Radical Catalysis in Coenzyme B_{12}-Dependent Isomerization (Eliminating) Reactions

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I. Introduction

Coenzyme B_{12} or adenosylcobalamin (AdoCbl) (Figure 1A), a naturally occurring organometallic compound that contains a unique Co−C σ bond, serves as a cofactor for enzymatic radical reactions. Since Barker's discovery of the light-sensitive coenzyme form of vitamin B_{12} (Figure 1C)-related corrinoid for the interconversion of glutamate and 3-methylaspartate in 1958,[1] AdoCbl has fascinated many investigators in a variety of fields by its peculiar function that resides in the peculiar, beautiful structure. The paradigm for the action of AdoCbl-dependent enzymes was put forth as early as in 1960.[2] Thus far, about 10 enzymes requiring AdoCbl as coenzyme have been reported that catalyze carbon-skeleton rearrangements, heteroatom eliminations, and intramolecular amino group migrations.[3,4] An elegant minimal mechanism was established by biochemical studies on diol dehydratase[5,6] and ethanolamine ammonia-lyase,[7] and it has now been accepted as a general mechanism for all the AdoCbl-dependent rearrangements.[3,4] However, it should be noted that most of the essential details of the mechanism remained unclear because three-dimensional structures of AdoCbl-dependent enzymes were not available until recently.[8−12] How do the enzymes form catalytic radicals at the active sites? How do the enzymes utilize and control the high reactivity of the radicals for catalysis? From the perspective of enzyme mechanism, these are the essential problems common to all the radical-containing and radical-forming enzymes, including AdoCbl-dependent enzymes.

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Tetsuo Toraya was born in Fukui, Japan, on May 1, 1945. He studied chemistry and biochemistry at Kyoto University. He received his Ph.D degree under the direction of Prof. Saburo Fukui in 1973 and was appointed Assistant Professor at the same university. He worked as a postdoctoral fellow with Prof. Robert H. Abeles at Brandeis University from 1977 to 1978 and was then promoted to Associate Professor at Kyoto University. In 1989, he became Professor of Bioscience and Biotechnology at Okayama University. He received the Young Investigator Award from the Japanese Biochemical Society in 1980 and the Award of the Vitamin Society of Japan in 1988. His research interests are focused on the structures, mechanisms, and regulation of vitamin B_{12}-dependent and radical enzymes, molecular chaperones, and proteins involved in cellular signal transduction.

In the concluding remarks of my review on diol and glycerol dehydratases published in 1999,[4] I wrote as follows: "In the next decade, it will likely become possible to discuss, on the basis of three-dimensional structures of proteins, how the enzymes activate the coenzyme's Co−C bond and how they deal with reactive radical intermediates. Although elucidation of the three-dimensional structures of the enzymes would not be a goal, it would provide us with the most important information concerning their mechanisms of action. Perhaps the functions of these interesting enzymes will become fully understood in the not-so-distant future on the basis of their exact structures and site-directed mutagenesis." As predicted, the X-ray crystallographic analyses of several cobalamin enzymes that have been performed in the last half decade opened quite a new horizon in our understanding of the mechanisms of their action. Many problems concerning the mechanism of action of AdoCbl have been solved or are being solved on the basis of the X-ray structures of the enzymes. In this sense, we are now in the most exciting time to understand the functions of B_{12} and B_{12}-enzymes.

This review will focus on AdoCbl-dependent enzymes that catalyze isomerization leading to the elimination of water or ammonia with special emphasis on the recent progress in the structural and mechanistic studies. They include diol dehydratase, glycerol dehydratase, and ethanolamine ammonia-lyase. Diol dehydratase and ethanolamine ammonia-lyase are the enzymes that had been most extensively studied to establish the minimal mechanism of action of AdoCbl-dependent enzymes, because the reactions catalyzed by these enzymes are apparently the simplest among them. Earlier mechanistic studies are not reviewed here because there are extensive reviews available.[5−7,13−22] Recent biochemical, biophysical, mutational, and crystallographic studies as well as theoretical calculations on the enzymes will be focused not only to identify the radical intermediates but also to understand the molecular mechanisms of activation of the Co−C bond toward homolysis, abstraction of a specific hydrogen atom from a

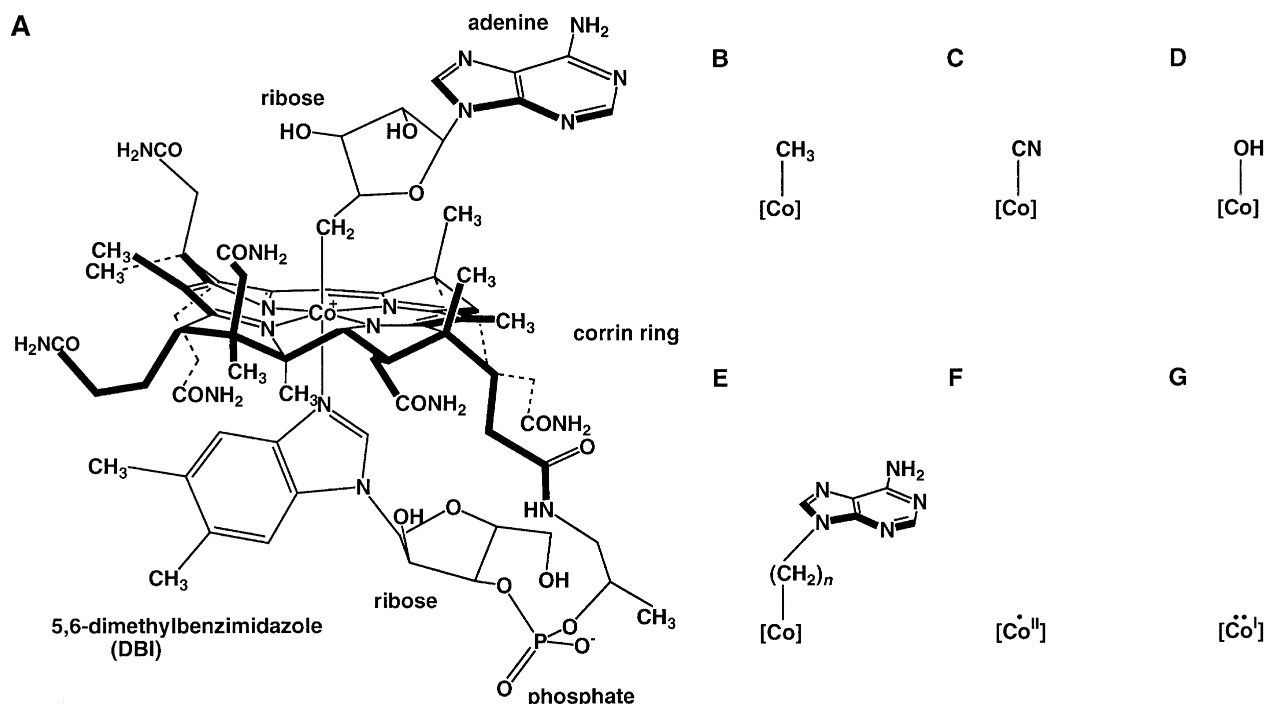


Figure 1. Coenzyme B_{12} and some of its analogues. (A) Three-dimensional structure of adenosylcobalamin (AdoCbl). (B) Methylcobalamin (MeCbl). (C) Cyanocobalamin (CN-Cbl). (D) Hydroxocobalamin (OH-Cbl). (E) Adeninylalkylcobalamin ($n = 2$, adeninylethylcobalamin (AdeEtCbl); $n = 3$, adeninylpropylcobalamin (AdePrCbl); $n = 5$, adeninylpentylcobalamin (AdePeCbl)). (F) cob(II)alamin. (G) cob(I)alamin. [Co] in panels B−G represents the corrin ring of cobalamins.

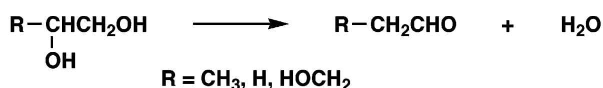
substrate and its recombination to a particular product, and transition state stabilization in the radical rearrangements or the 1,2-migrations of group X in radical intermediates. Studies on the functions of K^+ and the active-site amino acid residues in enzymatic catalysis are also described. On the basis of these studies, the refined catalytic mechanism of the enzymes as well as the concept and the energetic feasibility of enzymatic radical catalysis will be stated here. The structure–function studies of AdoCbl is out of the scope of this review (see refs 13–17).

Since the enzymes that catalyze by a radical mechanism tend to be easily inactivated, there is another very important point to be investigated. That is, do the enzymes become reactivated after they undergo inactivation? Discovery and action mechanisms of some reactivating factors for AdoCbl-dependent enzymes are also reviewed here. Their similarities to Hsp70-family molecular chaperones are summarized in this review. But the details of investigations on the metabolic roles of these enzymes are not extensively reviewed here (see refs 13, 14, 16, and 17 for reviews).

II. AdoCbl-Dependent Enzymes That Catalyze Isomerization (Eliminating) Reactions

A. Diol Dehydratase

Diol dehydratase or propanediol hydro-lyase (EC 4.2.1.28) catalyzes the conversion of 1,2-propanediol and other 1,2-diols to the corresponding aldehydes in the presence of AdoCbl:[23,24]



Abeles and co-workers found that the glycerol-grown cells of *Klebsiella oxytoca* (formerly *Aerobacter aerogenes* and *Klebsiella pneumoniae*) ATCC 8724 convert glycerol, 1,2-propanediol, and 1,2-ethanediol to β-hydroxypropionaldehyde, propionaldehyde, and acetaldehyde, respectively,[25] and isolated the enzyme from glycerol-grown cells.[23] Strangely, purified enzyme catalyzed the dehydration of glycerol at less than 5–10% of the rate with 1,2-propanediol. Later, this enigma was solved by the finding that glycerol serves as a potent mechanism-based inactivator as well as a good substrate.[24] The enzyme requires a certain monovalent cation, such as K^+, as an essential cofactor for catalytic activity.[23,26] It consists of three kinds of subunits, designated α, β, and γ.[27,28] Diol dehydratase is inducibly formed by some genera of *Enterobacteriaceae*, e.g., *Klebsiella* and *Citrobacter*, when they grow anaerobically in complex media containing 1,2-propanediol.[29–32] *Salmonella typhimurium*,[33] *Propionibacterium*,[30,34] *Flavobacterium*,[35] *Nocardia*,[36] and *Lactobacillus*[37] species also produce diol dehydratase. *Clostridium glycolicum* produces diol dehydratase, but this enzyme is a membrane-associating, noncobalamin radical enzyme.[38–40] The enzyme was purified from *K. oxytoca*[23,41] and *Lactobacillus brevis*.[37] The three sequential genes encoding diol dehydratase were cloned from *K. oxytoca*,[27] *K. pneumoniae*,[42] and *S. typhimurium*.[43] High-level expression and purification of the recombinant enzyme have been achieved with *K. oxytoca*[28] and *S. typhimurium*[44] enzymes.

B. Glycerol Dehydratase

Glycerol dehydratase or glycerol hydro-lyase (EC 4.2.1.30) also catalyzes the conversion of glycerol to β-hydroxypropionaldehyde and of 1,2-diols to the corresponding aldehydes in the presence of AdoCbl.[45] The enzyme was first found by Smiley and Sobolov in extract of an acrolein-forming *Lactobacillus* sp.[46] and by Pawelkiewicz and co-workers in *K. pneumoniae* (formerly *A. aerogenes*).[45] The enzyme was highly purified from *K. pneumoniae*[47,48] and *Lactobacillus reuteri*.[49] Glycerol and diol dehydratases are isofunctional, but the former prefers glycerol to 1,2-propanediol, whereas the latter prefers 1,2-propanediol to glycerol as substrates.[29] They are similar in the subunit structures but immunologically distinct enzymes.[29] They are different from each other in the binding affinity for AdoCbl,[31] monovalent cation-selectivity pattern[29] as well. Glycerol dehydratase is produced by some genera of *Enterobacteriaceae*, e.g., *Klebsiella* and *Citrobacter*, when they grow anaerobically on glycerol.[29–31] *Clostridium* species also produces this enzyme.[50] The three sequential genes of *K. pneumoniae*,[51] *Citrobacter freundii*,[52] and *Clostridium pasteurianum*[53] encoding glycerol dehydratase were cloned. Overexpression and purification of recombinant *K. pneumoniae*[12] and *C. freundii*[54] enzyme have been established.

C. Ethanolamine Ammonia-Lyase

Ethanolamine ammonia-lyase (EC 4.3.1.7) or ethanolamine deaminase catalyzes the conversion of ethanolamine to acetaldehyde in the presence of AdoCbl:[55]

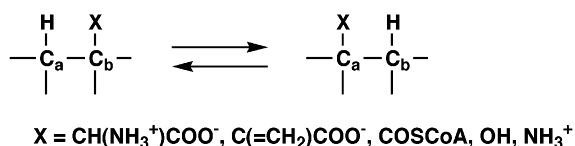
The enzyme was first discovered by Bradbeer in choline-fermenting *Clostridium* sp.[56] and then shown to be present in many bacteria in which exogenous vitamin B_{12} is required for growth on ethanolamine. They include *Klebsiella aerogenes*,[57] *Escherichia coli*,[58] *S. typhimurium*,[59] and *Bacillus megaterium*.[59] The enzyme was purified to homogeneity from *Clostridium* sp.[60] and *E. coli*.[61] The subunit structure of both clostridial[62] and *S. typhimurium*[63] enzyme is $\alpha_6\beta_6$. The cobamide bound to ethanolamine ammonia-lyase of *Clostridium* sp. was identified as pseudo-B_{12} (adeninylcobamide).[64] The number of functional active sites per enzyme molecule (n) reported is 2[65] or 6.[66] The latter value consistent with the subunit structure has been supported recently[67,68] and accepted widely. Ethanolamine and vitamin B_{12} are both required for enzyme induction; neither is effective by itself.[69] Ethanolamine ammonia-lyase was detected in a triethanolamine-fermenting *Acetobacterium* sp.[70] as well. Large and small subunits of the enzyme are encoded by the *eutB* and *eutC* genes in the *eut* operon of *S. typhimurium*,[71] *Rhodococcus*

sp.,[72] and *E. coli*.[73] Overexpression and purification of recombinant ethanolamine ammonia-lyase of *S. typhimurium*[63] and *E. coli*[73] have been achieved.

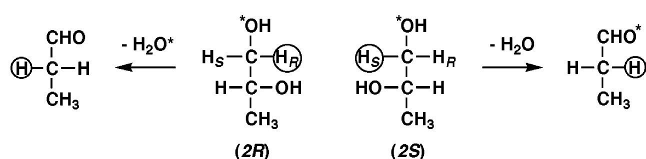
III. Biochemical Studies

A. Stereochemistry and Minimal Mechanism

Substrate specificity of diol dehydratase has been extensively reviewed and is thus summarized here only briefly (see refs 13, 16). Interestingly, diol dehydratase is not stereospecific—that is, it catalyzes the conversion of both (*R*)- and (*S*)-1,2-propanediols to propionaldehyde. This unusual property has been explained by assuming the two different modes of substrate binding[74] with different catalytic efficiency ($k_{cat}(R)/k_{cat}(S) = 1.8$) and binding affinity ($K_m(R)/K_m(S) = 3.2$).[75] Glycerol dehydratase also acts on both (*R*)- and (*S*)-1,2-propanediols, but relative activity ($k_{cat}(R)/k_{cat}(S) = 2.5$) and affinity ($K_m(R)/K_m(S) = 1.5$) for each enantiomer are different from those of diol dehydratase.[12] Although the enzymatic reactions requiring AdoCbl are seemingly quite different, all of them except for ribonucleotide reduction share a common feature:[3,4] That is, they are intramolecular

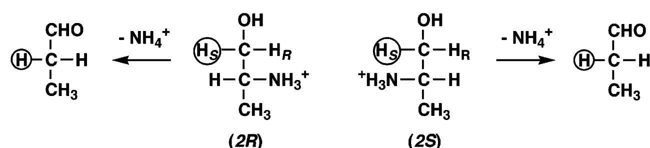


group-transfer reactions in which a hydrogen atom migrates from one carbon atom of the substrate to an adjacent carbon atom in exchange for group X that moves in the opposite direction. In the diol dehydratase reaction, X is the OH group on C2, and a water molecule is subsequently eliminated from a *gem*-diol. This common feature was first shown with diol dehydratase. The stereochemistry of the diol dehydratase reaction established by the labeling experiments of Abeles's and Rétey's groups are summarized as follows:



It was shown that the initial migration of an OH group from C2 to C1 is stereospecific, and the dehydration of a *gem*-diol undergoes steric control by the enzyme, with only one of the two OH groups on the prochiral center being eliminated.[76] [^{18}O]- and unlabeled propionaldehydes are formed from [1-^{18}O]-(*S*)- and [1-^{18}O]-(*R*)-1,2-propanediols, respectively. The hydrogen atom moves to the adjacent carbon atom without exchange with solvent protons.[77] Abeles and co-workers demonstrated that the *pro-S* and *pro-R* hydrogen atoms on C1 of (*S*)- and (*R*)-1,2-propanediols, respectively, migrate to C2.[78] The migrating OH group is replaced by the hydrogen atom with an accompanying inversion of the configuration at C2.[74,78] In contrast, ethylene glycols stereospecifically labeled with deuterium and tritium are converted to acetaldehyde with racemization, suggesting rapid internal rotation in the product radical with this substrate before hydrogen back-abstraction.[79]

The conversion of ethanolamine to acetaldehyde by ethanolamine ammonia-lyase takes place with the transfer of hydrogen from the carbinol carbon to the amino carbon without exchange with water.[80] The oxygen atom of the substrate is retained in the product as the carbonyl oxygen. It remains unknown whether the amino group is directly eliminated from C2, or migrates to C1 and is then eliminated. (*S*)-2-Aminopropanol is deaminated by the enzyme with the retention of configuration at C2, whereas (*R*)-2-aminopropanol reacts with its inversion:[81]



Tritium from [5′-^{3}H]AdoCbl is transferred to the 2-*pro-S* position of propionaldehyde and to the 1-*pro-S* position of 2-aminopropanol. These results can be explained by assuming that the rotameric intermediate formed from the (*S*)-enantiomer is favored in equilibration. The racemization during the deamination of much better substrate ethanolamine[82] is explained by the torsion symmetry of the trigonal intermediates arising from this substrate. (*S*)-2-Aminopropanol shows lower K_m and higher V_{max} values than the (*R*)-enantiomer.[83] The rate-limiting step for the former is the same as that for ethanolamine, namely, the transfer of hydrogen from the cofactor to the product, while there are two rate-limiting steps with the latter.

Abeles and co-workers demonstrated that the enzyme-bound AdoCbl serves as an intermediate hydrogen carrier, first accepting a hydrogen atom from C1 of the substrate to C5′ of the coenzyme and then, in a subsequent step, giving a hydrogen back to C2 of the product.[84−87] Intramolecular and intermolecular hydrogen transfers are difined as the transfers from C1 of a substrate molecule to C2 of the same and different molecules, respectively. If the intermediate hydrogen carrier binds only one hydrogen atom, hydrogen transfer must be intramolecular. If the number of hydrogens accepted is more than two, intermolecular hydrogen transfer might be also possible. It was established experimentally that hydrogen transfer is not necessarily intramolecular but occurs by an intermolecular process as well through an enzyme-bound intermediate in which the hydrogen abstracted from the substrate and the two hydrogens on C5′ of AdoCbl all become equivalent.[84,88,89] From these results, 5′-deoxyadenosine was postulated to be an intermediate.

