GD) and the secondary structure of diol dehydratase. Gaps are indicated by hyphens. (Thick line), α helix; (thick right arrow), β strand; (zigzag line), 3_{10} helix; (rectangle), missing region in the β or γ subunit. Kox, *K. oxytoca*; Kpn, *K. pneumoniae*; Sty, *S. typhimurium*; Cfr. *C. freundii*; Cps, *Clostridium pasteurianum.*

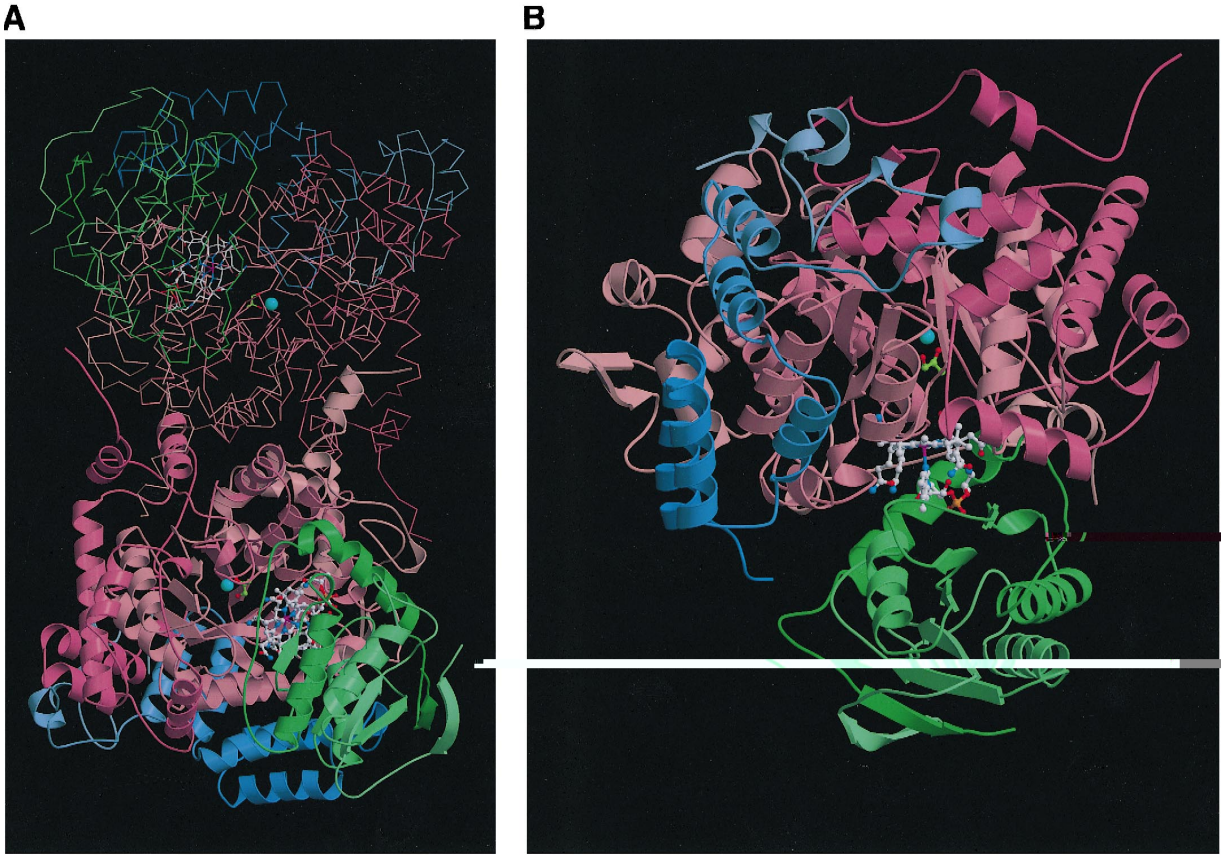


Fig. 3. The overall and a heterotrimer structures. Drawn with MOLSCRIPT [52] and RASTER3D [53] using the coordinates obtained by Shibata et al. [17]. (A) $(\alpha\beta\gamma)_2$ (overall structure). (B) $\alpha\beta\gamma$ heterotrimer unit. Pink, green, and blue colors are used for the α, β, and γ subunits, respectively, darkening continuously from the N-terminal to the C-terminal sides. Carbon atoms and bonds in cobalamin and 1,2-propanediol are shown in white and greenyellow, respectively, and K^+ in cyan.

The topology of the β subunit is quite unique. A Rossmann-fold-like structure is observed in the central part of the subunit and may play an important role in the contact with the lower axial ligand of cobalamin. The remaining parts mainly consist of many α helices and a few anti-parallel β strands and surround the Rossmann-fold structure. Surface representations of the α and β subunits viewed from the β and α subunits, respectively, are shown in Fig. 5A and B.

3.3. Mode of cobalamin binding

One of the striking features in the structure of diol dehydratase is that the 5,6-dimethylbenzimidazole (DBI) nucleotide moiety of the enzyme-bound cobalamin is coordinated to the cobalt atom in the corrin ring [17]. 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 [58,59]. In contrast, the X-ray structures of methionine synthase cobalamin-binding domain [40], methylmalonyl-CoA mutase [41] and glutamate mutase [42], together with EPR studies on these enzymes [40,60,61], revealed that the DBI ligand is displaced from the cobalt atom and accommodated in an extended form as a so-called ''nucleotide tail'', and instead, the imidazole group of cer-

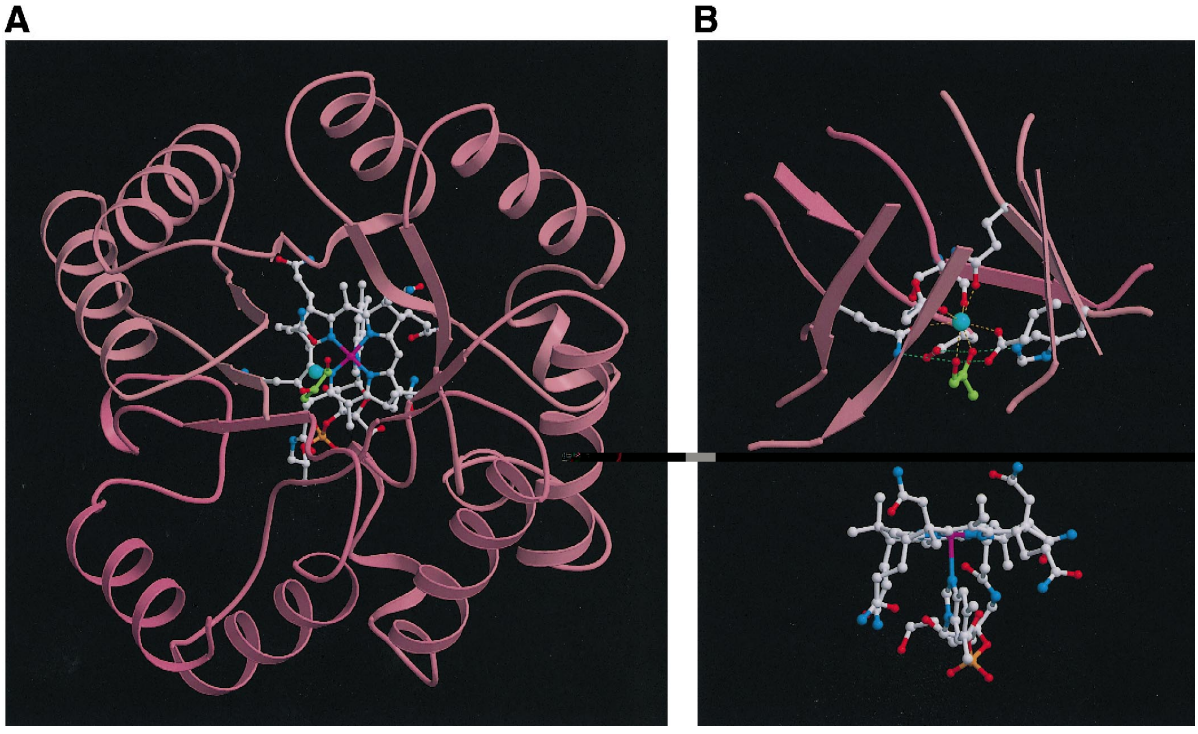


Fig. 4. Structure of the active site inside the $(\beta/\alpha)_8$ barrel. Drawn with MOLSCRIPT [52] and RASTER3D [53] using the coordinates obtained by Shibata et al. [17]. (A and B) Views from the directions perpendicular (A) and parallel (B) to the plane of the corrin ring of cobalamin. The eight β strands composing the barrel (β_4–β_{11} in Fig. 2) are colored in pink, darkening continuously from the N-terminal to the C-terminal side. Carbon atoms and bonds in the substrate and K^+ are shown in greenyellow and cyan, respectively.

tain histidine residue in the above-mentioned sequence DxHxxG of proteins is ligated to the cobalt atom in these enzymes. The presence of this motif is not a necessary condition for the ‘‘base-off’’ mode of cobalamin binding with histidine ligation, because a corrinoid protein lacking this motif was reported to bind 5-hydroxybenzimidazolylcobamide in this mode [62]. 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 reaction, as well [63]. Therefore, it was concluded that there are at least two types of B_{12} proteins whose ancestors are different.

3.4. Binding and conformation of cobalamin

The interactions of cobalamin with side chains of amino acid residues are rather complicated. For accommodating cobalamin, the side chains of a group of amino acid residues provide a space, which is mainly surrounded by hydrophilic groups [17]. Five amide groups out of six peripheral side chains of the corrin ring form hydrogen bonds with amino acid residues in the α and β subunits. The ribose moiety and the phosphate group of the nucleotide ligand are mainly surrounded by side chains of hydrophilic residues in the β subunit. A salt bridge between phosphate oxygen and ε-NH_3^+ group of Lysβ135 is involved in the enzyme–coenzyme interactions. Loose hydrophobic contacts are also observed between the DBI moiety and some hydrophobic amino acid residues in the β subunit. The importance of the phosphate group of cobalamin for the tight binding of AdoCbl to apoenzyme was established with adenosylcobinamide methyl phosphate, which tightly binds to the enzyme and brings about mechanism-

based inactivation [64]. There are no significant interactions between apoenzyme and the functional groups of the α-ribose moiety except for the hydrogen bonding between Proβ155 and 2′-OH. This is consistent with our finding that a series of analogs of AdoCbl in which α-ribose is replaced by an oligomethylene group are active as coenzymes [65].