The formation of cob(II)alamin and an organic radical intermediate during catalysis was observed with diol dehydratase,[90−94] glycerol dehydratase,[95] and ethanolamine ammonia-lyase[96−98] by optical and electron paramagnetic resonance (EPR) spectroscopies. The high-field doublet and the low-field broad signals of EPR spectra observed with either of the three

enzymes were assigned to an organic radical and low-spin Co(II) of cob(II)alamin, respectively. The doublet signal was interpreted to arise from a weak interaction of the organic radical with Co(II).[99,100] The formation of an organic radical intermediate at a kinetically competent rate was demonstrated.[92] EPR spectroscopic determination showed that spin concentrations of the organic radical and Co(II) in the diol dehydratase reaction are 0.58 and 0.50 mol/mol of active site.[94] Optical spectra indicate that approximately 77% and 90% of the active sites contain cob(II)alamin during the steady state of the reactions of diol dehydratase with 1,2-propanediol[94] and of ethanolamine ammonia-lyase with (*S*)-2-aminopropanol,[68] respectively. These results undoubtedly indicate that these reactions proceed by a radical mechanism. The kinetic experiments with labeled substrates and AdoCbl, together with spectral and EPR measurements, established a minimal mechanism for diol dehydratase[5,6,13,94] and ethanolamine ammonia-lyase[7,21] (Figure 2). The salient features are

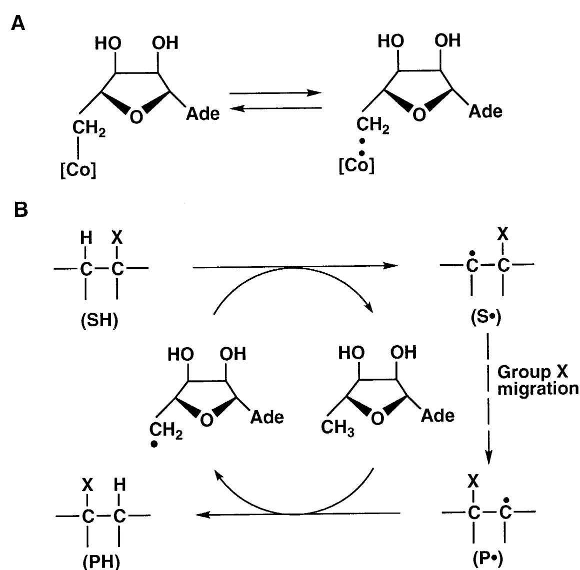


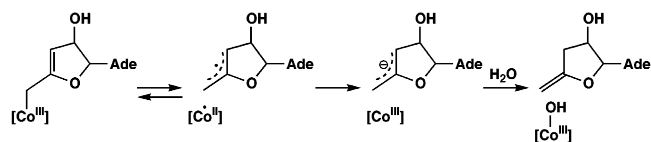
Figure 2. Minimal mechanism of AdoCbl-dependent rearrangements. (A) Homolysis of the Co−C bond of enzyme-bound AdoCbl. (B) Adenosyl radical-catalyzed rearrangements. [Co], cobalamin; X, a generic migrating group.

as follows. The interaction between the apoenzyme and the coenzyme leads to the activation of the Co−C bond of the coenzyme for homolytic cleavage, 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 holoenzyme 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 back from 5′-deoxyadenosine. This leads to the formation of the final product and regeneration of the coenzyme. This minimal mechanism seemed beautiful, but the essential details of the reaction mechanism remained largely unclear until recently.

Formation of radical intermediates in the reactions catalyzed by methylmalonyl-CoA mutase,[101−105] 2-methyleneglutarate mutase,[106,107] and glutamate mutase[107−110] was also shown by EPR. The signal is postulated to represent a strongly coupled cob(II)-alamin-organic radical pair. EPR spectroscopy of AdoCbl-dependent enzymes has been extensively reviewed in 1982 by Pilbrow[100] and more recently by Gerfen.[111] It is thus evident that all the AdoCbl-dependent rearrangements proceed by the common radical mechanism shown in Figure 2.[3,4]

B. Structure−Function Studies of Coenzyme and Mode of Cobalamin Binding

The structure−function relationship of AdoCbl was most extensively studied with diol dehydratase (for reviews, see refs 13−16). A lot of coenzyme analogues in which one of the structural components of the coenzyme is substituted by a closely related group were synthesized and tested for coenzymic activity and binding affinity for the enzyme. The essential roles of each component of the coenzyme in diol dehydratase can be briefly summarized as follows. The corrin ring including the peripheral amide side chains[94] and the adenine ring of the adenosyl group[93,112,113] are required for tight binding to the apoenzyme, and the ribosyl moiety of the adenosyl group is required for transmitting strains to the Co−C bond.[114] Thus, it was concluded that these components are indispensable for Co−C bond activation, i.e., catalytic radical formation, upon binding of AdoCbl to the apoenzyme.[14,15] The presence of adenosyl group-binding sites in the apoenzyme was demonstrated biochemically with diol dehydratase.[115] The phosphate group in the nucleotide moiety is necessary for the tight binding to the apoenzyme,[116] the ribose moiety as a spacer,[117] and the 5,6-dimethylbenzimidazole (DBI) moiety for controlling the reactivity of the cobalt atom.[116−119] In conclusion, the nucleotide moiety is important for the continuous progress of a catalytic cycle, i.e., for catalytic turnovers or control of radicals.[15,17] Recently, the mechanism-based inactivation of diol dehydratase by 3′,4′-anhydroAdoCbl has been reported.[44] Even under anaerobic conditions, cob(III)alamin and 3′-deoxy-4′,5′-anhydroadenosine are formed, indicating that the inactivation is caused by one electron transfer from cob(II)alamin to the allylic radical intermediate:



The coenzyme analogues having imidazole or pyridine as a Co-coordinating base cause mechanism-based inactivation of diol dehydratase[116−119] and ethanolamine ammonia-lyase,[119] forming Co(II)-containing species in the inactivation. Thus, $[^{15}N_2]$-imidazolyl analogues of the coenzyme is a useful probe to identify the Co-coordinating base in AdoCbl-dependent enzymes.[12,120,121] With the unlabeled imi-

dazolyl analogue and diol dehydratase, each line of the hyperfine octet due to the interaction of the unpaired spin of Co(II) with the cobalt nucleus ($I = {}^7/_2$) (coupling constant, 10.7 mT) showed superhyperfine splitting into triplets due to interaction with the ^{14}N nucleus ($I = 1$) of a nitrogenous base coordinating to Co(II) in the lower axial position (coupling constant, 2.0 mT) (Figure 3). Essentially the same

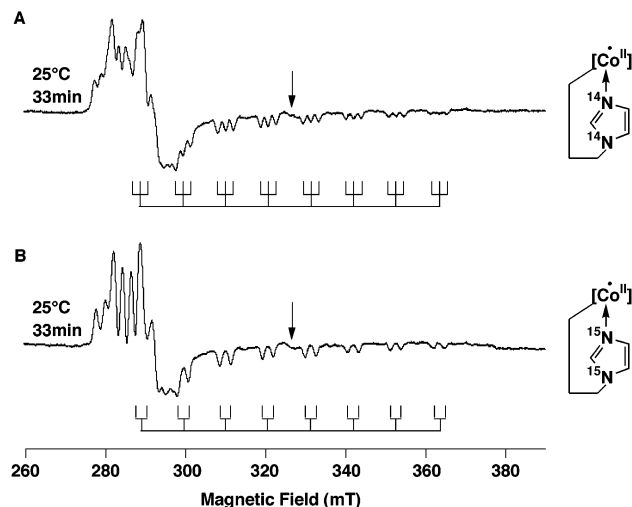


Figure 3. EPR spectra of the diol dehydratase-bound cob(II)inamide imidazolylpropyl phosphate. (A) Unlabeled, (B) [*imidazole*-$^{15}N_2$]-labeled. The Co(II)-containing species were formed by mechanism-based inactivation with 2-methyl-1,2-propanediol. Measured at 143 K. The arrows correspond to $g = 2.0$.

spectrum was obtained in the combination of unlabeled imidazolyl analogue and ^{15}N-labeled apoenzyme. With the [*imidazole*-$^{15}N_2$]-labeled analogue and unlabeled apoenzyme, the hyperfine lines (coupling constant, 10.7 mT) showed superhyperfine splitting into doublets (coupling constant, 2.7 mT). The ratio of the coupling constant with the ^{14}N nucleus (A_{14N}) to that with the ^{15}N nucleus (A_{15N}) was 0.741, which is in good agreement with the theoretical value that can be calculated as follows:

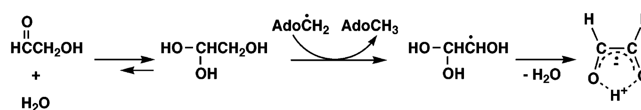
$$A_{14N}/A_{15N} = \gamma_{14N}/\gamma_{15N} = 0.713 \text{ (theoretical)}$$

where γ is a gyromagnetic ratio. Essentially the same results were obtained with glycerol dehydratase[12] and ethanolamine ammonia-lyase[73] as well. Thus, it is evident that these enzymes bind cobalamin in the so-called "base-on" mode—that is, with DBI coordinating to the cobalt atom. Using [*5,6-dimethylbenzimidazole*-$^{15}N_1$]AdoCbl, Rétey and co-workers reached the same conclusions with diol dehydratase,[122] ethanolamine ammonia-lyase,[123] and class II ribonucleotide reductase.[124] In ethanolamine ammonia-lyase, it was demonstrated by X-band electron spin−echo envelope modulation (ESEEM) spectroscopy that cob(II)alamin is a pentacoordinate, α-axial-liganded complex during catalysis.[125] In contrast, it was demonstrated by EPR that methionine synthase,[126] methylmalonyl-CoA mutase,[127] and glutamate mutase[128] bind cobalamin in the "base-off/His-on" mode—that is, the DBI ligand is displaced from the cobalt atom, and instead, the imidazole group of the His residue in the part of cobalamin-binding motif (Asp-X-His-X-X-Gly) is ligated to the cobalt atom. This motif is conserved in all the enzymes catalyzing carbon-skeleton rearrangements,[129−132] methionine synthase,[133] and lysine 5,6-aminomutase,[134] but not in the enzymes catalyzing isomerization (eliminating) reactions[27,51,71] and class II ribonucleotide reductase.[135] It is thus widely accepted that there are two types of B_{12}-proteins, i.e., base-on and base-off/His-on proteins, whose ancestors are probably different.[17]

Recently, the details of the enzyme−cobalamin interactions (methionine synthase,[126] methylmalonyl-CoA mutase,[8] diol dehydratase,[9] glutamate mutase,[10] class II ribonucleotide reductase,[11] and glycerol dehydratase[12]) as well as the enzyme−adenine moiety interaction (methylmalonyl-CoA mutase,[136,137] diol dehydratase,[138] and glutamate mutase[139]) were revealed by X-ray structures. The apparent inconsistency between diol and glycerol dehydratases and methylmalonyl-CoA mutase in the requirements of coenzyme activity for base coordination[140,141] can be explained from their differences in the mode of cobalamin binding.[8,9] In methylmalonyl-CoA mutase, AdoCbi-GDP is 25% as active as AdoCbl as a cofactor, although it reconstitutes the holoenzyme in the base-off/His-off form.[142] This result indicates that the lower axial ligand apparently plays a minor role in the methylmalonyl-CoA mutase reaction. Strong inhibition by ω-(9-adeninyl)alkylcobalamins was observed with ribonucleotide reductase,[143] diol dehydratase,[93] and ethanolamine ammonia-lyase.[144] Among [ω-(adenosin-5′-*O*-yl) alkyl]cobalamins that mimic the post homolysis intermediate state of AdoCbl, C5 and C6 analogues showed the strongest inhibition for diol dehydratase[54] and methylmalonyl-CoA mutase,[145] respectively. 2′-DeoxyAdoCbl showed 1−2% of activity of AdoCbl for methylmalonyl-CoA mutase.[146] The AdoCbl analogues with an α-adenosyl group[147] and with an imidazole group in place of DBI[148] were reported to be 9.7% and 100%, respectively, as active as that of AdoCbl for ribonucleotide reductase of *Lactobacillus leichmannii*.

C. Inactivation by Substrate Analogues

It has been known for a long time that diol dehydratase undergoes suicide inactivation by glycolaldehyde[149] and chloroacetaldehyde.[150] The inactivation is accompanied by irreversible formation of cob(II)alamin and 5′-deoxyadenosine. Recently, the product from glycolaldehyde was identified by EPR as the *cis*-ethanesemidione anion radical.[151] Formation of the same radical is observed by EPR when ethanolamine ammonia-lyase is treated with glycolaldehyde as well.[151] The mechanism-based inactivation of both enzymes by glycoladedehyde is thus postulated to result from formation of this stable radical, which cannot react further to abstract a hydrogen atom back from 5′-deoxyadenosine:



The distance between the radical and Co(II) in both enzymes was estimated to be ~11 Å by the analysis of electron spin–spin coupling between them.

Ethanolamine ammonia-lyase is inactivated by incubation of holoenzyme with aminoacetaldehyde[152] and ethylene glycol.[153] Inactivation of the holoenzyme by the latter forms 5′-deoxyadenosine, acetaldehyde, and an unidentified corrinoid, although the mechanism of inactivation is not yet clear. Some N-substituted ethanolamine analogues also serve as inhibitors or inactivators for the enzyme.[154] The enzyme undergoes mechanism-based inactivation by hydroxyethylhydrazine.[67,155] Equimolar quantities of 5′-deoxyadenosine, cob(II)alamin, hydrazine cation radical, and acetaldehyde are products of the inactivation:

$$H_2N-\overset{+}{N}H_2-CH_2CH_2OH \xrightarrow{Ado\dot{C}H_2 \to AdoCH_3} H_2N-\overset{+}{N}H_2-CH_2\dot{C}HOH$$

$$\longrightarrow H_2N\overset{+}{\dot{-}}NH_2 \quad CH_2=CH-OH \ (\downarrow CH_3CHO)$$

Removal of cob(II)alamin from the protein by acid ammonium sulfate fractionation restores the enzyme activity. Hydrazine cation radical is a stable radical. Thus, the mechanism-based inactivation results from irreversible Co-C bond cleavage of the cofactor and the tight binding of cob(II)alamin to the active site.

Lysine 5,6-aminomutase undergoes substrate-dependent mechanism-based inactivation in the presence of AdoCbl and PLP, forming cob(III)alamin and 5′-deoxyadenosine.[156] This inactivation is postulated to be caused by electron transfer from cob(II)alamin to some intermediates. A novel type of inactivation of glutamate mutase by 2-methyleneglutarate has recently been reported that involves the addition of the adenosyl radical to the *exo*-methylene group forming a tertiary carbon radical at C2.[157]

D. Magnetic Field Effects

Harkins and Grissom reported magnetic field effects on ethanolamine ammonia-lyase.[158] The V_{max}/K_m value was reduced by 25% by applying the magnetic field around 100 mT magnetic flux density. Magnetic field-dependent step was reported to be recombination of a transient spin-correlated radical pair that is formed in the reaction cycle of the enzyme.[159] However, no significant magnetic field effects were observed with either methylmalonyl-CoA mutase[160] or diol dehydratase (personal communication). They might be observed only when a set of fairly stringent requirements is satisfied.[159]

E. Identification of Organic Radical Intermediates

The radical intermediates present during steady-state turnover of substrates by ethanolamine ammonia-lyase were characterized by EPR.[161] The line shape is characteristic of a relatively weakly electron spin-coupled Co(II)-organic radical system. By using deuterated and ^{13}C-labeled substrates, the radical intermediates present during steady-state turnovers of 2-aminopropanol and ethanolamine were identified as the C1-centered substrate radical[98,162,163]

$$CH_3\underset{NH_3^+}{CH}CH_2OH \longrightarrow CH_3\underset{NH_3^+}{CH}\dot{C}HOH$$

and the C2-centered product radical,[161]

$$^+H_3NCH_2CH_2OH \longrightarrow {}^+H_3NCH_2\dot{C}HOH \longrightarrow \cdot CH_2CH\begin{matrix}OH\\NH_3^+\end{matrix} \text{ or } \cdot CH_2CHO$$

respectively. This difference was explained in terms of the contribution of a product radical trap for the radical-pair stabilization with ethanolamine as substrate.[161] Since the deamination of 2-aminopropnaol is reversible,[164] it is considered that there is no contribution of such product-radical trap with this substrate. The mechanism of propagation of the radical center among the cofactor, substrate, and product in the ethanolamine ammonia-lyase reaction was probed by pulsed electron nuclear double resonance (ENDOR) spectroscopy.[162] The amino group on C2 of the radical from (*S*)-2-aminopropanol is approximately eclipsed with respect to the half-occupied *p* orbital at C1. This radical was used in ENDOR experiments. From the dipole–dipole coupling, the distance between C1 of the radical and C5′ of the labeled cofactor component was estimated to be 3.4 ± 0.2 Å. It was thus suggested that C5′ of the adenosyl radical moves ~7 Å from its position as part of AdoCbl to a position where it is in contact with C1 of the substrate which lies ~10–12 Å[163,165,166] from the Co(II) of cob(II)alamin. It was also demonstrated by X-band two-pulse ESEEM spectroscopy that the unpaired electron on C1 of the substrate radical and C5′ are separated by 3.2 Å, suggesting that C5′ of the adenosyl radical directly mediates radical migration between cobalt in cobalamin and the substrate/product site over a distance of 5–7 Å in the active site of this enzyme.[167] These estimations of movement of C5′ during catalysis are based on the assumption that C1, C5′, and Co(II) are collinear. Geometry of reactant centers determined by using orientation-selection-ESEEM spectroscopy demonstrated that this is true—namely, C5′ is located close to the Co-(II)–C1 axis (7.8 Å from Co(II) and 3.3 Å from C1).[166] This conclusion is consistent with the ribosyl rotation model that we proposed from the three-dimensional structure of the diol dehydratase–adeninylpentylcobalamin (AdePeCbl, Figure 1E, $n = 5$) complex.[138]

In the AdoCbl-dependent glutamate mutase reaction, EPR-observed radical was identified in a similar way as the 4-glutamyl radical that interacts with Co-(II) from the distance of ca. 6.6 Å:[110]

$$^-O_2CCH_2CH_2\underset{NH_3^+}{CH}CO_2^- \longrightarrow {}^-O_2C\dot{C}HCH_2\underset{NH_3^+}{CH}CO_2^-$$

The radical formed by glutamate mutase from L-2-hydroxyglutarate, a very poor substrate, was iden-

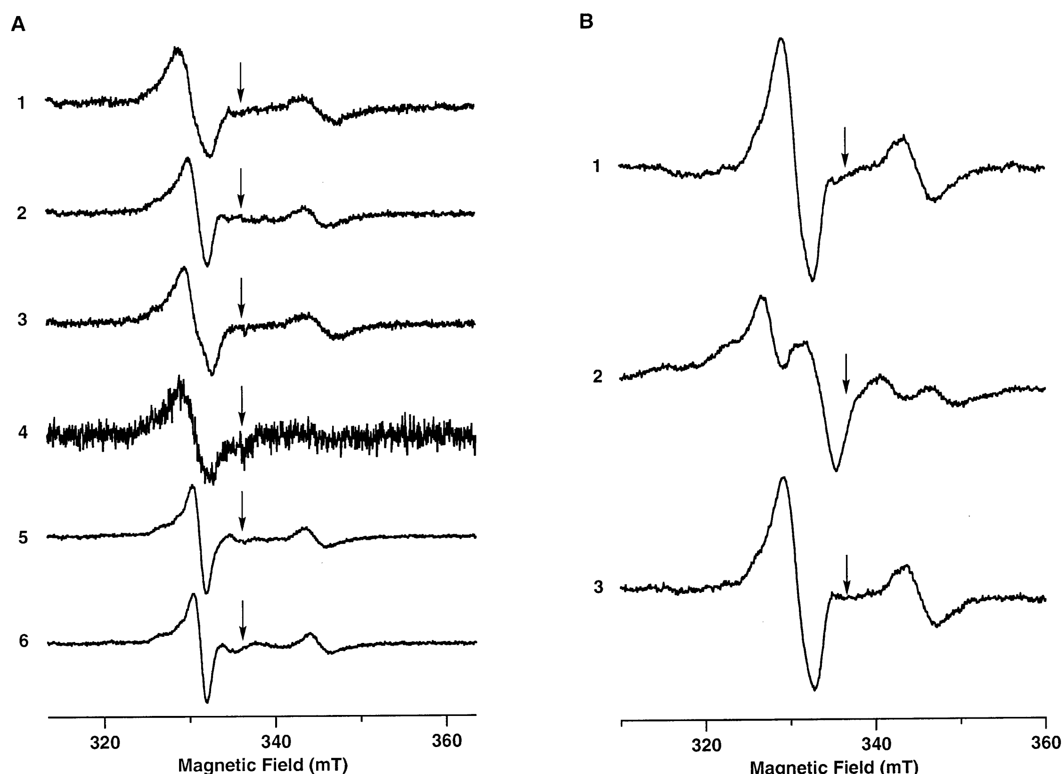
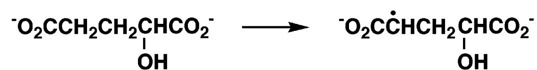
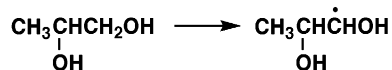


Figure 4. The doublet signals in the EPR spectra of reacting holodiol dehydratase with isotopically labeled substrates. X-band (9.4 GHz): 145K. (A) Unlabeled (1), 1,1-dideuterated (2), 2-deuterated (3), 3,3,3-trideuterated (4), 1,1,2-trideuterated (5), and 1,1,2,3,3,3-hexadeuterated (6), 1,2-propanediols. (B) Unlabeled (1), $1-^{13}C$-labeled (2), and $2-^{13}C$-labeled (3) 1,2-propanediols. The arrows correspond to $g = 2.0$. (Reprinted with permission from ref 19. Copyright 2002 The Japan Chemical Journal Forum and Wiley Periodicals, Inc.)

tified as its C4-centered radical:[168]



The formation of a radical pair consisting of Co(II) of cob(II)alamin and an organic radical intermediate during catalysis of diol dehydratase[91,92] and glycerol dehydratase[95] was observed by EPR. The high-field doublet and the low-field broad signals arise from a weak interaction of an organic radical with low-spin Co(II) of cob(II)alamin.[99,100] The distance between the organic radical and the Co(II) center, estimated from simulations including both exchange and dipolar coupling, is equal to or greater than 10 Å.[165] Recently, several deuterated and ^{13}C-labeled 1,2-propanediols were synthesized, and the EPR spectra were measured using them as substrates.[169] Among the spectra of reacting holoenzyme with them, significant line-width narrowing of the doublet signal was observed with the substrates deuterated on C1 (Figure 4). A distinct change in the hyperfine splitting was seen with [1-^{13}C]1,2-propanediol. Thus, the organic radical intermediate observed by EPR was identified as the C1-centered substrate-derived radical:



F. Isotope Effects

The deuterium kinetic isotope effect (KIE) (k_H/k_D) on the overall diol dehydratase reaction is 10,[78] indicating that breaking of the C−H bond is rate determining in the reaction. 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.[88] This unusually large isotope effect, corresponding to a k_H/k_D of 28, for the latter step suggests the involvement of hydrogen tunneling in the reaction. However, the exact reason for this large effect remains unclear at present.

In ethanolamine ammonia-lyase, ^3H at 5′-CH_2 of AdoCbl is discriminated against by a factor of ~100 in transfer to product.[170] This tritium isotope effect is much larger than that expected from the deuterium KIE on the overall reaction (~7). A paper appeared that reports some experimental data that show the presence of a second acceptor at the active site[171] and a rationalization of these exceedingly large KIEs. Hydrogens on this acceptor do not exchange with water during the course of the reaction, but are released to the solvent when the enzyme is denatured. However, no supporting data for such solvent-exchangeable pool of ^3H in ethanolamine ammonia-lyase have been reported after that. Recent results obtained reinforced the concept that the adenosyl radical is the species that abstracts hydrogen atoms from the substrate in ethanolamine ammonia-lyase.[155] The kinetics of reformation of the Co−C bond in the final phase of the reaction has also been examined in the ethanolamine ammonia-lyase reaction.[68] With unlabeled substrates, the rate of the Co−C reformation is independent of the number of substrate molecules turned over in the steady-state phase, but substrate deuterium KIEs were observed with deu-

Table 1. Deuterium Kinetic Isotope Effects on the Rates of Co−C Bond Homolysis and Overall Reactions

substrate	$k_h{}^a \times 10^{-2}$ at 4°C (s^{-1})	k_H/k_D	k_{cat} at 37°C (s^{-1})	k_H/k_D	ref
diol dehydratase (*K. oxytoca*)					179
1,2-propanediol	8.0 ± 1.0		366		
[1,1-D_2]1,2-propanediol	2.4 ± 0.3	3−4		10	
1,2-ethanediol	9.5 ± 3		220		
[1,1,2,2-D_4]1,2-ethanediol	2.4 ± 1	3−6			
ethanolamine ammonia-lyase[b,c] (*S. typhimurium*)					68
ethanolamine	>3.00		80 ± 2		
[1,1-D_2]Ethanolamine	0.29 ± 0.01	>10	15.6 ± 1.4	5.1 ± 0.5	
(*S*)-2-aminopropanol	0.74 ± 0.07		0.27 ± 0.03		
[1,1-D_2](*S*)-2-aminopropanol	0.24 ± 0.01	3.1 ± 0.3	0.054 ± 0.003	5.0 ± 0.6	

[a] k_h represents the rate constant for the Co−C bond homolysis. [b] k_{cat} values are calculated based on six active sites present in each $\alpha_6\beta_6$ oligomer.[67] [c] Temperature not given.

terated substrates and they are maximal after ∼2 turnovers. With 5′-deuterated coenzyme and deuterated substrate, the isotope effects on the Co−C bond reformation is independent of the number of substrate molecules that are turned over. These results indicate that the pool of exchangeable hydrogens in the holoenzyme is two—consistent with the numbers of hydrogens in the C5′ methylene group of AdoCbl.

In methylmalonyl-CoA mutase, the k_H/k_T for the hydrogen transfer from AdoCbl to substrate and product is 4.9, suggesting that hydrogen transfer is only partly rate-limiting.[172] The identical partitioning of tritium, regardless of the substrate used, shows that the rearrangement of the substrate radical into the product radical is not rate limiting. The involvement of protein radical intermediates in the methylmalonyl-CoA mutase-catalyzed rearrangement is very unlikely.[172] The possibility of a protein radical intermediate was examined with glutamate mutase as well, but the rate constants for the loss of tritium from the coenzyme and the appearance of tritium in substrate 3-methylaspartate are very similar, suggesting that a protein radical is unlikely.[173] The tritium isotope effect for the transfer of tritium from the coenzyme to product are 21 for the formation of glutamate and 19 for the formation of methylaspartate.[174]

G. Coupling of the Co−C Bond Cleavage to Hydrogen Abstraction

Although the adenosyl radical is presumed to be a catalytic radical in AdoCbl-dependent reactions, its intermediary formation has never been directly demonstrated in any AdoCbl-dependent enzymatic reactions. Probably, this is because the half-life of this radical is too short to be detected, since it is a primary carbon radical. Therefore, the rate of its formation upon the Co−C bond cleavage is usually estimated from the rate of spectral changes of the enzyme-bound coenzyme. Substrate deuterium KIE on the Co−C cleavage rate was first shown with methylmalonyl-CoA mutase,[175] glutamate mutase,[176] and then with ethanolamine ammonia-lyase,[68] although k_H/k_D values vary from 3 to 35. Since substrate binding sites of these enzymes are far from the coenzyme Co−C bond, a direct concerted mechanism is unlikely. Thus, it is considered that the Co−C bond homolysis is kinetically coupled with hydrogen abstraction. In glutamate mutase, the rate of substrate radical formation was examined by measuring the apparent rate of 5′-deoxyadenosine formation and shown to be sufficiently fast for this step to be kinetically competent.[177] The rate was very similar to that measured for the Co−C bond of AdoCbl, in support of the kinetic coupling of its homolysis to substrate hydrogen abstraction.

The ribonucleotide reductase of *L. leichmanni* catalyzes the exchange of tritium from [5-T]AdoCbl with solvent. Cob(II)alamin is formed in this exchange reaction. When the rate constants for Co−C bond homolysis were measured in D_2O, with [5′-D_2]-AdoCbl in H_2O, and with [5′-D_2]AdoCbl in D_2O, solvent, cofactor, and combined solvent and cofactor KIEs were 1.6, 1.7, and 2.7, respectively.[178] These results suggest that the enzyme catalyzes Co−C bond homolysis in a concerted fashion. The k_{cat} values measured with *S. typhimurium* ethanolamine ammonia-lyase for unlabeled and deuterated ethanolamines and (*S*)-2-aminopropanol[68] are listed in Table 1. Deuterium KIEs observed for overall reactions are similar to those reported for these substrates with the clostridial enzyme. Substrate deuterium KIEs on the rate of the Co−C bond homolysis (k_h) could be accounted for if the homolysis of Co−C bond is concerted or coupled to abstraction of a hydrogen atom from the substrate. EPR spectra indicate that the substrate radical is located ∼12 Å away from Co-(II) of cob(II)alamin in ethanolamine ammonia-lyase.[162] Since this distance appears to be incompatible with a direct concerted reaction, kinetic coupling is much more likely.

The rate of cob(II)alamin formation in diol dehydratase was determined by stopped-flow analysis with 1,2-propanediol and 1,2-ethanediol as substrates at 4 °C (Table 1).[179] It should be noted that the Co−C bond undergoes homolysis at a rate much faster than the catalytic turnover rates with these substrates, suggesting that the Co−C bond homolysis is not a rate-determining step for the overall reaction. The values of deuterium KIE for the Co−C bond homolysis indicate that the Co−C bond cleavage is coupled with the hydrogen abstraction from C1 of the substrate and that the hydrogen abstraction is partially rate-determining for the Co−C bond homolysis. The X-ray structure revealed that a direct concerted mechanism is of course not possible in diol dehy-

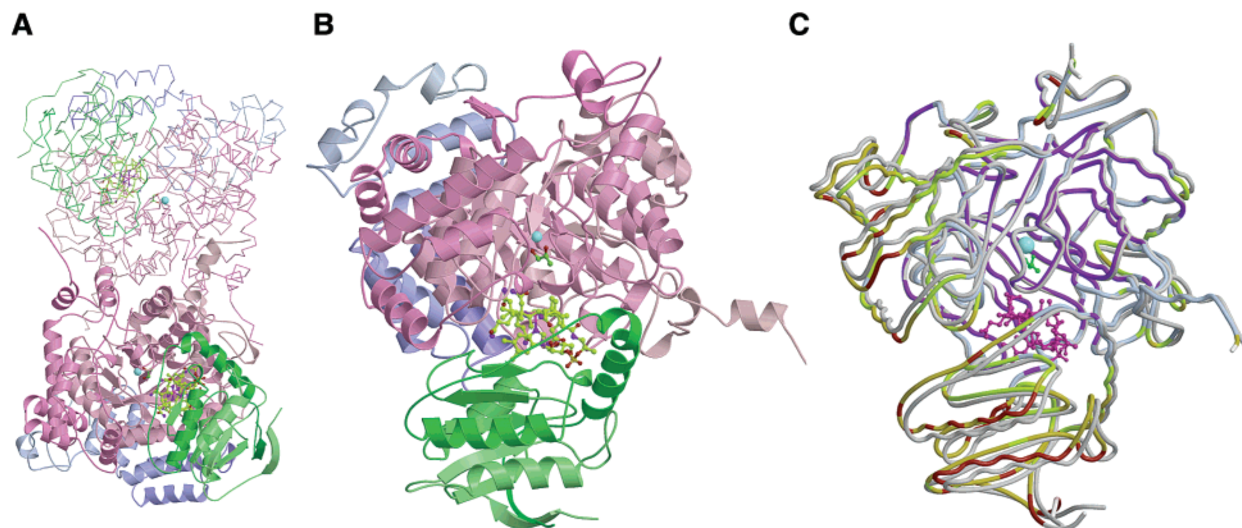


Figure 5. Crystal structures of diol dehydratase and glycerol dehydratase. (A) Overall structure of diol dehydratase $(\alpha\beta\gamma)_2$. (B) Heterotrimer unit $\alpha\beta\gamma$. Pink, green, and blue colors indicate the α, β, and γ subunits, respectively, darkening continuously from the N-terminal to the C-terminal sides; cyan for K^+. (C) The Cα traces of the $\alpha\beta\gamma$ heterotrimer unit of glycerol dehydratase. The corresponding traces of diol dehydratase are drawn in gray. Cobalamin, 1,2-propanediol, and K^+ are shown as ball-and-stick models. Drawn with MOLSCRIPT[285] and RASTER3D.[286]

dratase. Thus, it can be concluded that the Co−C bond homolysis is kinetically coupled with the hydrogen abstraction from the substrate.

In the absence of substrate, less than 1% of the diol dehydratase-bound coenzyme is in the dissociated form.[169] The addition of substrates to holoenzyme increases the steady-state concentrations of cob(II)-alamin and an organic radical intermediate to 50−60%.[94] In ethanolamine ammonia-lyase, the steady-state concentration of cob(II)alamin reaches at least 90%.[68] These results suggest that the equilibrium of the Co−C homolysis is shifted to the direction of dissociation by its thermodynamic coupling with hydrogen abstraction from substrates.

IV. Structural Studies

A. Overall Structures and Mode of Cobalamin Binding

Diol dehydratase of *K. oxytoca* purified from overexpressing *E. coli* was crystallized as the complex with cyanocobalamin (CN-Cbl, Figure 1C) and racemic 1,2-propanediol,[180] and its crystal structure was solved using the technique of multiple isomorphous replacement by Yasuoka and co-workers.[9] Figure 5A shows the overall structure of the complex viewed from a noncrystallographic 2-fold axis. The enzyme exists as dimer $(\alpha\beta\gamma)_2$ of the heterotrimer consisting of the α, β, and γ subunits. Two α subunits form dimer α_2 to which two β and two γ subunits are bound separately. Figure 5B depicts the structure of the $\alpha\beta\gamma$ heterotrimer unit. Cobalamin is bound between the α and β subunits in the base-on mode, that is, with DBI coordinating to the cobalt atom. The upper ligand of cobalamin is oriented in the direction of the α subunit, whereas the lower nucleotide ligand interacts with the β subunit. This is the first crystallographic indication of the base-on mode of cobalamin binding and support the conclusion obtained by EPR.[120−122] The substrate bound was assigned to the (*S*)-1,2-propanediol.[9]

Very recently, the crystal structure of the glycerol dehydratase·cobalamin complex was reported.[12] This enzyme is isofunctional with diol dehydratase, and its overall structure is very similar to that of diol dehydratase (Figure 5C), although the identities of it's α, β, and γ subunits with those of diol dehydratase are 71%, 58%, and 54%, respectively.[51] Although the complex was crystallized in the presence of racemic 1,2-propanediol, the electron density map of the substrate bound in the active site was assigned to the (*R*)-enantiomer.[12]

The recently reported crystal structures of class II ribonucleotide reductase[11] and glycerol dehydratase[12] are also indicative of the base-on mode of cobalamin binding to these enzymes, as expected from the EPR studies.[12,124] However, class II ribonucleotide reductase contains the cobalamin-binding region that shares no structural similarity with those of diol and glycerol dehydratases, although it uses similar chemistry for catalysis. It is more similar to related structural elements in class I ribonucleotide reductase.[181] On the other hand, the crystal structures also revealed that methionine synthase,[126] methylmalonyl-CoA mutase,[8] and glutamate mutase[10] are base-off/His-on proteins whose folding is quite different from that of the base-on enzymes. In glutamate mutase, it was shown by NMR spectroscopic methods that the B_{12}-binding subunit (MutS)[182] is structured in such a way, as to be able to trap the nucleotide segment of the base-off form of AdoCbl.[183] In contrast to the partial activity of AdoCbi-GDP which binds to methylmalonyl-CoA mutase in the base-off/His-off form,[142] His610 mutants of the enzyme are essentially inactive, suggesting that His610 may play a role in organizing a high-affinity cofactor-binding site or in catalyzing the displacement of DBI.[184] The base-off/His-on proteins share the consensus sequence "Asp-X-His-X-X-Gly", which constitutes a part of the cobalamin-binding motif containing the Co-coordinating His residue, whereas the base-on pro-

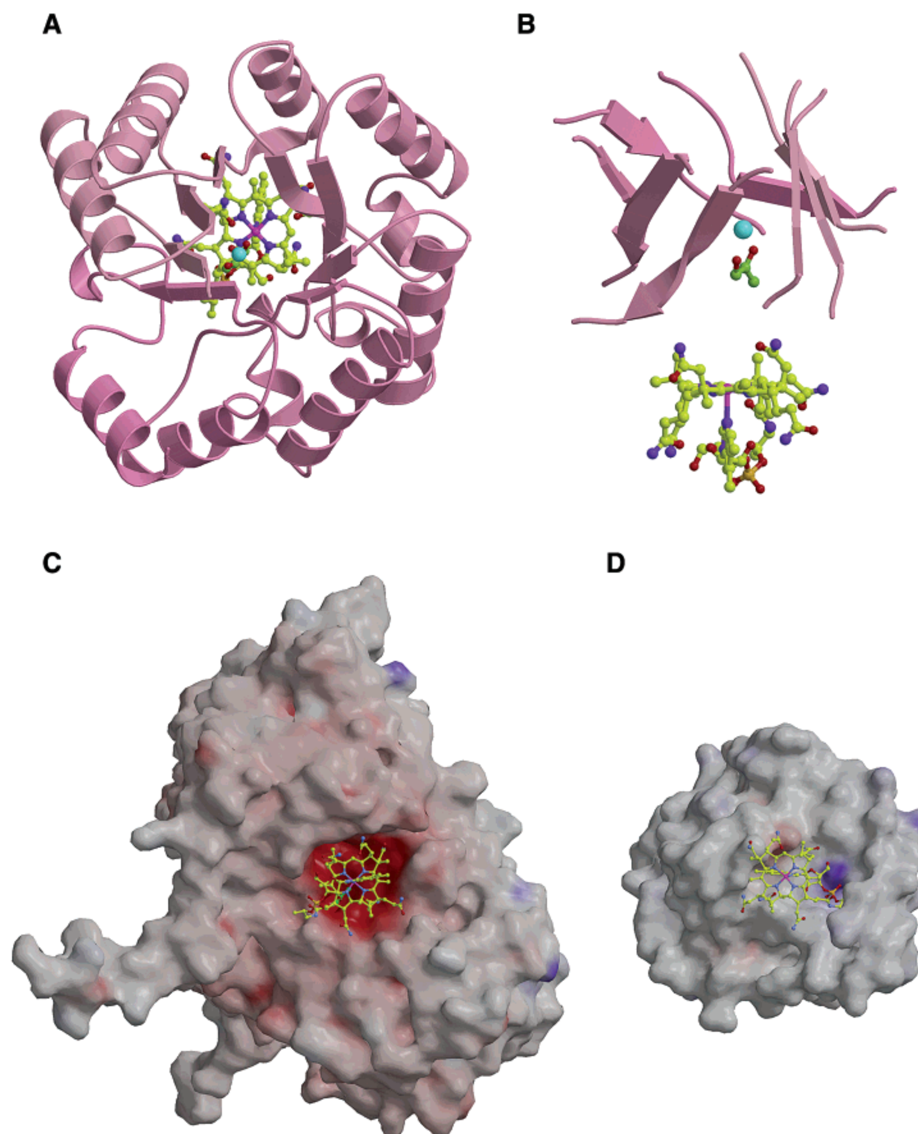


Figure 6. Active site of diol dehydratase in a $(\beta/\alpha)_8$ barrel and surface representations with electrostatic potential distributions. (A) $(\alpha/\beta)_8$ barrel (TIM barrel). (B) Side view of the active site in the TIM barrel. The barrel is colored in pink, darkening continuously from the N-terminal to the C-terminal side. Carbon atoms and bonds in the substrate and K^+ are shown in yellow-green and cyan, respectively. (C) α subunit viewed from the β subunit. (D) β subunit viewed from the α subunit. C and D were drawn with GRASP [287] and RASTER3D.[286] Red and blue areas show regions with negative and positive charges, respectively. The carbon atoms and bonds of cobalamin and 1,2-propanediol are colored in yellow and green, respectively.

teins do not. Therefore, it can be considered that B_{12}-proteins are grouped at least three superfamilies whose ancestors are different.