The bond distance between Co and N-3 of DBI of the enzyme-bound cobalamin is about 2.50 Å [17]. Such an elongated Co—N bond was reported with methylmalonyl-CoA mutase [41] and recently with glutamate mutase [42] as well, in clear contrast to the normal Co—N bond length in free cobalamins [66–69] as well as in the methionine synthase-bound methylcobalamin [70]. These 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° [17]. This value is close to that reported with methylmalonyl-CoA mutase [41]. The dihedral angle is 17° for free CN-Cbl [66]. Therefore, it is quite reasonable to assume that the tight interactions between enzyme and coenzyme's amide side chains through hydrogen bonding make the corrin ring almost planar. In the diol dehydratase-bound cobalamin, no strong interactions between amino acid residues and the DBI moiety are found. Thus, the Co—N bond would be lengthened by steric repulsion between the flattened corrin ring and the bulky DBI moiety [17].

3.5. Active-site cavity in a TIM barrel

Substrate 1,2-propanediol is bound inside the TIM barrel, coordinating to K^+, but away from the cobalt atom of cobalamin (Fig. 4) [17]. Although racemic 1,2-propanediol was used for purification and crystallization of the enzyme, we assigned the *S*-enantiomer to the electron density map, because the *S*-enantiomer is preferred in binding by the enzyme [71,72]. Active site residues interacting with substrate 1,2-propanediol and K^+ are shown in Fig. 6. Besides coordination to K^+, the O(2) atom of 1,2-propanediol is also bonded to COO^- of Aspα335 and Nε2 atom of Hisα143 by hydrogen bonding, and the O(1) to COO^- of Gluα170 and Nε1 of Glnα296. The methyl group and other carbon atoms of 1,2-propanediol have some hydrophobic contacts with nearby amino acid residues [17]. These hydrophilic and hydrophobic interactions seem to hold the substrate in the active site. 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. 4). Although the position of the adenosyl group of the coenzyme has not yet been determined, there is enough room for accommodating the adenosyl group above the corrin ring. Therefore, the bottom part of the barrel above the corrin ring must be the active site of this enzyme. A similar TIM barrel structure containing active sites was reported with methylmalonyl-CoA mutase [41] and glutamate mutase [42], although the mode of cobalamin binding and the reactions catalyzed are quite different from diol dehydratase. 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 [73] in his concept of ''negative catalysis'', or escape out of the active site. Such protection can be achieved by their spatial isolation inside the barrel. Thus, the TIM barrel 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. 5, the active site of the enzyme inside the TIM barrel is located in the cavity in the central region of the α subunit [17]. K^+ binds to the negatively charged inner part of the cavity, and cobalamin covers the cavity to isolate the active site from solvent molecules. The center of the barrel behind K^+ is filled by large side chains of amino acid residues. In contrast, it was reported that the

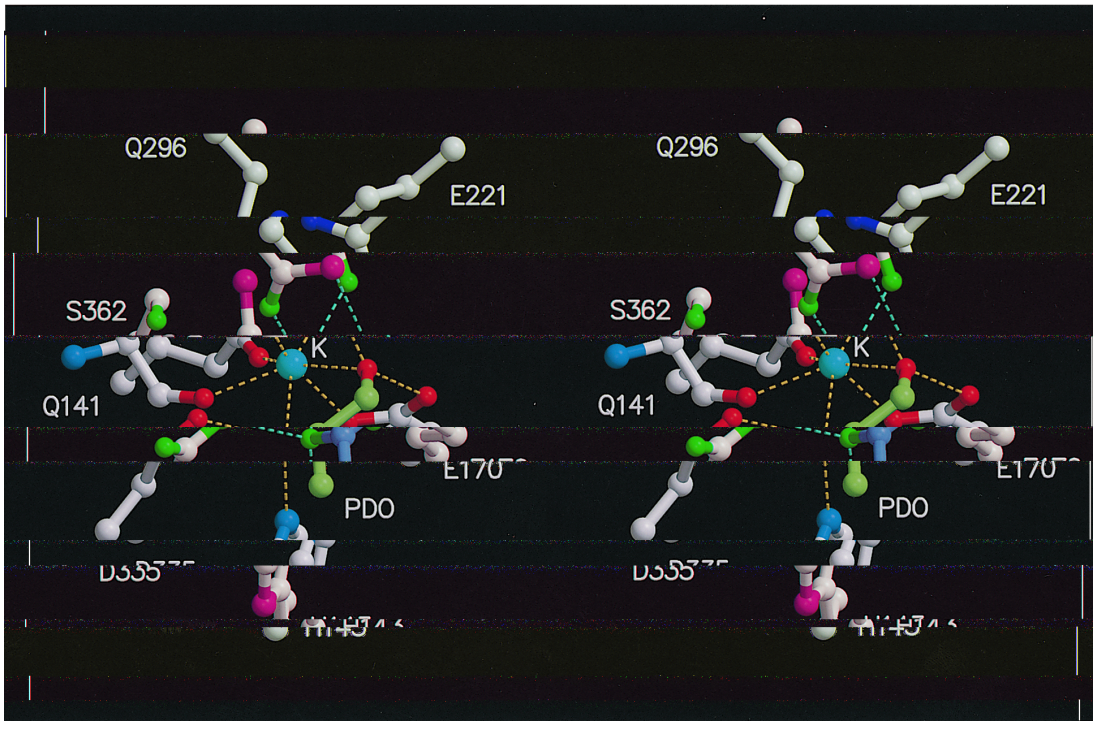


Fig. 6. A stereoview of the active-site residues interacting with substrate and K^+. Drawn with MOLSCRIPT [52] and RASTER3D [53] using the coordinates obtained by Shibata et al. [17]. Carbon atoms and bonds in the substrate and K^+ are shown in yellowgreen and cyan, respectively.

substrate is bound to methylmalonyl-CoA mutase, penetrating the TIM barrel [41].