B. Active Site and Conformation of Bound Cobalamins

If the N-terminal and C-terminal regions of the α subunit are deleted and only the central region is shown for simplicity, a $(\beta/\alpha)_8$ barrel-like structure called TIM (triose phosphate isomerase) barrel emerges (Figure 6A,B).[9,138] The substrate propanediol and the essential cofactor K^+ are bound inside the barrel, indicating that the active site is in the cavity inside the TIM barrel. Figure 6C,D represents the molecular surfaces of the α and β subunits with electrostatic potential distribution. K^+ binds to the negatively charged inner part of the cavity and is directly coordinated by the two OH groups of the substrate. The corrin ring of the bound cobalamin covers the cavity to isolate the active site from the bulk of water, with the upper axial ligand directed toward the inside.[9,138] The active site of glycerol dehydratase is quite similar to that of diol dehydratase.[12] The active sites in TIM barrels have been reported with methylmalonyl-CoA mutase[8] and glutamate mutase[10] as well, although the reactions catalyzed are quite different. Such a characteristic architecture may be a common molecular apparatus for radical reactions catalyzed by AdoCbl-dependent enzymes because the active site inside the barrel would be suitable for protecting the reactive radical intermediates from side reactions with solvent molecules. Thus, spatial isolation of the radical intermediates in the active-site cavity is the common strategy for the so-called “negative catalysis” of the Rétey’s concept.[185]

Table 2. Conformations of Free and Enzyme-Bound Cobalamins

enzyme	corrinoid	temperature (K)	upper axial ligand	axial Co−N (Å)	Co−N plane[a] (Å)	fold angle[b] (deg)	ref
free	cob(II)alamin	r.t.	−	2.16	+0.15	11.5	*c*
	CN-Cbl	88	CN	2.01	+0.02	14.1	*d*
	CN-(Im)Cba	88	CN	1.97	0.00	7.3	*d*
	OH-Cbl	100	OH	1.93	+0.05	17.5	*e*
	MeCbl	298	CH_3	2.19	+0.02	14.8	*f*
	AdoCbl	277	Ado	2.21	−0.01	10.0	196
	AdePrCbl	163	AdePr	2.21			197
diol dehydratase	cob(II)alamin[g]	277	−	2.50	−0.01	4.45	9
	CN-Cbl	100	CN	2.18	+0.07	2.92	138
	AdePeCbl	100	AdePe	2.22	+0.04	1.74	138
	CN-Cbl (-substrate)	100	CN	2.25		5.1	201
glycerol dehydratase	cob(II)alamin[g]	100	−	2.48		5.5	12
methylmalonyl-CoA mutase	cob(II)alamin[g]	95	−	2.50	+0.11	7.18	8
glutamate mutase	CN-Cbl	103	CN	2.29	+0.06	6.29	10
	Me-Cbl	103	CH_3	2.35	+0.07		
methionine synthase	Me-Cbl	277	CH_3	2.24	−0.29	3.18	126

[a] Distance from Co to mean plane of the pyrrole nitrogens. A positive distance means a displacement toward the lower axial ligand. [b] The fold angle along the Co−C10 line is defined as the dihedral angle between two linked conjugated planes in the corrin ring: C4−C6, C9, C10, N21, N22; and C10, C11, C14−16, N23, N24 (Kratky, C.; Kräutler, B. In *Chemistry and Biochemistry of B_{12}*; Banerjee, R., Ed.; John Wiley & Sons: New York, 1999; pp 9−41.) [c] Kräutler, B.; Keller, W.; Kratky, C. *J. Am. Chem. Soc.* **1989**, *111*, 8936−8939. [d] Kräutler, B.; Konrat, R.; Stupperich, E.; Färber, G.; Gruber, K.; Kratky, C. *Inorg. Chem.* **1994**, *33*, 4128−4139. [e] Kratky, C. et al., & Kräutler, B. *J. Am. Chem. Soc.* **1995**, *117*, 4654−4670. [f] Rossi, M.; Glusker, J. P.; Randaccio, L.; Summers, M. F.; Toscano, P. J.; Marzilli, L. G. *J. Am. Chem. Soc.* **1985**, *107*, 1729−1738. [g] Most part of the enzyme-bound CN-Cbl or AdoCbl was presumably reduced or decomposed to cob(II)alamin.

Although the enzyme was crystallized as the complex with CN-Cbl, no electron density corresponding to the CN group was observed when the structure was determined at 4 °C.[9] The electron density for the CN group was partly observed when data collection was made at cryogenic temperatures.[138] Thus, the structure determined at 4 °C was reassigned as that of the enzyme·cob(II)alamin complex. It was concluded that the Co−CN bond is cleaved by X-ray irradiation during data collection with diol dehydratase.[138,186] Kratky and co-workers also reported that free and glutamate mutase-bound CN-Cbl is reduced to cob(II)alamin by X-ray irradiation.[187] Table 2 summarizes the conformation of the enzyme-bound cobalamins so far reported. The corrin ring of bound cobalamin is almost flattened upon binding to enzymes. The Co−N(DBI) bond distances for cob(II)alamin and CN-Cbl are 2.50 and 2.18 Å, respectively. These are lengthened upon binding to the enzyme by 16% and 18%, respectively.

It has been shown by nonenzymic model studies that a long Co−N bond would preferentially destabilize Co(III) relative to Co(II) of cobalamin[188] and inhibit Co−C heterolysis as well as accelerate Co−C homolysis.[189] However, the Co−N bond of the enzyme-bound AdePeCbl is not elongated at all. Therefore, oscillation of DBI between the two positions might take place during catalysis, and the lengthening the Co−N bond upon binding of cob(II)alamin to apoenzyme might stabilize Co(II) of cobalamin and thus enables continuous progress of radical reactions. Supporting evidence for the latter possibility was provided by the finding that coenzyme analogues with no base or with less bulkier bases coordinating to the cobalt atom serve as effective mechanism-based inactivators for both diol dehydratase and ethanolamine ammonia-lyase.[116−119] In contrast, the rate of the Co−C bond homolysis is essentially unaffected by the bulkiness of the Co-coordinating base for diol dehydratase, indicating that the labilization of the Co−C bond through a ground state mechanochemical triggering is not likely in diol dehydratase.[119] These conclusions were supported by recent theoretical calculations. Recent combined density functional theory (DFT) and molecular mechanics study showed that control of the $Co-N_{axial}$ bond length is important primarily to inhibit the Co−C bond heterolysis, but not important for Co−C bond homolysis.[190] The DFT study by another group showed that, in the homolytic cleavage leading to cob(II)-alamin, the dissociation process is essentially unaffected by changes in the position of the lower axial ligand, although the heterolytic cleavage leading to cob(I)alamin and cob(III)alamin is heavily favored by long and short $Co-N_{axial}$ distances, respectively.[191]

The distances between the cobalt atom and C1 and C2 of 1,2-propanediol are 8.37 and 9.03 Å, respectively, which agree reasonably well with that predicted from the EPR spectra.[165] Figure 7A shows the interaction of the active-site amino acid residues with the substrate and K^+. O1 is hydrogen-bonded to $-COO^-$ of Gluα170 and Nϵ2 of Glnα296, and O2 to $-COO^-$ of Aspα335 and Nϵ2 of Hisα143. K^+ is 11.7 Å apart from the cobalt atom and hepta-coordinated by the two hydroxyls of the substrate and the five oxygen atoms from the five active-site residues. Besides coordination to K^+, the O2 and O1 atoms of 1,2-propanediol are connected by hydrogen bonds to respective two amino acid residues. The direct ion−dipole interactions between K^+ and the substrate is the most striking feature and novel among all of the protein structures so far reported. Quite similar interactions between the substrate and the active-site residues are present in glycerol dehydratase as

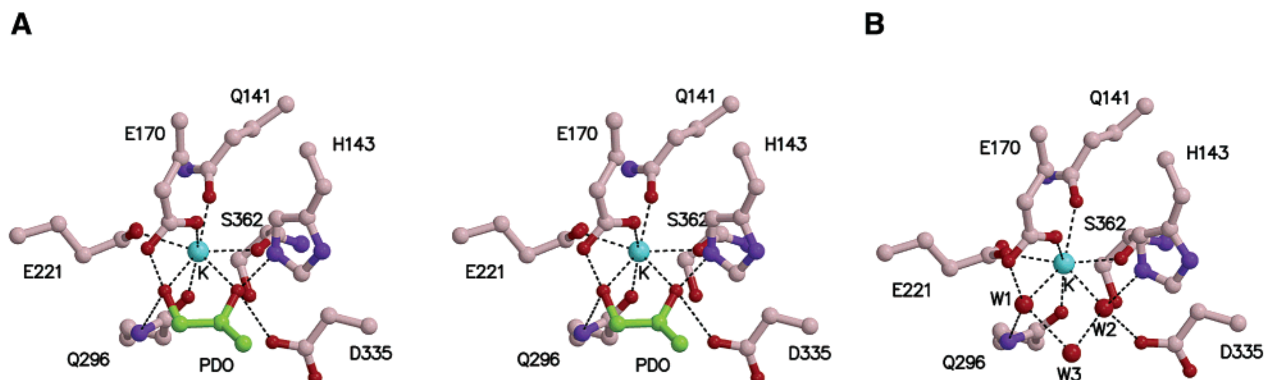


Figure 7. Interactions between active-site residues, substrate and K^+. (A) With substrate (stereoview). (B) Without substrate. The carbon atoms and bonds of the substrate are colored in yellow-green, and water molecules in red. Residue numbers in the α subunit.

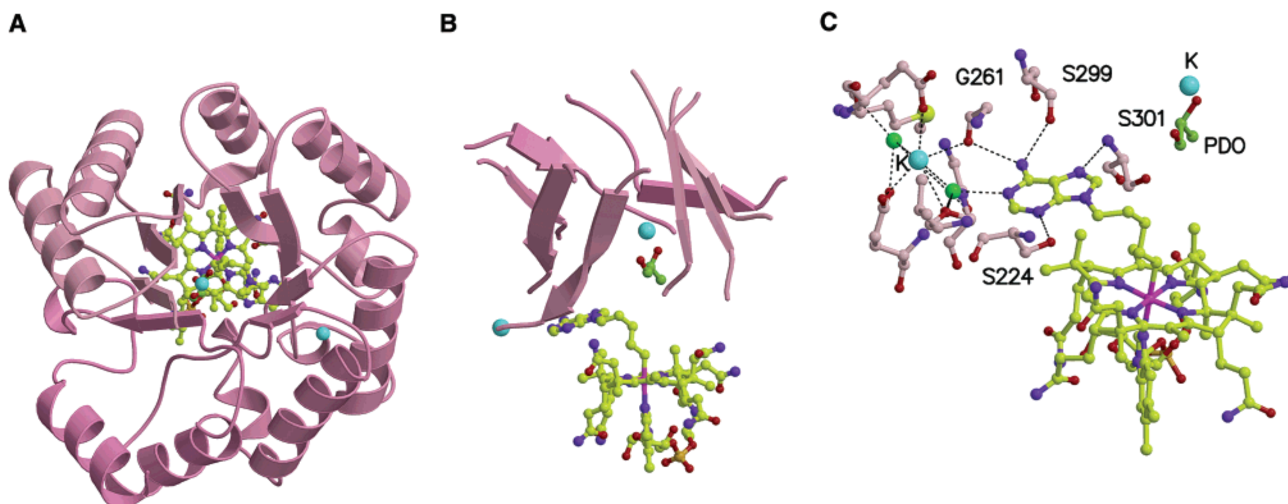


Figure 8. Identification of the adenine ring-binding site of diol dehydratase. (A) $(\alpha/\beta)_8$ barrel (TIM barrel) of the enzyme-AdePeCbl complex. (B) Side view of the active site in the TIM barrel. (C) Interaction of the adenine moiety with amino acid residues and a water molecule (green). The other color indications are the same as those in Figure 6A,B. Residue numbers in the α subunit.

well.[12] Such peculiar structures of the substrate-binding sites suggest that K^+ may play an important role in the catalysis of these enzymes.

C. Identification of the Adenine-Binding Pocket

Adeninylethylcobalamin (AdeEtCbl, Figure 1E, $n = 2$) undergoes Co−C bond cleavage upon binding to apodiol dehydratase, whereas adeninylpropylcobalamin (AdePrCbl, Figure 1E, $n = 3$) and other longer chain homologues do not.[192] On the basis of these results, the model of activation of the coenzyme Co−C bond by apoenzyme was proposed. That is, the "adenine-attracting effect" of apoenzyme is a major element that weakens the Co−C bond. The presence of the binding site for the coenzyme's adenosyl group in AdoCbl-dependent enzymes was suggested by the structure−function studies of AdoCbl[54,93,112,145] and demonstrated directly with diol dehydratase by using radioactive 5′-deoxyadenosine.[115] The effects of 5′-deoxyadenosine on the visible[193,194] and EPR[195] spectra of the enzyme-bound cobalamins also suggest that the interaction among apoenzyme, cobalamin, and 5′-deoxyadenosine may perturb the electronic properties by introducing a distortion into the coring ring.

To examine the above-mentioned model of the Co−C bond activation,[192] identification of the exact adenine-binding site of diol dehydratase was attempted by using AdePeCbl (Figure 1C, $n = 5$) as a probe. This analogue is totally inactive as a coenzyme but is bound more tightly than AdoCbl itself.[93] The crystal structure of the complex of the enzyme with this coenzyme analog[138] revealed that the overall structure of the enzyme·AdePeCbl complex is quite similar to that of the complex with CN-Cbl. As shown in Figure 8A,B, the adeninylpentyl group is located in the TIM barrel above the corrin ring of the cobalamin. As in free AdoCbl[196] and AdePrCbl,[197] the adenine ring is bound parallel to the corrin ring, but with the other side facing pyrrole ring C. As shown in Figure 8C, the adenine moiety of this analogue is trapped by a hydrogen bond network with main chain NH, main chain C=O, water, and a side chain OH group. This structure of the adenine-binding pocket is in good agreement with the hypothetical hydrogen bonding scheme between the adenine ring and the apoenzyme that we deduced from the structure−function studies of the coenzyme with diol dehydratase.[112]

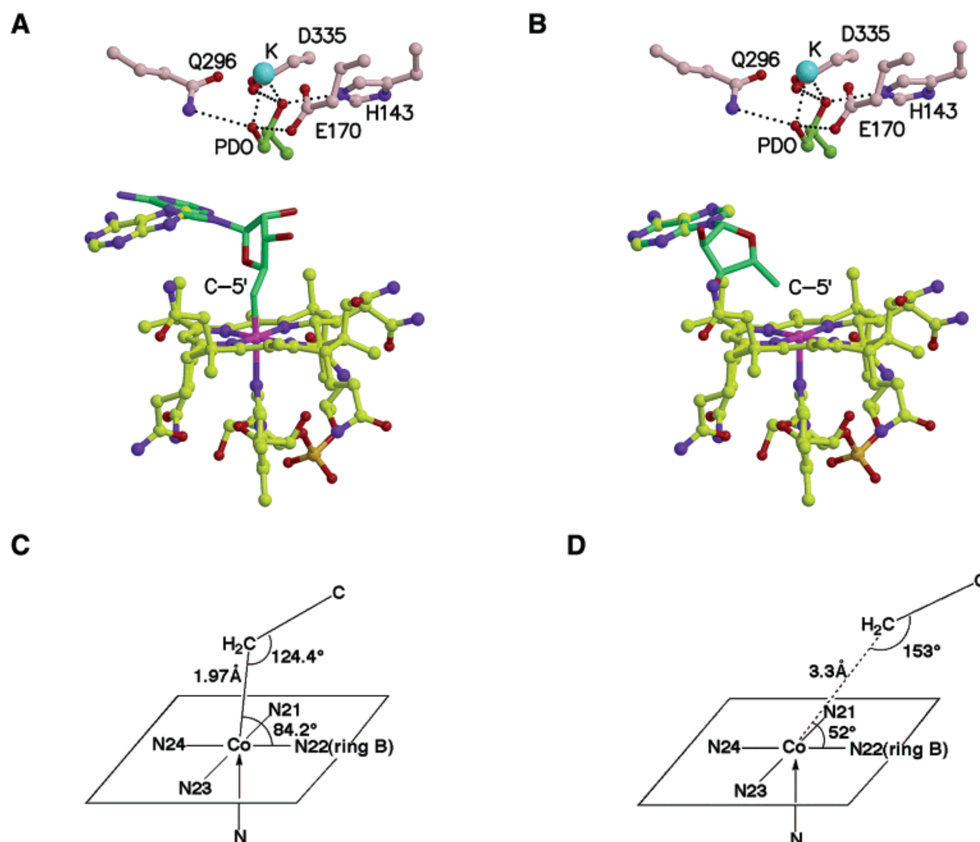


Figure 9. Steric strain model of the Co−C bond cleavage by diol dehydratase. (A) Superimposition of AdoCbl over that of enzyme-bound AdePeCbl at the cobalamin moiety without cleavage of the Co−C bond. (B) The same superimposition at both the cobalamin moiety and the adenine ring with the Co−C bond cleaved and the Co−C distance kept at a minimum ("proximal" conformation). Green stick model represents the adenosyl group of AdoCbl. The other color indications are the same as those in Figure 7. Residue numbers in the α subunit. Panels C and D are schematic representations of steric strains in panels A and B, respectively.

Coenzymic activity and binding affinity of coenzyme analogues in which the adenine moiety of the adenosyl group is substituted by a closely related base have been extensively studied with diol dehydratase.[93,112,113] The formation of cob(II)alamin as an intermediate in the catalytic reaction is spectroscopically observed with active coenzyme analogues but not with inactive ones. It was therefore concluded that the importance of the hydrogen bonds between apoenzyme and nitrogen atoms in the adenine moiety for manifestation of catalytic function and for activation of the Co−C bond decreases in the following order: N7 > 6-NH_2 > N3 > N1.[112] The extremely low affinity of inosylcobalamin ($K_i \sim 22$ μM) and 1,N^6-ethenoadenosylcobalamin ($K_i \sim 35$ μM) for apoenzyme can be explained by their lack of hydrogen bonds at N1 and 6-NH_2.[138] The relatively high coenzyme activity (36% that of AdoCbl) and high binding affinity of 3-isoadenosylcobalamin is rationalized by similar hydrogen bonds at N1, 6-NH_2, N9, and N7 of this analogue, instead of at N7, 6-NH_2, N3, and N1 of AdoCbl. A total number of possible hydrogen bonds at the adenine nitrogen atoms correlates roughly with relative coenzyme activity and binding affinity for the apoenzyme.[138] Thus, it is evident that the structure of the adenine-binding pocket provides a good molecular basis for the strict specificity of the enzyme for the adenosyl group of the coenzyme. Although the crystal structure reveals the presence of hydrogen bonds, it does not show the extents of their contributions to the enzyme catalysis. Thus, this shows that structural and biochemical studies are complementary to each other.

D. Steric Strain Models of Activation and Cleavage of the Co−C Bond

How does the coenzyme's Co−C bond become activated upon the coenzyme binding to the apoenzyme? In a modeling study, the structure of the cobalamin moiety of AdoCbl was superimposed on that of AdePeCbl in the substrate-bound form of diol dehydratase·AdePeCbl complex.[138] As shown in Figure 9A, the adenine moiety of AdoCbl is positioned in a different direction from the adenine-binding pocket. The adenine ring of the coenzyme would be accommodated to the adenine-binding pocket because the maximal binding energy would be obtained by doing so. However, it is not possible to superimpose both the cobalamin moiety and the adenine ring on the enzyme-bound AdePeCbl without cleavage of the Co−C bond. If they are superimposed with the Co−C bond cleaved and the Co−C distance kept at a minimum, C5′ moves to the position shown in Figure 9B. Thus, it is evident that marked strains must be induced. As compared with free AdoCbl (Figure 9C), the Co−C distance has to be elongated to 3.3 Å, and the Co−C bond has to lean toward the nitrogen atom of pyrrole ring B (Figure 9D). This would lead to marked angular distortions of the C5′-Co−N22 bond angle to 52° and the Co−C5′-C4′ bond angle to

153°. Therefore, it can be concluded that the tight enzyme–coenzyme interactions at both the cobalamin moiety and the adenine ring produce both angular strains and tensile force that inevitably break the Co–C bond. These may be entities of the activation of the coenzyme's Co–C bond by apoenzyme. This conclusion accounts for why the Co–C bond of AdeEtCbl is cleaved upon binding to the apoenzyme as well.[193]

Since the Co–C bond of AdoCbl is sufficiently weak (homolytic bond dissociation energy, ~30 kcal/mol),[198,199] only a modest labilization by interaction with apoprotein would be required for homolysis. The rate acceleration of the Co–C bond cleavage with diol dehydratase in the presence of substrate was calculated to be $10^{12\pm1}$-fold.[92,179,200] This acceleration corresponding to $\Delta\Delta G^{\ddagger}$ of 16–19 kcal/mol at 37 °C would be achieved by utilizing the coenzyme and substrate binding energies. This value of $\Delta\Delta G^{\ddagger}$ seems reasonable because there are 12 hydrogen bonds and a salt bridge between the apoenzyme and cobalamin and four hydrogen bonds and two coordinate bonds to K^+ between the enzyme and 1,2-propanediol.[9] It is likely that $\Delta\Delta G^{\ddagger}$ reflects the extent of steric strain. The steric strain imposed upon coenzyme binding to the enzyme is completely relieved by Co–C bond cleavage. The enzyme is flexible, and it may be usually easier for the enzyme to move loops and side chains around a bit than to distort a covalent bond. But it should also be noted that the *pro-S* CH_3 group on C12 of the corrin ring props up the adenine ring and prevents its dropping. This would be the reason the carboxymethyl group on C12 of precorrin-6B has to be decarboxylated by CobL (CbiT) in the B_{12} biosynthesis.

In the crystal structure analysis of glutamate mutase·AdoCbl complex in the presence of glutamate, one conformer with the ribose in a C2′-*endo* conformation is postulated to contain an activated AdoCbl molecule.[139] Since C5′ in this conformer is positioned 3.1–3.2 Å above the cobalt atom, it is clear that its Co–C bond is highly strained or cleaved in this enzyme as well.

E. Substrate Binding Triggering for the Co–C Bond Homolysis

Substrate triggering is an important mechanism for radical-catalyzed reactions by which the O_2-sensitive catalytic radicals are formed only in the presence of substrate. In the absence of substrate, the rate acceleration of the Co–C bond homolysis is equal to or greater than $10^{6\pm1}$-fold.[179] It is therefore likely that the Co–C bond homolysis of the coenzyme is facilitated in two steps—rate acceleration upon binding of the coenzyme to apoenzyme and further rate enhancement upon binding of substrate to the holoenzyme. The rate enhancement equal to or smaller than 10^6 fold by substrate binding corresponds to $\Delta\Delta G^{\ddagger}$ at most 8.8 kcal/mol at 37 °C. This energy would be supplied by the substrate binding energy (four hydrogen bonds and two coordinate bonds to K^+).[9] Substrate-induced conformational changes of enzymes were investigated to understand how substrate binding triggers the Co–C bond homolysis in AdoCbl-dependent enzymes. The recently

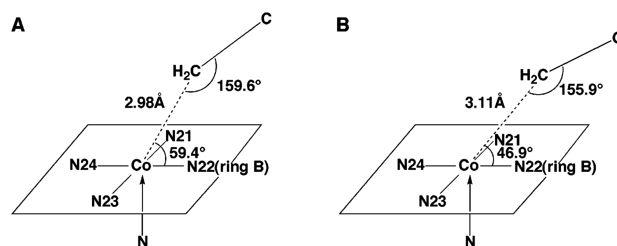


Figure 10. Effect of substrate on the activation of the Co–C bond. (A) Without substrate. (B) With substrate. Panels A and B are schematic representations of steric strains from modeling studies with the substrate-free and substrate-bound enzyme·CN-Cbl complex, respectively.

reported crystal structure of the substrate-free form of the diol dehydratase·CN-Cbl complex (Figure 7B)[201] revealed that two water molecules hydrogen-bonded to a third water molecule are coordinated to K^+ in place of the two hydroxyls of the substrate, and that K^+ in the substrate-binding site of the substrate-free enzyme is also hepta-coordinated. By comparison of the structures of substrate-bound and substrate-free forms, it is evident that, upon the substrate binding, cobalamin is tilted so that pyrrole rings A and D are significantly lifted up toward the substrate-binding site, whereas rings B and C are lifted up only slightly. As a result, the β subunits are tilted by ~3°.

Substrate binding triggers the Co–C bond homolysis in AdoCbl-dependent enzymes. In a modeling study, the structures of both the cobalamin moiety and the adenine ring of the coenzyme were superimposed onto those of the diol dehydratase-bound CN-Cbl and the adenine ring-binding pocket, respectively. It was demonstrated that the distortions of the Co–C bond in the substrate-free form (Figure 10A) are already marked but less than those in the substrate-bound form (Figure 10B).[201] It is thus strongly suggested that, in the case of diol dehydratase, the Co–C bond becomes largely activated (labilized) upon the coenzyme binding to apoenzyme even in the absence of substrate, and that its homolysis is triggered by the substrate-induced conformational changes of the enzyme. Thermodynamic coupling of the Co–C bond homolysis with hydrogen abstraction from the substrate shifts the equilibrium to its dissociation.[179]

Among the other AdoCbl-dependent enzymes, the structures of substrate-free forms are available with methylmalonyl-CoA mutase[136] and very recently with glycerol dehydratase.[202] In contrast to the rather small conformational change of diol dehydratase upon the substrate binding, substrate binding induces a marked conformational change in the case of methylmalonyl-CoA mutase—that is, the so-called TIM (triose phosphate isomerase) barrel is split apart. Such a large change needs larger substrate-binding energy and might thus be inducible only by the binding of a large substrate, such as CoA derivatives. Evans and co-workers proposed that substrate binding to methylmalonyl-CoA mutase induces the marked conformational change that was suggested to destroy the binding site for the adenosyl group, displacing it from the cobalt atom and forming the radical species.[136] Resonance Raman studies of me-

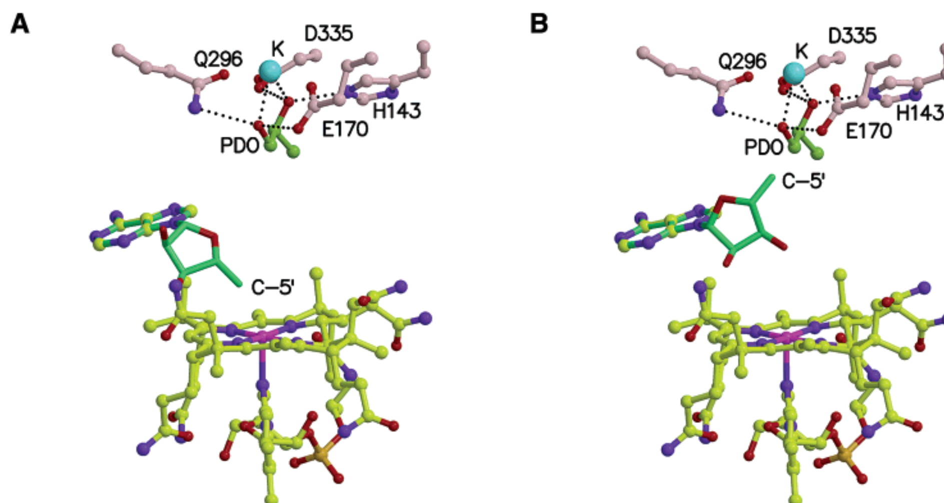


Figure 11. Ribosyl rotation model of access of the adenosyl group to substrate. (A) The same as Figure 9B ("proximal" conformation). (B) Superimposed as in panel A, but with the ribose moiety of the adenosyl group rotated around the glycosidic linkage so that C5′ is closest to C1 of the substrate ("distal" conformation). The other color indications are the same as those in Figure 9.

thylmalonyl-CoA mutase indicated that Co-adenosyl tilting in the enzyme-bound AdoCbl in the presence of substrate is important for the Co−C bond activation[203] and that the Co−C bond is left unaffected upon the coenzyme binding to methylmalonyl-CoA mutase in the absence of substrate.[204] The EXAFS results show small decreases in the average distance from cobalt to the corrin equatorial nitrogens and no significant change in the Co−C bond length upon cofactor binding to methylmalonyl-CoA mutase.[205] Thus, the change in coordination of the nitrogenous axial ligand does not significantly contribute to a trans effect in the ground state. It was concluded that in the absence of substrate the Co−C bond stays unaffected and that the substrate binding to the enzyme plays an important role in weakening of the Co−C bond.

The holoenzymes of diol dehydratase[149] and glycerol dehydratase[206] are very sensitive to oxygen in the absence of substrate, which is postulated to be due to the irreversible cleavage of the partially activated Co−C bond of holoenzymes by reaction with oxygen, whereas the holoenzyme of methylmalonyl-CoA mutase is not. Such different effects of substrate binding on the Co−C bond activation might be reminiscent of the difference in the substrate-induced conformational changes between these enzymes.[136,201] That is, significant activation of the Co−C bond in the absence of substrate might not be necessary for methylmalonyl-CoA mutase because the binding of its large substrate can induce a marked conformational change of the enzyme using a large binding energy that leads to homolysis of the Co−C bond. In this context, substrates of diol dehydratase are too small to get such a large binding energy for facilitation of the Co−C bond homolysis. The coordination of substrates to K^+ might be important for increasing the substrate binding energy as well in this enzyme.

F. Ribosyl Rotation and Pseudorotation Models for Radical Transfer from Coenzyme to Substrate

How can the C5′-centered radical of the adenosyl group abstract a specific hydrogen atom from the substrate? The X-ray structure of the diol dehydratase·AdePeCbl complex indicates that C1 of the substrate is 6.6 Å apart from the radical center C5′ (Figure 11A).[138] This distance is too far for the radical transfer from the adenosyl radical to the substrate. Structural data[8−11] and EPR spectra of reacting holoenzymes[99,110,162,163,165,166,207] suggested that organic radical intermediates (or a thiyl radical in ribonucleotide reductase) are 6−12Å away from Co(II) in this and other AdoCbl-dependent enzymes. This means that such a distance problem is not specific for diol dehydratase but is general for all the AdoCbl-dependent enzymes. This problem was solved with diol dehydratase by a modeling study.[138] The radical center C5′ is far from C1 of the substrate just after the Co−C bond cleavage ("proximal" conformation) (Figure 11A), but it should be noted that the ribosyl moiety becomes rotatable around the glycosidic linkage after the Co−C bond cleavage. The original idea of rotation of the ribose group about the glycosidic linkage was proposed by Glusker and co-workers.[208] The location of the adenine-binding pocket in the crystal structure of the enzyme·AdePeCbl complex has made the modeling studies possible.[138] If the ribose moiety rotates 94° counterclockwise, the radical center comes closest to the substrate ("distal" conformation) (Figure 11B). The distances from C5′ to C1 and C2 of the substrate are 2.0 and 3.3 Å, respectively. As described below, the specific hydrogen atom on C1 to be abstracted is on the same side as the adenosyl group and close to C5′, whereas the other hydrogen atom is positioned in a different direction and far from the radical center. This enables the specific hydrogen abstraction from C1. Thus, the "ribosyl rotation" model of radical transfer from adenosyl radical to substrate can account for both the problem of distance between the catalytic radical and the substrate and the stereospecificity in the hydrogen abstraction.[138] This model was supported by the ENDOR and ESEEM studies on ethanolamine ammonia-lyase.[162,163,166,167]

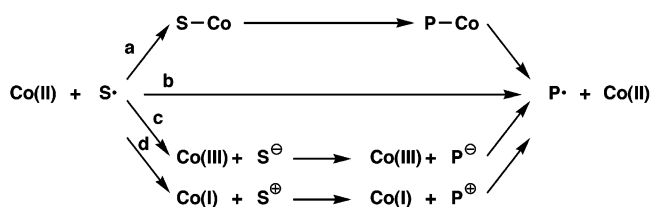
In the crystal structure analysis of glutamate mutase·AdoCbl complex in the presence of glutamate,

electron density corresponding to the adenosyl group was explained by a superposition of two species: one with the ribose in a C2′-*endo* and the other in a C3′-*endo* conformation.[139] C5′ in the former is positioned 3.1−3.2 Å above the cobalt atom, whereas C5′ in the latter is 4.5 Å apart from the cobalt atom and is within van der Waals distance from the substrate. It was thus postulated that the former is an activated AdoCbl molecule with a highly strained Co−C bond and the latter is a 5′-deoxyadenosine formed by hydrogen abstraction of the adenosyl radical from the substrate. This "ribose pseudorotation" model of Kratky and co-workers[139] might be applicable to methylmalonyl-CoA mutase in which the cobalt-substrate distance is shorter than that in diol dehydratase and ethanolamine ammonia-lyase.

V. Pathway of the Group X Migration

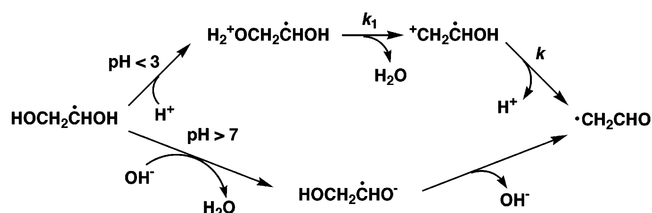
A. Model Studies

In a formal sense, several pathways can be considered for the 1,2-shift of group X or the rearrangement of a substrate radical to a product radical:



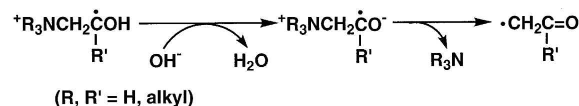
It is difficult to get direct evidence in support of a certain mechanism from biochemical experimentation, but X-ray structures as well as model reactions provide some clues. The transalkylation pathway (a) by a $\sigma-\pi$ rearrangement through a substrate-cobalamin and product−cobalamin adducts was postulated in earlier reports,[88,209,210] but no experimental evidence for intermediary formation of these hypothetical organocobalamins is available yet. 2,3-Butanediol and 2-propanolamine served as substrates for diol dehydratase and ethanolamine ammonia-lyase, respectively.[24,211] In the transalkylation pathway, organocobalamins that are sterically equivalent to tertiary alkylcobalamins must be formed as intermediates in these reactions. More decisively, long distances between the radical intermediates and the cobalt atom estimated by EPR and structural studies eliminated the possibility that Co(II) of cob(II)alamin is directly involved in the rearrangement of the substrate radical to the product radical, such as a transalkylation pathway through organocobalamin intermediates. The rearrangement pathways through carbanion (c) or carbocation (d) intermediates that might be formed by electron transfer between organic radicals and Co(II) are also unlikely because of the same reason.

The direct rearrangement of a substrate radical to a product radical without interaction with Co(II) (b) is supported by model reactions of diol dehydratase.[212−216] Livingston and Zeldes reported that the 1,2-dihydroxyethyl radical is formed by reaction of the •OH radical with ethylene glycol and undergoes a proton-catalyzed dehydration to the formylmethyl radical in the presence of H_2SO_4:[212]



Bansal et al. investigated the pulse radiolysis of aqueous solutions of ethylene glycol at different pH value of the solution.[213] In the pH range from 3 to 7, only 1,2-dihydroxylethyl radical is observed. Below pH 3, the dehydration of this radical to formylmethyl radical is seen (pK = 0.74, k1 = 8.6 × 10^5 s^{-1}). In alkaline solutions, a rapid dehydration also occurs which is explained by the diffusion-controlled, OH^--catalyzed deprotonation of the 1,2-dihydroxylethyl radical and the fast dehydration of the radical anion. The pK_a values of the hydroxyl groups attached to a carbon radical were reported to be approximately five pH units lower than those in the corresponding alcohols.[214]

Amino alcohol radical rearrangements that model the ethanolamine ammonia-lyase reaction were also reported:



The radicals were formed from β-amino alcohols by the Ti(III)-H_2O_2 oxidation[217] or photolysis in the presence of H_2O_2.[218] These rearrangements proceed under neutral conditions in the absence of Co(II).

B. Theoretical Calculations

Golding and Radom first proposed on the basis of ab initio molecular orbital theory calculations that the barrier in the intramolecular 1,2-shifts of the OH group through a cyclic transition state is reduced by protonation of the migrating OH group.[216,219] Full protonation pathway is, however, unlikely because there are no such strongly acidic groups in proteins that could protonate the migrating hydroxyl. George et al. proposed that the protonated 1,2-dihydroxyethyl radical rearranges without activation to a hydrogen-bonded hydrate of the vinyl alcohol radical cation, $H_2O\cdots HOCH-CH_2]^{\bullet+}$, which ultimately forms acetaldehyde and H_2O after proton and H-atom transfer steps.[220] According to this predissociation mechanism, the oxygen atom of the product aldehyde would always be derived from that originally attached to the radical center. This is incompatible with the above-mentioned results of labeling experiments.[76]

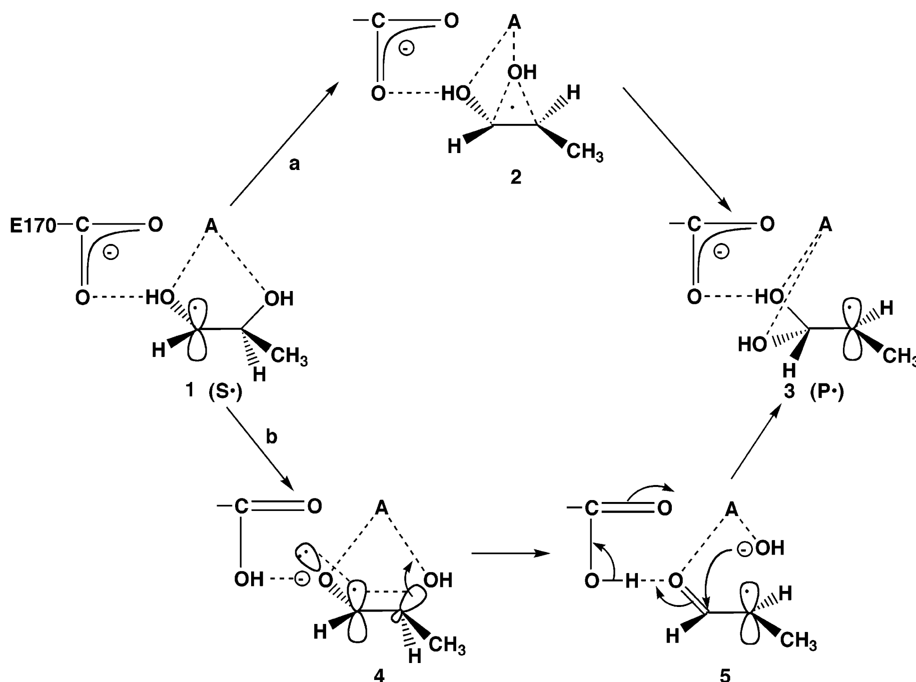


Figure 12. Possible pathways of the OH group migration from C2 to C1 in the diol dehydratase reaction. Involvement of conjugate acid (BH^+) of an active-site amino acid residue or Lewis acid (K^+) is postulated for A. In the case of BH^+, there would be no interaction with O1 of the substrate. (Reprinted with permission from ref 19. Copyright 2002 The Japan Chemical Journal Forum and Wiley Periodicals, Inc.)

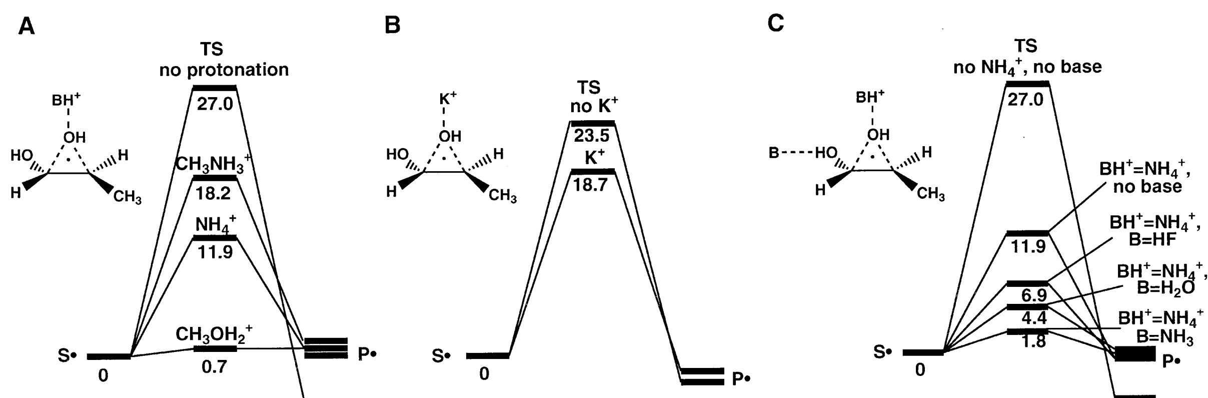


Figure 13. Energy diagrams obtained by DFT computations for the OH group migration from C2 to C1 in the diol dehydratase reaction. (A) Transition state stabilization by partial protonation of the migrating OH group. (B) Transition state stabilization by coordination of the migrating OH group to K^+. (C) Transition state stabilization by partial protonation-partial deprotonation. S•, substrate radical; P•, product radical; TS, transition state.

Figure 12 shows two possible pathways of the OH group migration from C2 to C1 that are consistent with the X-ray structure of diol dehydratase. Pathway a is a concerted pathway through a cyclic transition state.[216] The involvement of a hydrogen-bonded −COOH group as an additional cofactor in the rearrangement has been proposed.[221] Since the activities of diol dehydratase and ethanolamine ammonia-lyase are almost constant at pH 6.0−10.0[23] and 6.8−8.2,[60] respectively, the carboxyl groups in the active sites would exist as the $-COO^-$ under the conditions for the enzymatic reactions. Thus, involvement of not −COOH but conjugate acids of the active-site residues is more likely. Radom and Golding demonstrated that the activation energy for the OH group migration is lowered by partial protonation of the migrating OH group (Figure 13A).[222] From the active-site structure of diol dehydratase, conjugate acids of the active-site residues (BH^+) or Lewis acid (K^+) might lower the energy of the transition state. Alternative pathway b in Figure 12 is a stepwise OH abstraction/re-addition pathway through a radical anion and a ketyl radical.[223] In this pathway, K^+ might assist deprotonation of the OH group on C1 and facilitate elimination and addition of the OH group. Dehydration of 2-hydroxyglutaryl-CoA through a ketyl radical was postulated by Buckel to proceed by a radical mechanism.[224]

The possibility was tested by DFT computations with a protein-free, simplified model that K^+ might assist the OH group migration. Only a transition state for the concerted pathway was obtained,[225] and the presence of K^+ lowers the activation energy for the OH group migration, but only by 4.8 kcal/mol (Figure 13B).[226,227] In either model involving BH^+ or K^+, it seemed that the barrier heights for the transi-

Table 3. Kinetic Parameters and Deuterium Kinetic Isotope Effects for Overall Reaction and Mechanism-Based Inactivation with Mutant Diol Dehydratases

mutant enzyme	k_{cat} (s^{-1})	K_m(1,2-propanediol) (mM)	$k_{cat}/K_m \times 10^{-6}$ (s^{-1} M^{-1})	$k_{cat}/k_{inact} \times 10^{-4}$	k_H/k_D[a]	$k_{inact,H}/k_{inact,D}$[a]
wild type	367	0.043	8.5	73	10	1.1
Gln296Ala	155	10	0.016	1.6		
Gln141Ala	137	0.1	1.4	3.1		
Ser362Ala	97	0.08	1.2	32		
His143Lys	80	0.12	0.67	16	12	1.0
His143Gln	7.8	0.081	0.096	1.6	11	1.0
His143Ala	5.3	0.25	0.021	0.020	5-6	1.2
His143Leu	~0.7	0.22	0.003	~0.020	2	1.0
Glu170Ala	<0.01					

[a] Determined with [1,1-D_2]1,2-propanediol.

tion state are still too high and that an additional mechanism of transition state stabilization by the active-site residues must be considered. Computational results indicated that the presence of imidazolium is effective for transition state stabilization in the absence of K^+, but not effective in its presence.[227] In the protein, Lewis acidity of K^+ may be lowered by cancellation of its positive charge. Thus, the imidazolium group of Hisα143 may contribute to the stabilization of the transition state in the enzyme. Recently, Radom and Golding demonstrated by calculations that the barrier height for the transition state can be further lowered by partial deprotonation as well (Figure 13C).[228] According to their calculations, a Lewis acid, such as Li^+ or Na^+, alone is rather anticatalytic but facilitates the OH group migration in the presence of $HCOO^-$. They proposed a synergistic retro-push−pull catalysis or the partial protonation-partical deprotonation model assisted by Hisα143 and Gluα170, although they have not calculated with a model including K^+ or imidazole.