3.6. Access of substrate to the active site

The β_8-barrel of diol dehydratase constituted by eight β strands (β4–β11) (Fig. 2) has two cracks between β6 and β7 and between β10 and β11. The access of substrate to the active site through the former crack is blocked by the TIM-barrel helices and the γ subunit. The latter crack is much closer to the solvent and may provide a possible path for substrate uptake to the active site near pyrrole ring D of cobalamin. This path is covered only by a loop between β11 and the α-helix following it. Three asparagine residues and three glutamine residues located nearby might allow a 1,2-diol substrate, a neutral hydrophilic compound, to enter the active site. These features suggest that this is the most likely path for uptake of a substrate and release of a product. The TIM barrel of substrate-free methylmalonyl-CoA mutase splits apart to bind succinyl-CoA or methylmalonyl-CoA [74]. Diol dehydratase may not need such a large conformational change for the substrate binding, because its substrate is much smaller. The postulated entrance area of the substrate-uptake path is shown with the arrow in the surface representation of the α subunit (Fig. 5A).

3.7. K^+ in the active site

Essential K^+ deeply buried in the TIM barrel is coordinated by five oxygen atoms originated from Glnα141, Gluα170, Gluα221, Glnα296 and Serα362 with bond distances ranging from 2.19 Å to 2.48 Å (Fig. 6). The sixth and seventh coordination positions are occupied by O(1) and O(2) of substrate 1,2-propanediol with distances of 2.38 and 2.40 Å, respectively. The distance between K^+ and Co is 11.7 Å. Monovalent

cations with certain ionic radii, such as K^+, serve as essential cofactors for diol dehydratase [9,54]. Although NH_4^+ shows higher affinity for this enzyme than K^+, the bound K^+ is not changed with NH_4^+ under the crystallization conditions in the presence of substrate [75]. 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 [56,76], suggesting that K^+ bound in the barrel participates in the cobalamin binding 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 destroying the enzyme–coenzyme interactions.

K^+ is bound to the enzyme as a hepta-coordinated complex in the presence of substrate. Although the crystal structure of the substrate-free enzyme 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 of the enzyme, which may be important for switching the substrate-free form to the substrate-bound form.

4. Mechanism of action

4.1. Labeling experiments and isotope effects

Although the AdoCbl-dependent enzymatic rearrangements are seemingly quite different, they share a common feature — that is, 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] (Eq. 1). In the cases of diol and glycerol dehydratases, X is the hydroxyl group on C-2, and a water molecule is subsequently eliminated from a *gem*-diol, forming aldehydes or ketones. Using [1-^{18}O]1,2-propanediol, Rétey et al. [77] demonstrated that the initial migration of a hydroxyl group is stereospecific, and that the dehydration of the *gem*-diol is sterically controlled 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.

In the reaction, the hydrogen atom moves to the adjacent carbon atom without exchange with solvent protons [78]. Zagalak et al. [79] showed 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 C-2 configuration [79,80]. Using unlabeled and deuterated substrates, Abeles and Zagalak [81] and Frey et al. [82] demonstrated that the hydrogen transfer is not necessarily intramolecular, but occurs by an intermolecular process as well. With a tritiated substrate, it was shown 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 [82–85].

The deuterium isotope effect (k_H/k_D) for the overall reaction was 10 [79], 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 [86]. The reason why such an unusually large isotope effect ($k_H/k_T = 125$), corresponding to a k_H/k_D of 28, was obtained for the latter step remains unclear.

4.2. Reaction intermediates and a minimal mechanism

Frey et al. [82] and Essenberg et al. [86] showed by kinetic experiments 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. From these results, 5′-deoxyadenosine (AdoH) was postulated to be an intermediate. Its formation was actually demonstrated in inactivation reactions with several substrates [87–89] or coenzyme analogues [64,65,90]. Its reversible formation was indicated with ethanolamine ammonia-lyase [91].

The formation of cob(II)alamin, another intermediate, during catalysis was observed by optical [88,92–94] and electron paramagnetic resonance (EPR) [93,95–98] 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 cob(II)alamin, respectively [99,100]. The doublet signal was interpreted to arise from a weak exchange interaction of the organic radical with Co(II). A model proposed for the system suggested an interaction of the free radical with the Co(II) center at least 6 Å away [99]. The distances between Co and C-1 and C-2 of 1,2-propanediol are 8.37 and 9.03 Å, respectively [17]. These values are in reasonable agreement with the distance between the organic radical and Co(II) of cob(II)alamin, which was estimated by EPR. The distance of closest approach of water to Co(II) was estimated to be 10.2 Å [96]. The generation of an organic radical intermediate at a kinetically competent rate in the enzymatic reaction was also demonstrated by EPR spectroscopy [98]. Thus, it was established that the radical species formed by homolysis of the Co–C bond of AdoCbl is involved in the catalysis, and that hydrogen migrates as a hydrogen atom.

A minimal mechanism proposed for diol dehydratase [3,5,7] and common to other AdoCbl-dependent enzymes [4,6,8] is illustrated in (Fig. 7). The interaction between apoenzyme and coenzyme leads to the activation of the Co–C bond of the coenzyme for homolysis, forming cob(II)alamin 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

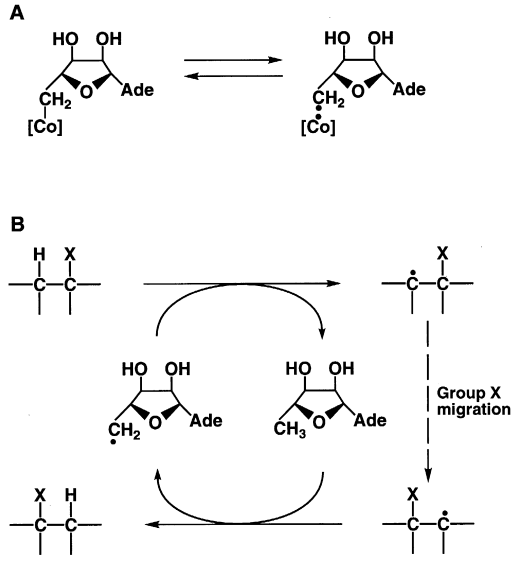


Fig. 7. The minimal mechanism for AdoCbl-dependent rearrangements. (A) Homolytic cleavage of the Co–C bond of enzyme-bound AdoCbl. (B) Adenosyl-radical-catalyzed enzymatic rearrangements. [Co], cobalamin; Ade, 9-adeninyl; X, a generic migrating group.

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 AdoH. The substrate radical rearranges to the product-derived radical, which then abstracts a hydrogen atom from AdoH. This leads to the formation of the final product and regeneration of the coenzyme.

4.3. Possible pathways of the hydroxyl group migration

In the minimal mechanism for diol dehydratase, the pathway of the hydroxyl group migration from C-2 to C-1 of the substrate-derived radical remained almost obscure, although several proposals have been made based on non-enzymatic model reactions and theoretical calculations [101–103]. The long distance between Co and substrate [17] eliminated a possibility that Co(II) of cob(II)alamin is directly involved in the rearrangement of a substrate-derived radical

to a product-derived one by transalkylation through organocobalamin intermediates [104]. Co(II) is considered as a ''spectator'' in the 1,2-shift of the hydroxyl group in the diol dehydratase reaction. The two pathways shown in Fig. 8 seem to be consistent with the X-ray structure of the enzyme.

In one pathway (a), it was postulated that the hydroxyl group migrates from C-2 to C-1 via a cyclic transition state. Based on ab initio calculations as well as model reactions, Golding and Radom [101] and Smith et al. [102] proposed that protonation of the migrating hydroxyl group facilitates the rearrangement. They pointed out that the barrier height for the transition state in the hydroxyl group migration is reduced by protonation [101] or even by partial protonation with NH_4^+ [102].

An alternative pathway (b) with a radical anion intermediate might also be possible. The pK_a values of the hydroxyl groups attached to a carbon radical were reported to be ~5 pH units lower than those of the corresponding alcohols [105]. Formation of such a radical anion intermediate may be favored by K^+ through electrostatic stabilization. These might ensure deprotonation of the hydroxyl group on C-1 by COO^- of Gluα170 for the *S*-isomer. The abstraction of the hydroxyl group from C-2 may be facilitated by the resulting oxyanion on C-1 through $\sigma-\pi$ 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 Bothe et al. [103], however, may be incompatible with the constant activity of diol dehydratase in the pH range from 6.0 to 10.0 [9].

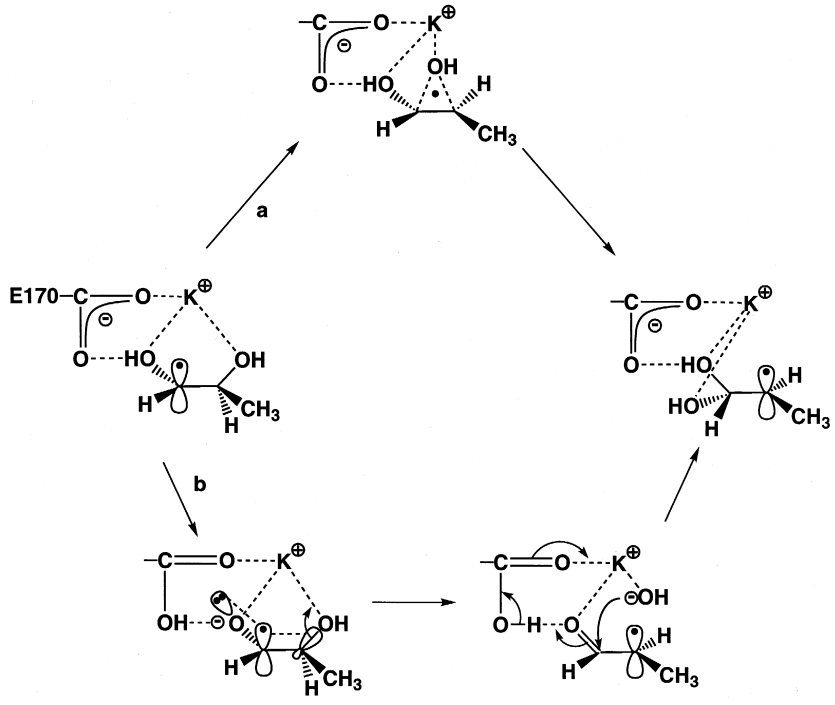


Fig. 8. Possible pathways of the hydroxyl group migration from C-2 to C-1. (a) Concerted mechanism via a cyclic transition state. (b) Stepwise mechanism via a radical anion intermediate. The reaction with (*S*)-1,2-propanediol is illustrated. –COO⊖, carboxylate group of Gluα170.