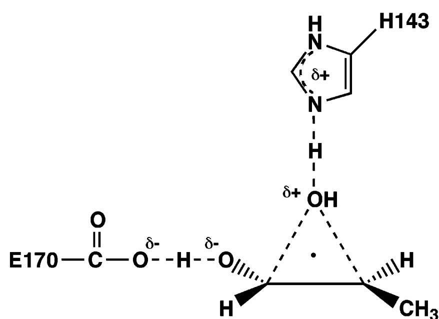
It was predicted from a MNDO SCF-MO theoretical study that AdoCbl-dependent rearrangements can proceed by three distinct mechanistic pathways: $-CH(NH_2)CO_2H$ by dissociation−recombination, in 1,2-diols by stepwise migration of a protonated OH group via an intermediate π−allyl complex, and acyl groups by either a concerted migration or dissociation−recombination.[229] The results of theoretical calculations by Radom, Golding, and co-workers support experimental evidence for the glutamate mutase-assisted interconversion of (*S*)-glutamate and (2*S*,3*S*)-methylaspartate through a fragmentation-recombination pathway.[230] In contrast, they reported that, for 2-methyleneglutarate mutase and methylmalonyl-CoA mutase, a cyclization/ring-opening (addition/elimination) pathway is energetically more favorable than the fragmentation/recombination pathway, and that protonation of the migrating group facilitates the rearrangement.[231] This difference is because of the absence of an unsaturated linkage in the migrating group in glutamate and the potential for greater relative stability of the separated fragments in the fragmentation/recombination pathway.[230] Protonation of the amino group of the glycyl fragment is unfavorable, and the enzyme may serve to reduce this by partial proton-transfer.

C. Roles of Active-Site Amino Acid Residues

The importance of each active-site residue in the α subunit of diol dehydratase was surveyed. Mutant enzymes, in which one of the active-site residues is mutated to Ala, were prepared by site-directed mutagenesis.[232] (Table 3). Among the residues interacting with the substrate and/or K^+, the Gluα221Ala and Aspα335Ala mutant enzymes do not form an $(\alpha\beta\gamma)_2$ complex, suggesting that these residues are important for correct folding. The Gluα170Ala mutant is totally inactive, and the Hisα143Ala mutant shows only a trace of enzyme activity, indicating that Gluα170 and Hisα143 are catalytic residues.[233] The Hisα143Ala and Hisα143Leu mutants undergo rapid mechanism-based inactivation. The k_{cat}/k_{inact} values show that these mutants become inactivated after only ca. 200 catalytic turnovers. It is thus evident that the interaction between Hisα143 and the OH group on C2 of the substrate is important for both catalytic activity and catalytic turnovers. The other mutant enzymes show partial activity. From the kinetic parameters shown here, it was also concluded that Glnα296 and Hisα143 are important for substrate binding and that Glnα296 and Glnα141 are also important for preventing the enzyme from mechanism-based inactivation.

Deuterium KIEs for the Hisα143Ala and Hisα-143Leu mutants were determined with the deuterated substrate [1,1-D_2]1,2-propanediol[233] (Table 3). Although deuterium KIE on k_{inact} was not observed, the deuterium KIEs for the overall reaction are approximately 5-6 and 2, respectively, which are much smaller than that observed for the wild-type enzyme. This indicates that the hydrogen abstraction step is fully rate-determining for the wild-type enzyme but partially rate-determining with the Hisα143Leu mutant enzyme. These results strongly suggest that the activation energy for the OH group migration is almost comparable with that for the hydrogen abstraction upon the Hisα143Leu mutation. Enzymatic activity of the other Hisα143 mutants seem to be correlated with the acidity of their conjugate acids in neutral solution. These findings provide experimental evidence for the roles of Hisα143 not only in protection of the radical intermediates from side reactions but also for the involvement of Hisα143 as the general acid in the transition state stabilization in the OH group migration. This conclu-

sion is consistent with the computational results.[227,228] The following is the predicted structure of the transition state:



The rates of Co−C bond homolysis in the presence of substrate with the Hisα143Lys and Hisα143Gln mutants were not much different from that with the wild-type enzyme. It was highly suggested that the rate of hydrogen abstraction from substrate is not significantly affected by the Hisα143 mutations.[233]

In methylmalonyl-CoA mutase, it has been reported that the loss of a single interaction involving the OH group of Tyr89 both affects the stability of radical intermediates and decreases the rate of interconversion of the substrate- and product-derived radicals.[234] The X-ray structure of methylmalonyl-CoA mutase revealed that His244 is hydrogen-bonded to the carbonyl oxygen of the migrating thioester group.[8] This hydrogen bond is crucial for the stability and reactivity of substrate-derived radical.[235] The His244Gly and His244Ala mutations not only result in an acute sensitivity of the enzyme to oxygen but also lead to an ∼300- and 1000-fold lowering, respectively, in the catalytic efficiency.[236,237] It was indicated from substrate-dependent tritium partitioning that the His244 assists radical rearrangement.[237] Banerjee and co-workers proposed that partial protonation by this residue may facilitate the rearrangement reaction[236] in support of the prediction by theoretical calculations.[231] Therefore, the roles of His244 in methylmalonyl-CoA mutase and Hisα143 in diol dehydratase are functionally very similar—that is, they are important for both stabilizing the transition states and protecting reactive radical intermediates against side-reactions, although the reactions catalyzed are quite different. In glutamate mutase, mutations were introduced in the "[14]Asp-X-His-X-X-Gly[19]" motif of the MutS subunit.[238] Mutations of either Asp14 to Ala, Glu and Asn or of His16 to Gly and Gln decrease k_{cat} by 1000-fold, and cob(II)alamin does not accumulate in the mutants, suggesting that the rate-determining step is altered. The affinity for AdoCbl is lowered, and the mutant enzymes coordinate to cobalt less well. In this enzyme, the NH_2 group of substrate is hydrogen-bonded to $-COO^-$ group of Glu171.[10] From the properties of Glu171 mutants, it was suggested that this residue acts as a general base to facilitate the formation of glycyl radical intermediate by deprotonating the amino group of the substrate.[239]

VI. Refined Mechanism of Action of Coenzyme B$_{12}$

A. Structural Rationalization of Stereochemical Courses of Diol Dehydratase Reaction

The X-ray structures of (*R*)- and (*S*)-1,2-propanediol-bound forms of diol dehydratase·CN-Cbl complexes revealed that the (*R*)- and (*S*)-enantiomers are bound to the active site in a symmetrical mode with respect to the plane including K^+, O1 and O2 (Figure 14A).[240] The C5′ radical center of the modeled adenosyl radical in the distal conformation[138] comes closest to C1 of the substrate on the same symmetrical plane. Among the three hydrogen atoms on C1 and C2, *pro-R* (1.45 Å) and *pro-S* (1.46 Å) hydrogens on C1 of (*R*)- and (*S*)-1,2-propanediols, respectively, are the closest to C5′ of the adenosyl radical. The angles of C4′-C5′-H_R and C4′-C5′-H_S are 137.6° and 128.5°, respectively. Thus, it is reasonable to predict that the adenosyl radical stereospecifically abstracts the *pro-R* hydrogen from C1 of the (*R*)-isomer and the *pro-S* hydrogen from C1 of the (*S*)-isomer, in accordance with the experimental results of Zagalak et al.[78] Both (*R*) and (*S*)-isomers are bound in staggered conformations. It is therefore reasonable to assume that the OH group in the radical intermediates migrates from C2 to C1 by a suprafacial shift (Figure 14B) to minimize the energy required. This results in the inversion of the configuration at C1, as proposed before.[81,228] Consequently, the 2-OH groups of the (*R*)- and (*S*)-isomers become the *pro-S* and *pro-R* OH groups of the resulting 1,1-*gem*-diol intermediates, respectively.

It is likely that the hydrogen bonds between the OH group on C2 and amino acid residues are maintained during the 1,2-OH group migration.[138] Thus, if viewed from the position of K^+, the C1−C2 bonds of the (*R*)- and (*S*)-isomers would rotate clockwise by about 60° and counterclockwise by about 70°, respectively, around the axis connecting K^+ and the center of the O1−O2 line in the step of 1,2-OH group migration. In a modeling study, the ribose moiety and the 1,1-*gem*-diol intermediates are rotated around the glycosidic linkage and the O1−O2 line, respectively, so that C2 comes closest to C5′ of the enzyme-bound 5′-deoxyadenosine. Again, the modeled 1,1-*gem*-diol intermediates from (*R*)- and (*S*)-enantiomers are considered to be bound at the active site in a symmetrical mode with respect to the plane including K^+, O1, and O2 (Figure 14C). It should be noted that the CH_3 group of 5′-deoxyadenosine is on the same symmetrical plane and thus accessible to C2, a new radical center, of both 1,1-*gem*-diol radicals from the distances of 2.58 Å and 2.55 Å for 1,1-*gem*-diol radicals from (*R*)- and (*S*)-1,2-propanediols, respectively. Therefore, the hydrogen back-abstraction (recombination) from the CH_3 group of 5′-deoxyadenosine by the product radical is structurally quite feasible. Since the CH_3 group of 5′-deoxyadenosine is positioned on the opposite side to the OH group that migrated from C2 to C1, the hydrogen recombination takes place with the inversion of the configuration at C2 with both isomers. This prediction is also just as expected from the experimental results.[74,78]

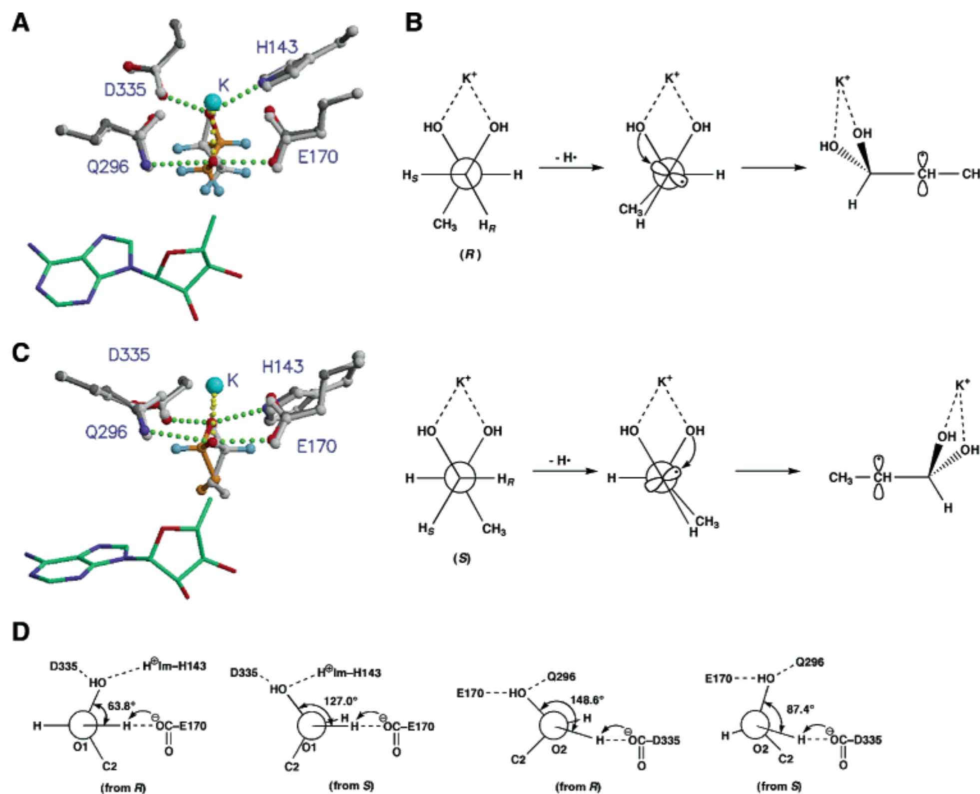


Figure 14. Binding of (*R*)- and (*S*)-1,2-propanediols to diol dehydratase and a modeling study of 1,1-*gem*-diol intermediates. (A) Superimposed models of the (*R*)- and (*S*)-1,2-propanediol-bound forms. Carbon atoms of (*R*)- and (*S*)-isomers are colored in orange and light gray, respectively. Hydrogen atoms are colored in sky blue. Active-site residues of the (*S*)-isomer-bound form are colored in light gray. (B) Suprafacial 1,2-shift of the OH group on C2 and the resulting rotation of the C−C−C backbone. (C) Models of 1,1-*gem*-diol intermediates formed from the (*R*)- and (*S*)-1,2-propanediols. Color codes are the same as in panel A. (D) Stereospecific elimination of the OH group in the dehydration of 1,1-*gem*-diol intermediates formed from the (*R*)- and (*S*)-1,2-propanediols.

The dehydration of the 1,1-*gem*-diol intermediates would be catalyzed by active-site residues serving as a proton acceptor and a proton donor. Exchanges of the hydrogen bonding partners for O1 and O2 during catalysis seem to be energetically less likely. Since the activation energy for the dehydration of 1,1-*gem*-diol to aldehyde is quite small, especially in the presence of acid or base catalyst, it was postulated that the $-COO^-$ groups of both Gluα170 and Aspα335 might function effectively as a base for the dehydration of a 1,1-*gem*-diol intermediate. In the dehydration, deprotonation of an OH group and elimination of the other OH group would proceed in a concerted manner because the energy for the dehydration is lowered by overlapping the orbitals of the bonds being cleaved and formed. From the torsion angles of $H-O-C1-O_{eliminating}$ in the modeled structures of 1,1-*gem*-diol intermediates (Figure 14D), it is suggested that the dehydration of the 1,1-*gem*-diols from the (*R*)- and (*S*)-isomers by the Aspα335-Glnα296 and the Gluα170-Hisα143 pairs, respectively, is energetically more favorable. This mechanism of stereospecific dehydration of 1,1-*gem*-diols is consistent with the finding of Rétey et al. that the oxygen atom originated from O1 of the (*R*)- and (*S*)-isomers is lost in water and is retained in the product as the C=O oxygen, respectively.[76]

B. Overall Mechanism for Diol Dehydratase

Figure 15 illustrates the refined overall mechanism that was proposed by us for diol dehydratase.[138,201,240] The reaction pathways with both (*R*)- and (*S*)-1,2-propanediols as substrates are shown here. The X-ray structure of the substrate-free enzyme (**1**) revealed that two oxygen atoms of two water molecules coordinate to the K^+ ion instead of the substrate OH groups. That is, K^+ in the substrate-free enzyme is also hepta-coordinated by five oxygen atoms from the active-site amino acid residues and two oxygen atoms of water molecules.[201] As judged from the modeling study on the Co−C distance and bond angles using the crystal structure of the substrate-free form of diol dehydratase, it is likely that major conformational changes of AdoCbl occur upon its binding to apoenzyme even in the absence of substrate, leading to the activation of the coenzyme Co−C bond. At this stage, however, only a small fraction ($<1\%$[169]) of the enzyme-bound coenzyme is in the dissociated form. In the absence of substrate, the adenosyl radical in the "proximal" conformation[138] (Figure 11A) and cob(II)-alamin would recombine rapidly even if they are formed, and thus the fraction of the enzyme-bound coenzyme in the dissociated form would be kept very small.

The addition of substrate to the holoenzyme brings about displacement of the two water molecules from K^+ by the two OH groups of the substrate (**1** → **2**).

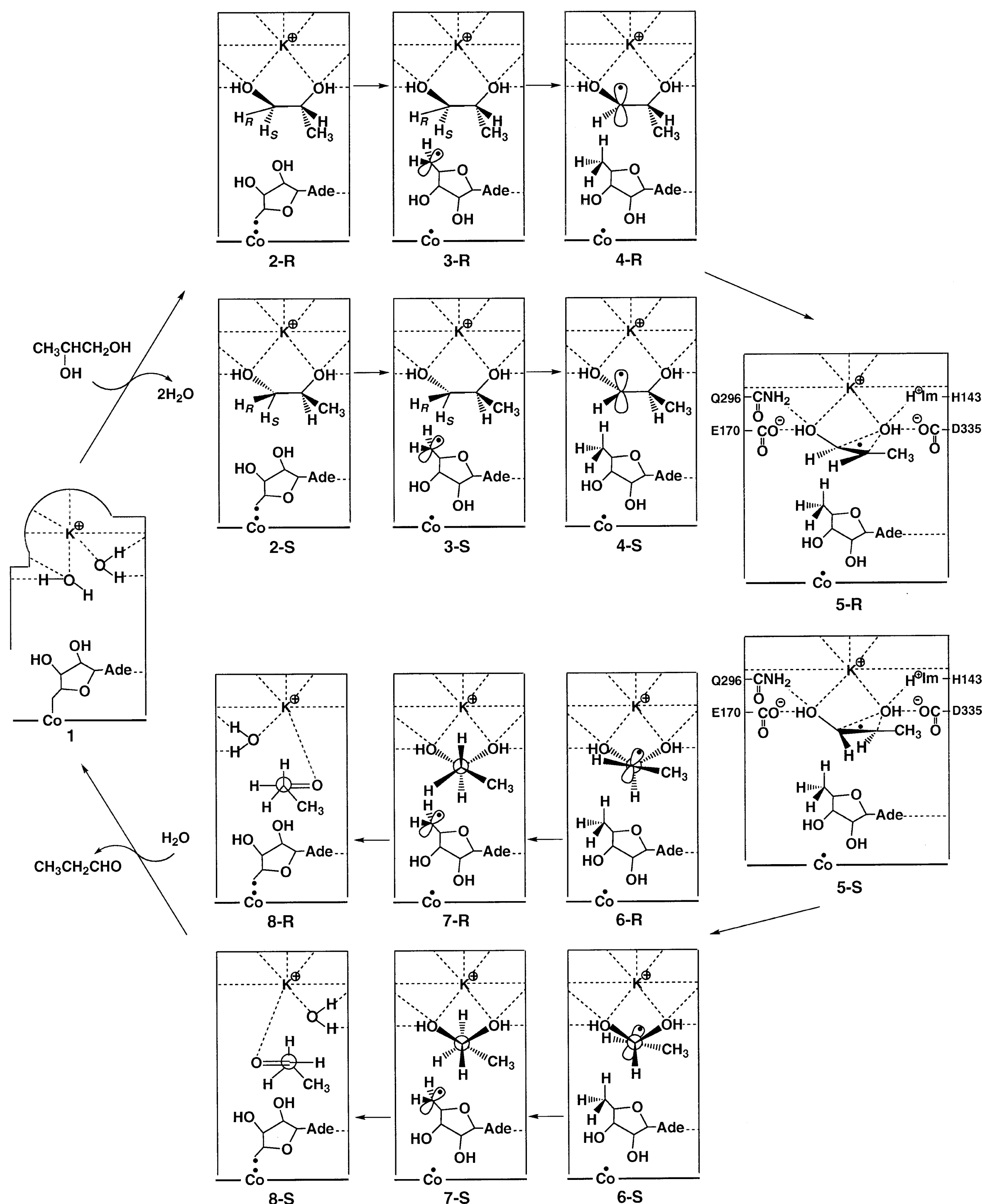


Figure 15. Overall mechanism and stereochemical courses of the diol dehydratase reaction with (*R*)- and (*S*)-1,2-propanediols as substrates. −Co−, cobalamin; Ade, 9-adeninyl group; H^+Im, imidazolium group of Hisα143.

Upon the substrate binding, rather small but distinct conformational changes take place that trigger the Co−C bond homolysis forming cob(II)alamin and the adenosyl radical in the proximal conformation (**2**). The adenosyl radical undergoes rotation of the ribose moiety counterclockwise by approximately 94° around the glycosidic linkage to the distal conformation (**3**) (Figure 11B).[138] The (*R*)- and (*S*)-isomers of substrate are bound to the active site in a symmetrical mode with respect to the plane including K^+ and the two oxygen atoms of the substrate. Since C5′, the radical center of the adenosyl radical, in the distal conformation comes on this symmetrical plane, it can abstract the specific hydrogen atoms on C1 of the enantiomeric substrates—namely, the *pro-R* and *pro-S* hydrogens of the (*R*)- and (*S*)-isomers, respectively. The substrate-derived 1,2-diol-1-yl radicals and 5′-deoxyadenosine are thus formed (**4**). Kinetic coupling of Co−C bond homolysis and hydrogen abstraction was suggested from the substrate deuterium KIE on the rate of Co−C bond homolysis.[179] According to our DFT computations with a protein-free, simplified model of the diol dehydratase system, the relative energy is lowered upon this hydrogen abstraction by

6.7 kcal/mol with the activation energy of 9.0 kcal/mol.[226,227] Thus, it is evident that the equilibrium is shifted in favor of Co−C bond homolysis by its thermodynamic coupling with hydrogen abstraction from the substrate.

The substrate-derived radicals undergo OH group migration from C2 to C1 by a concerted pathway through a cyclic transition state (**5**), resulting in rearrangement to the product-derived 1,1-diol-2-yl radicals (**6**). As judged from the results of site-directed mutagenesis and theoretical calculations, $-COO^-$ of Gluα170 and the imidazolium group of Hisα143 play important roles as a general base and a general acid, respectively, in the transition state stabilization. K^+ would be important not only for keeping substrates in proper position and orientation but also for increasing the substrate-binding energy. Because of the surrounding anionic groups, the Lewis acidity of K^+ is weakened, and it may be less important for stabilizing the transition state. The OH group of the substrate radicals would migrate by a suprafacial shift, the OH groups on C2 of the (*R*)- and (*S*)-isomers becoming the *pro-S* and *pro-R* OH groups on C1 of the product radical, respectively. Since hydrogen bonds are stronger than hydrophobic or van der Waals interactions, the OH groups would maintain their positions during catalysis, and the C1−C2 bonds of the (*R*)- and (*S*)-isomers would thus rotate clockwise by 60° and counterclockwise by 70° if viewed from K^+, respectively, around the axis connecting K^+ and the center of the O1−O2 line.

C2 of the 1,1-*gem*-diol radical (**6**), a new radical center, then comes close to the CH_3 group of 5′-deoxyadenosine and back-abstracts a hydrogen atom from it, producing a 1,1-*gem*-diols and the adenosyl radical (**7**). Since the CH_3 group of 5′-deoxyadenosine is positioned on the opposite side to the migrating OH group, this hydrogen recombination proceeds with the inversion of the configuration at C2. The stereospecific dehydration of the 1,1-*gem*-diols formed from the (*R*)- and (*S*)-isomers would be catalyzed by $-COO^-$ groups of Aspα335 and Gluα170, respectively. Propionaldehyde formed (**8**) loses binding affinities for K^+ and the active-site residues and is thus displaced from K^+ by a water molecule. This causes the conformational change back to the substrate-free form (**1**), which shifts the equilibrium to the direction of recombination of the adenosyl radical and cob(II)alamin. Consequently, the coenzyme is regenerated (**1**) and energy liberated upon re-formation of the Co−C bond would be utilized for ensuring the final steps of the reaction (**8**→**1**). This structure-based mechanism and the stereochemical courses of the diol dehydratase reaction account for all the results of the biochemical and mutational experiments with this enzyme reported so far.

VII. Enzymatic Radical Catalysis

A. Concept of Radical Catalysis

Although enzymes catalyze reactions in aqueous solution, X-ray structures of enzymes revealed that active sites are rather hydrophobic. Until recently, many enzymes have been reported to catalyze by radical mechanisms (for reviews, see refs 3, 4, 17, 19, 20, 185, and 241−250). These enzymes utilize the high reactivity of radicals to catalyze the reactions. Catalytic radicals are either the radicals derived from cofactors, such as AdoCbl[3,4,17,20,241,242] and *S*-adenosylmethionine,[20,241,245] or protein radicals.[241,246−250] 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, physiological conditions without enzymes. In most of these enzymes, the substrates become activated through the abstraction of a hydrogen atom by a radical species at the active site, forming a substrate-derived radical. Cleavage of a C−C, C−O, or C−N bond following the adjacent bond to a radical now takes place easily.[17] The AdoCbl-independent counterpart of diol dehydratase from *Clostridium glycolicum* is an iron-containing radical enzyme.[38−40] Ribonucleotide reductases[246−249] and lysine aminomutases[20,245] that use the same chemistry as that of diol dehydratase and ethanolamine ammonia-lyase, respectively, are all radical enzymes. These facts suggest that these reactions proceed only by assistance of catalytic radicals. Enzymatic radical catalysis can therefore be defined as the mechanism of catalysis by which enzymes catalyze chemically difficult reactions by utilizing the high reactivity of free radicals.

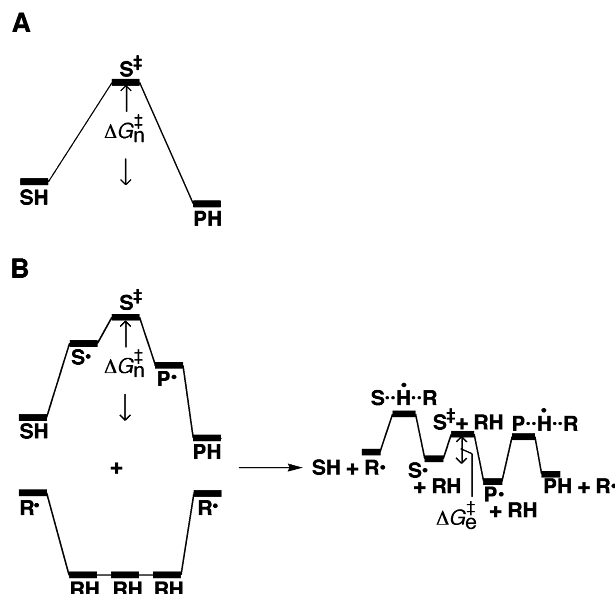


Figure 16. Principle of enzymatic radical catalysis represented by a potential energy diagram. (A) Without a catalytic radical. (B) With a catalytic radical. SH, substrate(s); PH, product(s); $S^‡$, transition state; R·, catalytic radical. Barrier heights are arbitrarily depicted. (Reprinted with permission from ref 19. Copyright 2002 The Japan Chemical Journal Forum and Wiley Periodicals, Inc.)

The concept of radical catalysis can be expressed by a simplified potential energy diagram shown in Figure 16.[17] The common feature of this type of reactions is that the conversion of a substrate to a product is thermodynamically possible but kinetically very difficult because of the extremely high energy of the transition state (Figure 16A). If a catalytic radical (R·) is introduced into the active site, it

becomes stabilized by abstraction of a hydrogen atom from the substrate, forming RH and a substrate-derived radical (S•) (Figure 16B). Thus, the substrate becomes easily activated by coupling with this hydrogen abstraction, resulting in marked decrease of the barrier height for the transition state. After rearrangement of S• to a product-derived radical (P•), P• abstracts a hydrogen atom back from RH to form a product (PH) and regenerates the catalytic radical (R•). In other words, the transition state ($S^{\ddagger}$) becomes much stabilized relative to the ground state (SH) by coupling with the hydrogen abstraction of R•. Consequently, the activation energy ($\Delta G_e^{\ddagger}$) is markedly decreased by the enzyme, which leads to a large rate enhancement of thermodynamically possible but kinetically difficult reactions. Thus, it was proposed that the enzymatic radical catalysis accelerates the reaction rate by dividing a single transition state with a high barrier height into three or more transition states with lower barrier heights.[17,19]

B. Energetic Feasibility

To prove the energetic feasibility of this mechanistic concept of enzymatic radical catalysis, the energetics of the complete pathway of the diol dehydratase reaction was evaluated by DFT computations using a protein-free, simplified model consisting of K^+, two hydrogen-bonded water molecules, an ethyl radical, and 1,2-propanediol.[226,227] For simplicity, the ethyl radical was employed as a model of the adenosyl radical. According to the energy diagrams along the reaction pathways, hydrogen abstraction from 1,2-propanediol by the adenosyl radical and hydrogen recombination from 5′-deoxyadenosine by a product radical requires the activation energies of 9.0 and 15.1 kcal/mol, respectively. The activation energy for the OH group migration was obtained to be larger than those for the hydrogen abstraction and recombination, which is obviously not consistent with the earlier conclusion that the hydrogen abstraction is the rate-determining step for the overall diol dehydratase reaction.[78,88] This computational result, together with the results of mutational experiments,[232,233] indicates that the energy of the transition state for the OH group migration must be lowered by its interaction with active-site residues. Thus, hydrogen abstraction from the CH_3 group of 5′-deoxyadenosine by the product radical would be the rate-determining step of the reaction. Two pathways can be considered in the aldehyde production processes in which the order of hydrogen recombination and dehydration is reversed. It should be noted that, if active-site residues are included in the model, all the diol intermediates and diol transition states are stabilized by hydrogen bonding with them, whereas an aldehyde radical and an aldehyde transition state are not. From the energetic viewpoint, it is more likely that hydrogen recombination takes place prior to dehydration. The strict stereoselectivity in the substitution of the OH group by a hydrogen atom on C2 also supports this idea because the 1,1-*gem*-diol radical can be more tightly fixed to K^+ and active-site residues and thus more easily controllable than the aldehyde radical.

DFT computations indicated that the activation energies for nonpolar processes, i.e., the hydrogen abstraction and recombination, are essentially not affected by coordination of the substrate and the radical intermediate to K^+.[226,227] Similar values of these activation energies were obtained in the computations with a "realistic" model that includes the active-site residues as well.[251] Since these energies can be considered to be suppliable by the substrate-binding energy, the computational results suggest that the hydrogen abstraction and recombination in the radical catalysis of diol dehydratase are energetically feasible.

The radical generation steps in carbon-skeleton rearrangement was shown by Radom and co-workers to be thermodynamically feasible with glutamate mutase[230] because BDEs for the models of 5′-deoxyadenosine, (*S*)-glutamate, and 3-methylaspartate were 99.8, 91.5, and 99.8 kJ/mol, respectively. Activation energies for each hydrogen abstraction step are, however, not reported.

VIII. Reactivation of Inactivated Holoenzymes

A. AdoCbl-Dependent Isomerizations (Eliminating) in Bacterial Metabolisms

1,2-Propanediol, 1,2-ethanediol, and glycerol are more reduced substrates than the corresponding carbohydrates, and thus they can be fermented by only a limited number of bacteria under anaerobic conditions without external electron acceptors. Upon fermentation of 1,2-propanediol or 1,2-ethanediol, they are converted to the corresponding acids and alcohols through aldehydes, acyl-CoA, and acyl phosphates (Figure 17A).[34,252] Diol dehydratase and other enzymes needed for the utilization of 1,2-propanediol as a carbon source are encoded by the *pdu* operon.[253−256] Diol dehydratase is inducibly formed when bacteria are grown anaerobically in the 1,2-propanediol-containing media,[29−32] and the metabolic role of diol dehydratase is to produce both an electron acceptor and a metabolic intermediate for incomplete oxidation of 1,2-diols.[13,14,16]

Glycerol is fermented anaerobically by bacteria with accumulation of 1,3-propanediol in the cultured broth. A so-called *dha* (dihydroxyacetone) system is responsible for the anaerobic dissimilation of glycerol; two enzymes constitute a branch for oxidation of glycerol to dihydroxyacetone phosphate and the other two enzymes including glycerol dehydratase serve as a branch for reduction of glycerol to 1,3-propanediol (Figure 17B).[257,258] Glycerol dehydratase and the other three key enzymes of the *dha* system are encoded by the *dha* regulon.[52,257−259] The expression of the *dha* regulon is induced by glycerol or dihydroxyacetone. The metabolic role of glycerol dehydratase is thus to produce an electron acceptor, β-hydroxypropionaldehyde, in the glycerol fermentation.[13,14,16] In some bacteria, glycerol dehydratase can be substituted by the isofunctional enzyme diol dehydratase, which is induced in a low level by glycerol.[30,32,257]

Ethanolamine ammonia-lyase is involved in the metabolism of ethanolamine to acetaldehyde.[56−58] In

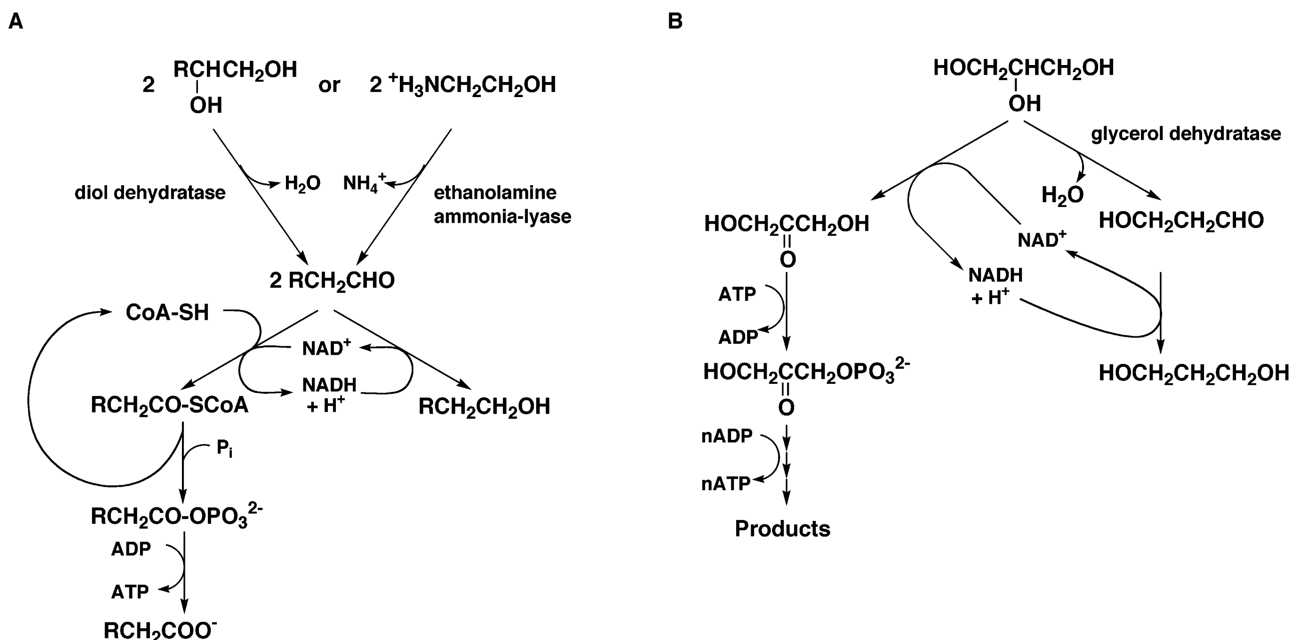


Figure 17. Metabolic roles of AdoCbl-dependent isomerization (eliminating) reactions in the fermentation of 1,2-diols, ethanolamine, and glycerol. (A) Diol dehydratase and ethanolamine ammonia-lyase. (B) Glycerol dehydratase. R = CH_3 or H in panel A.

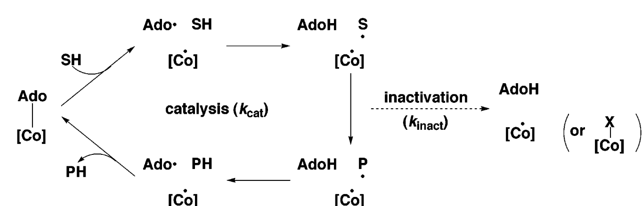


Figure 18. Mechanism-based inactivation of diol dehydratase by substrate and coenzyme analogues. Ado, adenosyl group; AdoH, 5′-deoxyadenosine; [Co], cobalamin; SH, substrate; PH, product; X, unidentified ligand to cobalamin.

clostridia, acetaldehyde serves as both a metabolic intermediate to acetate and an electron acceptor in the anaerobic fermentation of ethanolamine (Figure 17A).[56] In *E. coli* and other bacteria, ammonium ion formed from ethanolamine is utilized as a nitrogen source.[57,58] In *E. coli*[69,260] *and S. typhimurium*,[261] both ethanolamine and vitamin B_{12} are required for enzyme induction; neither is effective alone.

B. Mechanism-Based Inactivation of Diol and Glycerol Dehydratases by Glycerol and in Situ Reactivation

The dehydration of 1,2-propanediol by diol and glycerol dehydratases proceeds linearly with time, but the enzymes undergo mechanism-based inactivation by other substrates or coenzyme analogues during catalysis.[15,24,75] AdoCbl is converted to 5′-deoxyadenosine and either cob(II)alamin, hydroxocobalamin, or unidentified cobalamin in the inactivation (Figure 18). Most interestingly, glycerol, a physiological substrate for the enzymes, serves as a good substrate and a potent inactivator for both enzymes,[24,75,206] although diol dehydratase undergoes the inactivation by glycerol at a faster rate than glycerol dehydratase. Richards and co-workers reported that diol dehydratase distinguishes between "*R*" and "*S*" binding conformations, and that the enzyme·("*R*")-glycerol and the enzyme·("*S*")-glycerol complexes are primarily responsible for the product formation and the inactivation, respectively.[75] *R* and *S* binding conformations mean the conformations in which the *pro-R* and *pro-S* CH_2OH groups, respectively, are bound at the hydrogen-abstracting site. These correspond to the binding conformations for (*R*)- and (*S*)-1,2-propanediols, respectively. The slower rate of inactivation with glycerol dehydratase may be explained by its less marked preference toward (*S*)-1,2-propanediol.[12] The inactivation by glycerol is a sort of mechanism-based inactivation and is accompanied by irreversible cleavage of the Co−C bond of the enzyme-bound coenzyme. Since the modified coenzyme remains tightly bound to the apoenzyme, this results in inactivation of the enzyme. This inactivation seemed enigmatic, because glycerol is a growth substrate for the bacteria that produce these enzymes.

To solve this apparent inconsistency, it was first examined whether the inactivation by glycerol takes place in the cell or not, and whether a reactivating system is present in the cell or not. When permeabilized cells by toluene treatment, a so-called in situ system, are used for the enzyme assay instead of in vitro or in living cells, the inactivation by glycerol takes place in situ as well as in vitro, but the enzymes do not apparently undergo inactivation in situ in the presence of AdoCbl, ATP and Mg^{2+} (or Mn^{2+}).[262,263] Rapid reactivation takes place in situ when ATP and Mg^{2+} are added to the completely inactivated system in the presence of AdoCbl. It was shown with the in situ system that the inactivated holoenzymes of diol and glycerol dehydratases undergo rapid reactivation by exchange of the modified coenzyme for intact AdoCbl under the conditions. Interestingly, the in situ reactivation is observed when the enzyme was inactivated by an imidazolyl coenzyme analogue

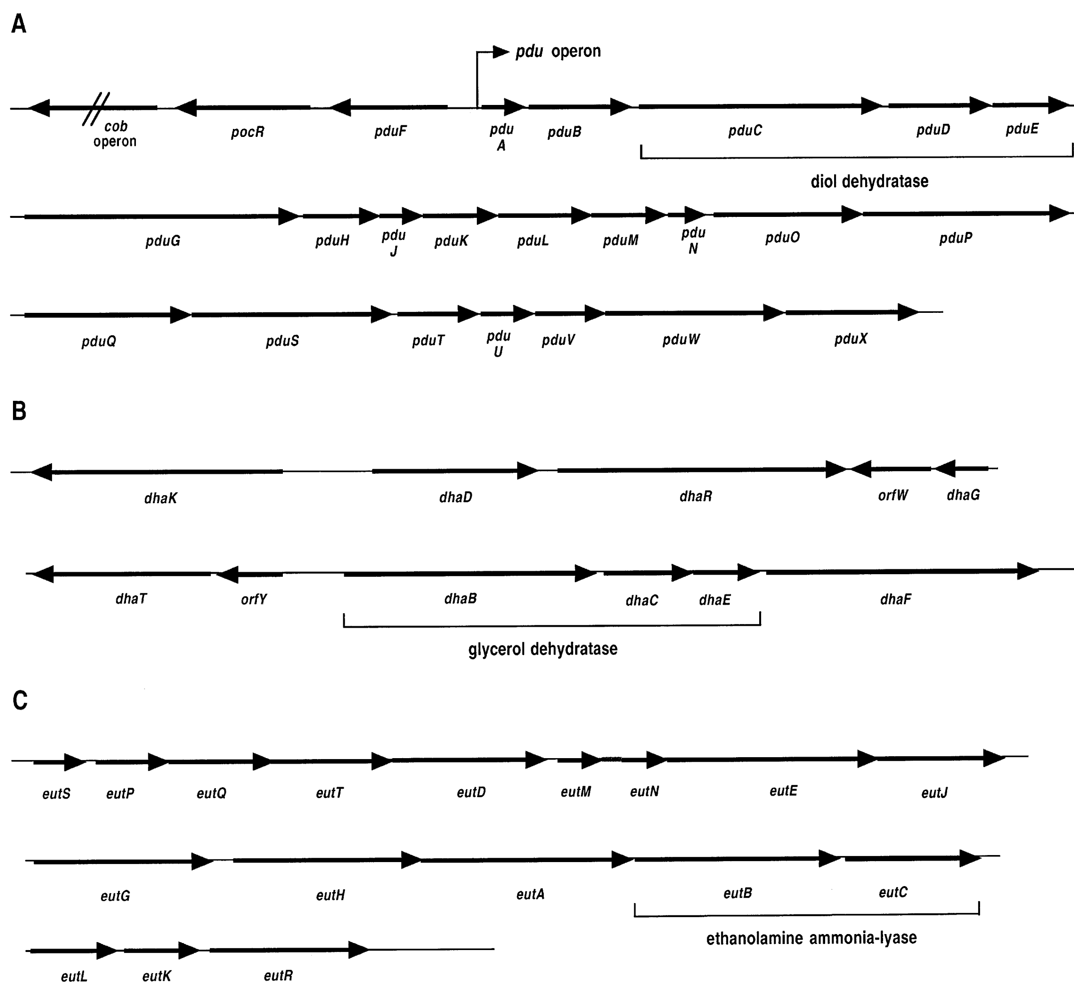


Figure 19. Gene organizations of the *pdu/cob* regulon of *S. typhimurium*,[43] the *dha* regulon of *C. freundii*,[52] and the *eut* operon of *S. typhimurium*.[288]

during dehydration of 1,2-propanediol as well.[264] Since the reactivation was detectable only in situ but not in vitro, it remained unclear for a long time whether a specific proteinaceous factor is involved in the reactivation, although some factor(s) necessary for the in situ reactivation was indirectly suggested to be inducible by glycerol.