4.4. Role of K^+ in the enzyme catalysis

It was difficult to determine from the three-dimensional structure of the active site, which pathway is more likely. Hence, the use of ab initio molecular orbital theory was attempted to investigate the energetics of the possible pathways of the hydroxyl group migration. We carried out density-functional-theory (DFT) computations [106] to determine whether K^+ can assist the 1,2-shift of the hydroxyl group in the substrate radical [107]. Between two pathways of the hydroxyl group migration — that is, a stepwise abstraction/re-addition reaction that can proceed via a direct hydroxide abstraction by K^+ (b) and a concerted hydroxyl group migration assisted by K^+ (a), only a transition state for the latter concerted mechanism was found from our DFT computations. The migration of the hydroxyl group requires 20.6 kcal/mol in the absence of K^+. Fig. 9 shows the potential energy diagram. In the initial stages of the reaction, the substrate replaces the K^+-bound H_2O to form a stable complex with K^+ with a net binding energy of 10.7 kcal/mol. The most notable geometric change in the complexation is the rotation of the dihedral angle of O–C-1–C-2–O in the substrate, due to the direct ion–dipolar interactions between K^+ and the two hydroxyl groups. The barrier height for

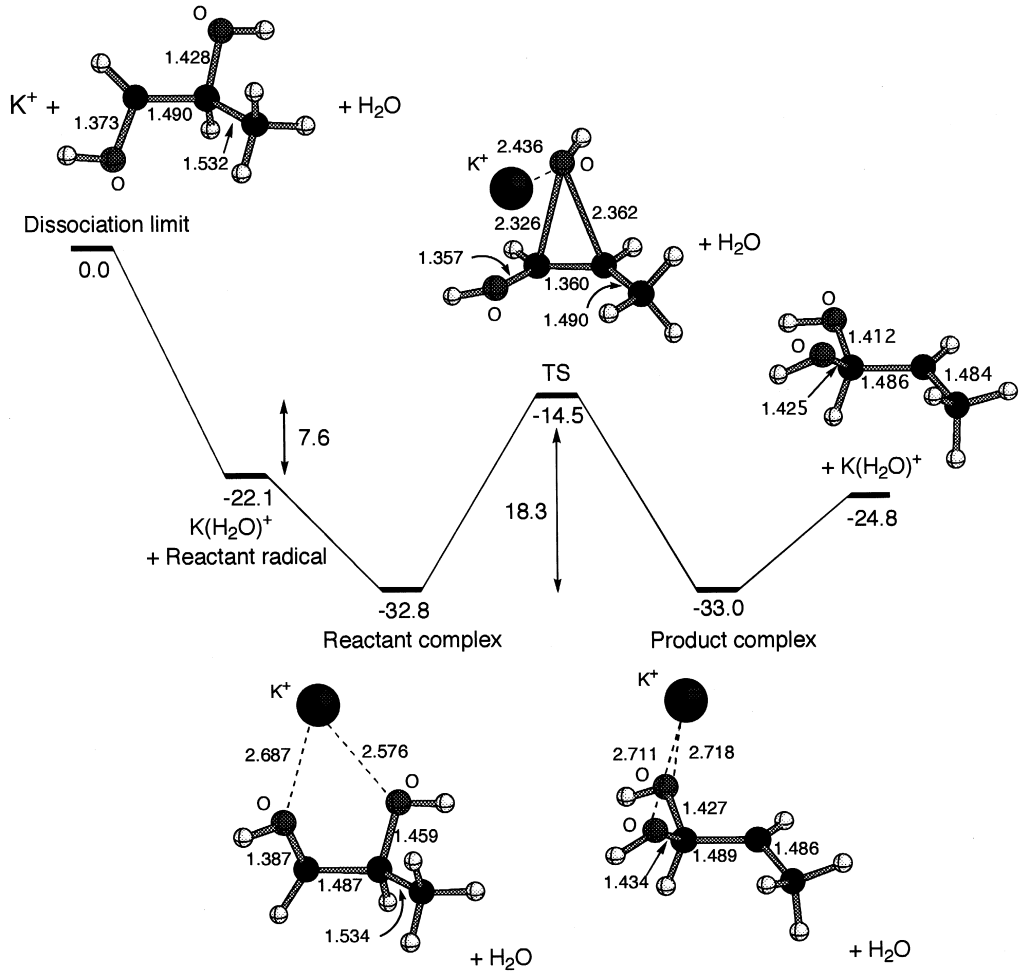


Fig. 9. Energy diagram for the migration of the hydroxyl group from C-2 to C-1 in the 1,2-dihydroxypropyl radical in the presence of K^+ [107]. Bond length in Å and energies in kcal/mol.

the transition state was computed to be 18.3 kcal/mol if measured from the stabilized K^+ complex. Decrease in the barrier height by 2.3 kcal/mol corresponds to 42-fold rate acceleration at 37°C by coordination of the migrating hydroxyl group to K^+. The hydroxyl group migration still requires high activation energy, but this might not be unreasonable, because the deuterium isotope effect indicates that the hydroxyl group migration is not rate determining [79]. Thus, it was concluded that the hydroxyl group migration will undergo only partial rate acceleration by coordination to K^+. If K^+ is assumed not to bind H_2O, catalytic turnovers would be difficult, because dissociation of the product radical complex to a dissociation limit requires high energies. The large binding energy released upon complexation of the substrate with K^+ would be utilized at least in part to facilitate the Co–C bond homolysis through the substrate-induced conformational change of the enzyme [107].

4.5. Proposed mechanism

A new overall mechanism for diol dehydratase, which we propose based on the computational results [107] as well as the three-dimensional structure of the enzyme [17] is illustrated in Fig. 10. As described above, it is likely that the binding of 1,2-propanediol to the active site of the substrate-free enzyme (**1**) converts a hexa-coordinated complex of K^+ into the hepta-coordinated complex (**2**) through the coordination of its two hydroxyl groups to K^+ displacing sixth ligand H_2O [17]. This would induce a conformational change of the protein, which triggers homolytic cleavage of the Co–C bond of AdoCbl, forming an adenosyl radical and cob(II)alamin.

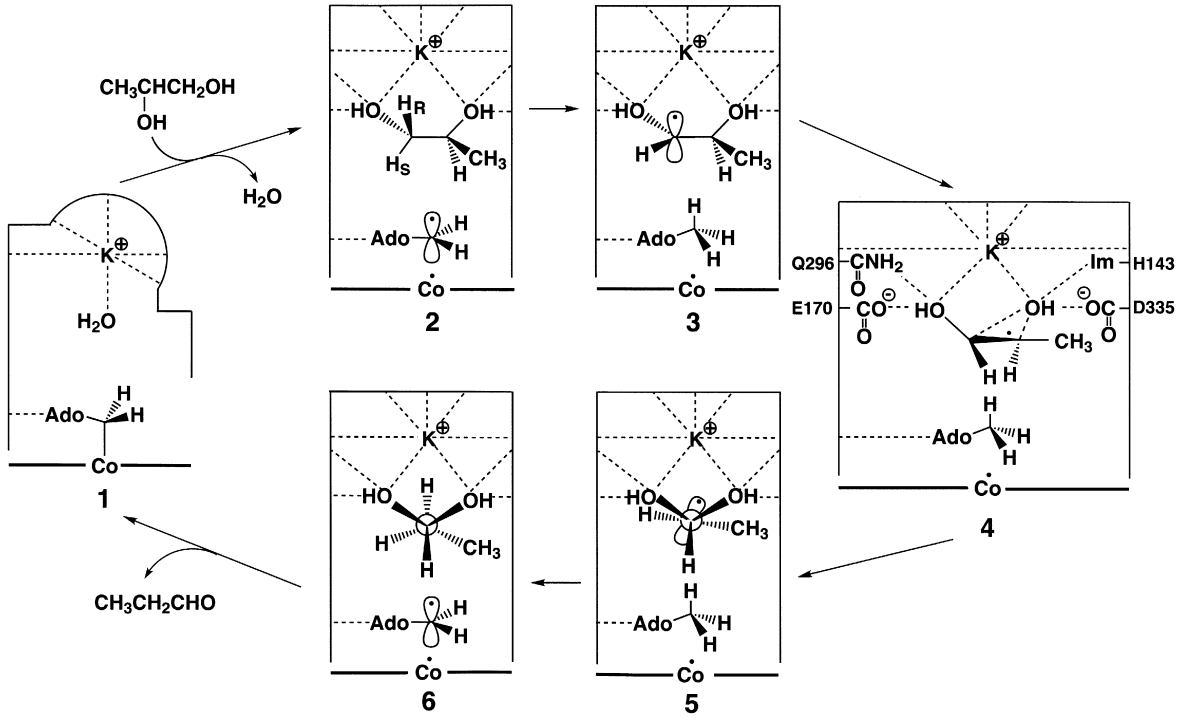


Fig. 10. The proposed mechanism for diol dehydratase. The reaction with (*S*)-1,2-propanediol is illustrated. –Co–, cobalamin; $AdoCH_2$, adenosyl group; Im, imidazole group of Hisα143.

Specific hydrogen atom of the substrate (*proS* and *proR* hydrogen atoms of *S*- and *R*-isomers, respectively) [79] is abstracted by the adenosyl radical, producing a substrate-derived radical and AdoH(3). Computational results indicate that the 1,2-shift of the hydroxyl group of the substrate radical takes place through the cyclic transition state [101,102,107]. 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. Smith et al. [102] 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. 6). 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 inconsistent with the constant activity of diol dehydratase at pH 6.0–10.0 [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-1 bond turning around through the transition state illustrated as **4** in Fig. 10. 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 AdoH, 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^+. Product aldehyde loses its affinity for K^+ because of the loss of the hydroxyl groups and is thus released from the active site. 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 cob(II)alamin to regenerate the coenzyme. A bond energy released upon re-formation of the Co—C bond ensures 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 demonstrated here that the monovalent cation is not a spectator but an active ''conductor'' in the enzyme catalysis.

4.6. Stereochemical course

In Fig. 10, the reaction with (*S*)-1,2-propanediol is illustrated. When enantiomers of 1,2-propanediol are used individually as substrates, the rate with the *R*-isomer is 1.7–1.8 times faster than that with the *S*-isomer [71,72,79]. But, the *S*-isomer reacts at a faster rate than the *R*-isomer in competitive reactions [108]. Jensen and Neese [108] 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. Among the diastereomers of 2,3-butanediol, only the *meso*-isomer serves as a substrate [109]. From these results, 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. In the cases of *S*- and *R*-isomers, the *proS* and *proR* hydrogens would be abstracted by the adenosyl radical, respectively [79]. We have to await an X-ray analysis of the complex with (*R*)-1,2-propanediol to explain the experimental observation [77] that the oxygen atom derived from C-1 is retained in the product from the *S*-isomer and lost into the solvent from the *R*-isomer.

4.7. Activation of the Co–C bond of AdoCbl

The Co–C bond of free AdoCbl is stable in the dark but becomes activated when the coenzyme is bound by the apoenzyme. This is important for the enzyme to generate a catalytic radical in the active site. Since the Co–C bond of AdoCbl is sufficiently weak, only a modest labilization by interaction with the apoprotein would be required for homolysis. The X-ray structure of diol dehydratase [17] as well as the structure–function studies of the coenzyme [64,93] revealed that the cobalamin moiety of AdoCbl is tightly bound to apoenzyme by specific interactions through peripheral amide side chains of the corrin ring and the phosphate group of the nucleotide moiety. As a result of enzyme–cobalamin interactions, the corrin ring becomes almost planar and the Co–N bond unusually lengthened by steric repulsion between the flattened corrin ring and DBI [17]. Although the extent of labilization is rather small without the adenosyl group [110], the enzyme-induced distortion of the corrin ring could lead to labilization of the coenzyme's Co–C bond of AdoCbl to homolysis rather than to heterolysis. Coordination of the axial base to the cobalt atom is not absolutely necessary for labilization of the Co–C bond [64], suggesting that a mechanochemical activation is less likely. 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 [111]. There is biochemical evidence for the presence of the adenosyl group-binding site in diol dehydratase [112], although this position has not yet been crystallographically determined. The strong attraction of the coenzyme's 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 [113].

In the absence of substrate, only a small fraction of the enzyme-bound coenzyme is in the dissociated form [92,95,96]. When the substrate is added to the system, most of the coenzyme undergoes homolysis of its Co–C bond at a kinetically competent rate [98]. This indicates that substrate-induced conformational change of the enzyme brings about further strain, which triggers homolytic cleavage of the Co–C bond. The rate acceleration of the Co–C bond cleavage with diol dehydratase in the presence of substrate was calculated to be 10^{11} to 10^{12}-fold [98,114]. In the absence of substrate, the rate acceleration of the bond cleavage was approximately 10^{6} to 10^{7}-fold (Yamanishi and Toraya, to be published). Therefore, the substrate enhances the rate of Co–C bond cleavage by a factor of 10^{4} to 10^{6}. This corresponds to $\Delta\Delta G^{\ddagger}$ of 6–9 kcal/mol. Such energy for the rate enhancement by substrate is considered to be supplied by the large binding energies released upon binding of the substrate to the enzyme. For diol dehydratase, the presence of K^{+} in the active site seems to be important for increasing the substrate binding energy.

4.8. Role of the DBI moiety of AdoCbl in the catalysis

For enzymatic radical catalysis, it is generally important how the enzymes control highly reactive radical intermediates. In diol dehydratase [17] as well as methylmalonyl-CoA mutase [41] and glutamate mutase [42], radical species could be protected from solvent molecules by spatial isolation inside the TIM barrel. It was also demonstrated with coenzyme analogues in diol dehydratase that 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 [64,65,115]. In this sense, cob(II)alamin is not simply a spectator but a ''stabilizer'' for organic radical intermediates. The apparent radical-stabilizing effect of the base correlates well with its bulkiness rather than basicity [11,12]. Therefore, the steric effect of the base seems important for stabilizing radical intermediates during catalysis.

Since diol dehydratase binds cobalamin in the base-on mode, it is likely that a bulky base exerts its effect by a so-called ''trans'' effect. It was demonstrated by X-ray crystallography [17] that the corrin ring of the enzyme-bound cobalamin is almost planar unlike those of free cobalamins, and that the Co—N bond is lengthened upon binding to diol dehydratase. These are also the cases in methylmalonyl-CoA mutase [41]. The Co—N bond is lengthened in glutamate mutase [42] as well, but not in methionine synthase [70], although the lower axial ligand in these enzymes is an imidazole group of certain histidine residues in common [40–42]. Therefore, it is likely that the homolysis of the Co—N bond is much more favored than its heterolysis by lengthening the Co—N bond, which enables continuous progress of radical reactions. It was suggested strongly that the reactivity of the cobalt atom is controlled by the Co—N bond distance. Because of steric repulsion between the flattened corrin ring and the base moiety, longer Co—N bond would be expected with a bulkier base like DBI. Thus, the role of the DBI moiety of AdoCbl in the enzyme catalysis is most likely to prevent a B_{12} enzyme from mechanism-based inactivation. This may be at least one of the reasons why most of the naturally occurring corrinoids contain a unique, bulky base DBI.

5. Concluding remarks

Recent solution of the crystal structure of diol dehydratase opened up a new horizon to us. It would become possible in the near future to understand on the basis of the three-dimensional structure how the enzyme activates and cleaves the Co—C bond of AdoCbl, although the binding site of the enzyme for the adenosyl group of the coenzyme is needed to be determined. The structure of the substrate-free enzyme also remains to be solved to understand the exact nature of the conformational change, which is induced by the substrate binding. The presumption and confirmation of the functional roles of the active-site residues by site-directed mutagenesis would lead us to the molecular engineering studies of this B_{12} enzyme in the future.

For better understanding of the mechanism of catalyses of B_{12} enzymes, I think, it is also important to investigate how the enzymes are reactivated when inactivated by extinction of free radical intermediates. Our studies of diol and glycerol dehydratases on this point are out of the scope of this review and will be summarized elsewhere [116].

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