C. Identification of the Genes Encoding Reactivating Factors for B_{12}-Enzymes

The glycerol-inactivated holodiol dehydratase in permeabilized cells of *E. coli* harboring a plasmid containing the diol dehydratase genes (*pddABC*) and their flanking regions undergoes rapid reactivation in the presence of free AdoCbl, ATP, and Mg^{2+}.[264] *E. coli* cells harboring a plasmid containing the enzyme genes alone do not show the reactivation. The results with a recombinant *E. coli* strain carrying a deletion mutant plasmid demonstrated that certain protein(s) encoded by the 3′-flanking region of the diol dehydratase genes are essential for the in situ reactivation. The two open reading frames in the 3′-flanking region were identified as the genes encoding a functional reactivating factor for inactivated diol dehydratase.[265] These genes of *K. oxytoca* were designated *ddrAB* because they are the genes for a “diol dehydratase-reactivating factor” (corresponding to *pduGH* in Figure 19A).

Homology searches revealed that polypeptides homologous to *K. oxytoca* DdrA and DdrB proteins are encoded by ORF4[51] or *dhaB4* and orf2b, respectively, of *K. pneumoniae* in the vicinity of the glycerol dehydratase genes (*gldABC* or *dhaB1B2B3*) (corresponding to *dhaBCE* in Figure 19B). By coexpressing these open reading frames with the glycerol dehydratase genes, they were identified as the genes encoding a functional reactivating factor for inactivated glycerol dehydratase. These genes of *K. pneumoniae* were named *gdrAB* genes after a “glycerol dehydratase-reactivating factor”.[266] The *dhaFG* genes of *Citrobacter freundii* that are homologous to *gdrAB* genes of *K. pneumoniae* were also demonstrated to be the genes for the reactivating factor for glycerol dehydratase.[267] Very recently, *E. coli eutA* was identified as the gene encoding the essential component of a functional reactivating factor for inactivated ethanolamine ammonia-lyase (Figure 19C).[268]

D. Discovery of Functional Reactivating Factors for B_{12}-Enzymes

Recombinant DdrA and DdrB proteins of *K. oxytoca* are co-purified to homogeneity from overexpressing *E. coli* harboring the expression plasmid for the *ddrAB* genes.[269] They exist as a tight complex with an apparent molecular weight of 150 000 whose subunit structure is most likely $(DdrA)_2(DdrB)_2$. The

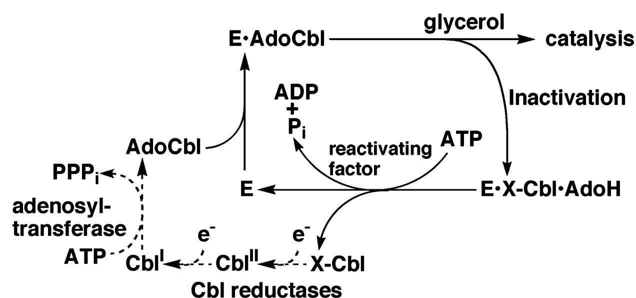


Figure 20. Reactivation of glycerol-inactivated holodiol dehydratase by the cobalamin exchange mechanism. E, apodiol dehydratase; AdoH, 5′-deoxyadenosine; Cbl^{II}, cob(II)alamin; Cbl^{I}, cob(I)alamin; X-Cbl, unidentified cobalamin formed upon mechanism-based inactivation by glycerol. Dotted lines represent the conversion of free X-Cbl to AdoCbl outside diol dehydratase.

purified factor actually functioned in vitro as a reactivating factor for glycerol-inactivated holodiol dehydratase in the presence of AdoCbl, ATP, and Mg^{2+}. O_2-inactivated holoenzyme and the inactive enzyme•CN-Cbl complex also undergo rapid reactivation and activation, respectively, by the factor under the same conditions. Figure 20 summarizes the function of the reactivating factor. When the holoenzyme undergoes inactivation by glycerol during catalysis or by O_2 in the absence of substrate, the enzyme-bound coenzyme loses the adenine moiety from its upper axial ligand through irreversible cleavage of the Co−C bond. It was shown that the reactivating factor mediates the ATP-dependent exchange of the enzyme-bound modified coenzyme or CN-Cbl with free intact AdoCbl. Intermediary formation of apoenzyme was demonstrated.[270] It was thus concluded that the function of the reactivating factor is to release a tightly bound adenine-lacking cobalamin from the enzyme, but not an adenine-containing cobalamin, such as AdoCbl or AdePeCbl. The modified coenzyme released from the enzyme active site is back-converted to AdoCbl by reductive adenosylation outside of the enzyme.

The GdrA and GdrB proteins of *K. pneumoniae* are overexpressed in *E. coli* and co-purified to homogeneity.[271] They also exist as a tight complex $(GdrA)_2(GdrB)_2$ and serve as the actual reactivating factor for glycerol-inactivated and O_2-inactivated hologlycerol dehydratase. The similar complex $(DhaF)_2(DhaG)_2$ of *C. freundii* was also shown to function as a reactivating factor for glycerol dehydratase.[267]

The reactivating factor for diol dehydratase efficiently cross-reactivates glycerol-inactivated glycerol dehydratase, whereas the reactivating factor for glycerol dehydratase hardly reactivates the inactivated diol dehydratase.[272] The latter result was confirmed with the glycerol dehydratase-reactivating factor of *C. freundii* as well.[267] From the results with hybrid reactivating factors, it was shown that the glycerol dehydratase-reactivating factor is much more specific for the dehydratase partner and that a large subunit of the reactivating factor principally determines the specificity for a dehydratase substrate.[272] It should be noted that the small subunits (DdrB and GdrB or B subunit) of both reactivating factors shows substantial homology to the β subunits of diol dehydratase and glycerol dehydratase.[265] When the conserved amino acid residues of the β subunit are mapped on the X-ray structure of diol dehydratase, they are localized not in the interface of the α and β subunits but in the certain part of the β subunit. It was thus suggested that the B subunit of the reactivating factors might be directly involved in the reactivation process by replacing the β subunit of the dehydratases in the interaction with cobalamin.[272]

E. Mechanism of Action of the Reactivating Factors

The mechanism of reactivation of holodiol dehydratase by its reactivating factor was investigated in vitro by using enzyme•CN-Cbl complex as a model of inactivated holoenzyme.[270] The factor showed low but distinct ATP-hydrolyzing activity. The turnover number is only 1.4 per min, and such an extremely low ATPase activity has been reported with molecular chaperones, such as chaperonine, as well.[273] The large subunit (A subunit) was shown to be responsible for the ATPase activity.[267] The factor forms a tight complex with apodiol dehydratase in the presence of ADP, but not in the presence of ATP.[270] When the factor is incubated with the enzyme•AdePeCbl complex, a nonactivatable complex, no complex formation was observed even in the presence of ADP. On the other hand, 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 enzyme•reactivating factor complex. The resulting tight complex is inactive even in the presence of added AdoCbl, but it dissociates upon incubation with ATP, forming apodiol dehydratase that are reconstitutable into catalytically active holoenzyme with added coenzyme. Thus, it was established that the reactivation of the inactivated holoenzyme by the reactivating factor in the presence of ATP and Mg^{2+} takes place in two steps: ADP-dependent cobalamin release and 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 conformation of the factor into the low-affinity form for diol dehydratase.

Figure 21A summarizes the mechanism of action of the reactivating factor.[270] The holoenzyme undergoes inactivation during catalysis accompanying irreversible cleavage of the coenzyme Co−C bond. The ADP-bound, high-affinity form of reactivating factor binds to the inactivated holoenzyme and causes its conformational change, which results in release of the modified coenzyme. The resulting tight complex is inactive, but it dissociates into the apoenzyme and the reactivating factor in the presence of ATP. The apoenzyme can thus be reconstituted into catalytically active holoenzyme. The reactivation and the ATP hydrolysis are not directly coupled, but the rates of inactivation by glycerol and ATP hydrolysis are almost equal. Thus, these two cycles are virtually synchronized. Figure 21B shows the mechanism how the modified coenzyme, that is, adenine-lacking co-

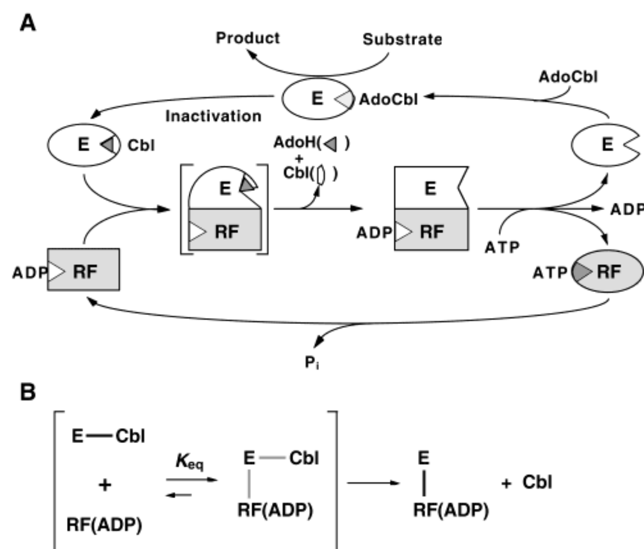


Figure 21. A proposed mechanism of reactivation of inactivated holodiol dehydratase by a reactivating factor. (A) Mechanism of the exchange of inactivated coenzyme for intact AdoCbl. (B) Possible mechanism of discrimination of the enzyme-bound adenine-lacking cobalamins from adenine-containing cobalamins. E, apodiol dehydratase; RF, reactivating factor; AdoH, 5′-deoxyadenosine; Cbl, cobalamin.

balamins, are discriminated from the adenine-containing ones upon release from the enzyme. It can be interpreted in terms of an equilibrium constant K in the formation of an intermediate ternary complex between the enzyme, cobalamin, and the reactivating factor. The presence of adenine moiety in the upper axial ligand in cobalamin would lower the K value by a factor of 1.7×10^4 when calculated from the K_D value for adenine.[112]

Exactly the same mechanism was demonstrated for the reactivation of inactivated glycerol dehydratase by the glycerol dehydratase-reactivating factor.[271]

F. Functional and Evolutionary Relationship of Reactivating Factors and Hsp70 Molecular Chaperones

So far, two reactivating factors for diol and glycerol dehydratases have been purified and their functions were confirmed in vitro. In addition, we identified a similar reactivating factor for ethanolamine ammonia-lyase as well.[274] The sequence of *glmL* (or *mutL*) gene that exists between *glmS* (*mutS*) and *glmE* (*mutE*) genes for clostridial glutamate mutase indicates that its product also shows fragmentary similarity to the DdrA and GdrA proteins.[109] This suggests that the product of this gene might be a component of the reactivating factor for glutamate mutase, although its function as a reactivating factor has not yet been confirmed. Therefore, such reactivating factors are not special but rather general for the AdoCbl-dependent enzymes. Each AdoCbl-dependent enzyme may have its own reactivating factor that plays a role in keeping the enzyme active. This seems reasonable because, in general, holoenzymes of AdoCbl-dependent enzymes tend to undergo inactivation during catalysis or by oxygen in the absence of substrate. Stadtman and co-workers[275] reported that β-lysine mutase (lysine 5,6-aminomutase) undergoes concomitant inactivation during catalysis, and that this inactivation is prevented by the addition of a sulfhydryl protein termed E2 and ATP. There might be possibilities that this phenomenon is due to reactivation of the inactivated holoenzyme and that E2 might be a kind of reactivating factor for β-lysine mutase.

In the mechanism illustrated in Figure 21B, the reactivating factors bind to the target proteins and induce their conformational change through tight complex formation with them. This results in release of the tightly bound, modified coenzyme, and the reactivating factors themselves do not become a constituent of the final products. Thus, they meet the criteria of molecular chaperones. Their extremely low ATPase activity is not unusual for molecular chaperones. Therefore, the reactivating factors for AdoCbl-dependent enzymes can be considered as a new type of molecular chaperons that participate in the reactivation of inactivated enzymes. When the amino acid sequences of main subunits of these reactivating factors are compared with those of molecular chaperones, neither of the reactivating factors showed overall similarity. But, all share three regions consisting of the 15−18 amino acid residues that show high fragmentary sequence similarities with those of the ATPase domain of *E. coli* DnaK protein and the other Hsp70 group molecular chaperones (Figure 22).[265,276,277] These conserved regions constitute the part of ADP-binding site of human Hsp70.[278,279] Therefore, it is highly suggested that the reactivating factors and the Hsp70 family of molecular chaperones are evolved from a common ancestor protein, and that the ATP/ADP-switching mechanism might be conserved between them.

Very recently, the crystal structure of the *K. pneumoniae* reactivating factor for glycerol dehydratase (glycerol dehydratase reactivase) has been reported by Liao et al.[280] The factor is an A_2B_2 heterotetramer (Figure 23A). The A subunit has an elongated structure consisting of the ATPase domain, the insert domain, the linker domain, and the swiveling domain (Figure 23B). It contains structural features resembling both GroEL and Hsp70 families of molecular chaperones. The B subunit is a small and globular protein whose fold resembles that of the enzyme β subunit, except that it lacks some AdoCbl-binding elements, as expected from the homology analysis.[265] On the basis of the structure, it was proposed that the damaged cofactor is removed from the inactivated holoenzyme by the "subunit swap" mechanism.[280] This hypothesis is consistent with the earlier finding that neither the β subunit (component F) nor the αγ complex (component S) of diol dehydratase alone binds cobalamins.[281] The crystal structure of the *K. oxytoca* diol dehydratase-reactivating factor has almost been determined.[282] As expected from the sequence similarity and the results of cross-reactivation experiments, the structure is very similar to that of the glycerol dehydratase-reactivating factor. The conserved regions between the reactivating factors and Hsp70 family molecular chaperones

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Kox DdrA   4 IAGIDIGNSSTEVAL 18···409 LAILDLGAGSTDASIINP 426···549 IPFVVLVGGSSLDFEV 564
Kpn GdrA   4 IAGIDIGNATTEVAL 18···411 LAILDLGAGSTDAAIVNA 428···551 IAFVVLVGGSSLDFEI 566
Eco EutA   8 SVGIDIGTTTTQVIF 22···148 VLNIDIGGGTANYALFDA 165···445 DIGTPLFGGSVVPVTV 460
Cco GlmL   4 YLLLDFGSTYTKLTA 18···253 LIVVDIGGATTDIHSIAD 270···389 VKTVIGTGGVLVHSKN 424
Eco DnaK   4 IIGIDLGTTNSCVAI 18···190 IAVYDLGGGTFDISIIEI 207···334 IDDVILVGGQTRMPMV 349
hum Hsp70  6 AVGIDLGTTYSCVGV 20···195 VLIFDLGGGTFDVSILTI 212···333 IHDLVLVGGSTRIPKV 348
               ••* *  •  •  •            *•*•••  •   •            •  • ••**•     •
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Figure 22. Fragmentary sequence homology between reactivating factors and Hsp70 family molecular chaperones. Kox DdrA, *K. oxytoca* DdrA;[265] Kpn GdrA, *K. pneumonia* GdrA;[266] Eco EutA, *E. coli* EutA;[268] Cco GlmL, *Clostridium cochlearium* GlmL;[109] Eco DnaK, *E. coli* DnaK;[276] Hum Hsp70, human Hsp70.[277]

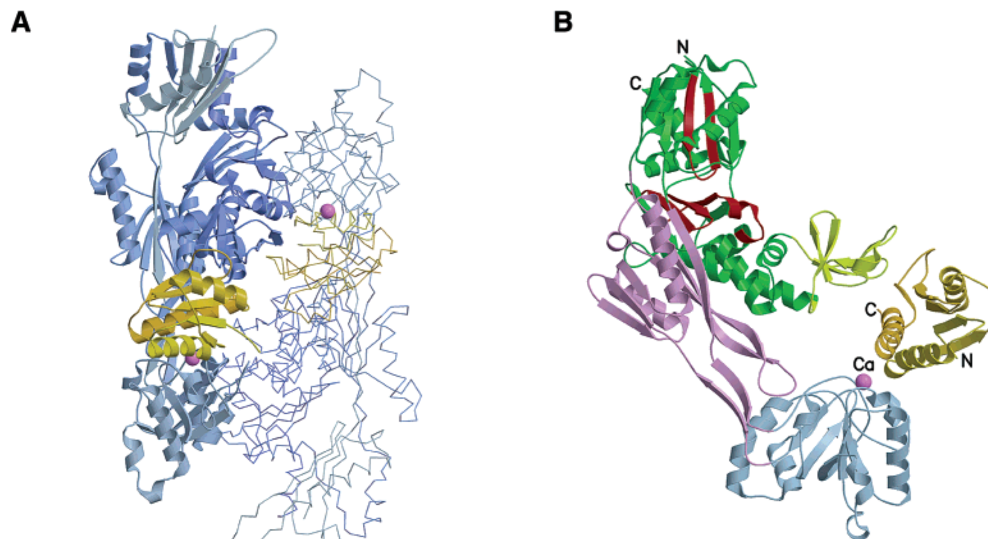

Figure 23. Crystal structures of glycerol dehydratase-reactivating factor. (A) Overall structure A_2B_2 ($\alpha_2\beta_2$). Blue and gold colors indicate the A (α) and B (β) subunits, respectively, darkening continuously from the N-terminal to the C-terminal sides. (B) Heterodimer unit AB ($\alpha\beta$). Violet for Ca^{2+}. The ATPase domain, linker domain, swiveling domain, and insert domain are colored green, plum, skyblue, and yellow, respectively. Color code for the B (β) subunit is the same as that for panel A. The conserved regions between reactivating factors and Hsp70 family molecular chaperones are shown in red. Drawn with MOLSCRIPT[285] and RASTER3D[286] using the coordinates obtained by Liao et al.[280]

(Figure 22) correspond to the ADP-binding site and thus must constitute an ADP/ATP switch. Crystal structures of the reactivating factors in complexes with ADP and ATP or its analogue are waited to reveal the conformational changes induced by ATP hydrolysis.

IX. Concluding Remarks

Several important conclusions for coenzyme B_{12} catalysis as well as enzyme mechanisms have been reached by recent investigations. First, steric strain models of the Co−C bond activation and homolysis have been proposed based on the X-ray structures. In these models, steric strains induced by tight interactions of the coenzyme to apoenzyme at the cobalamin moiety and the adenine ring are postulated to be important, although the nature of the strain differs among enzymes. The fact that substrate binding triggers the Co−C bond cleavage was also explained with diol dehydratase by the increased extent of steric strains that is induced upon substrate binding. Second, two models of radical transfer from coenzyme to substrates have been proposed to account for how the coenzyme-derived adenosyl radical comes close to the substrates and abstracts a hydrogen atom stereospecifically. The ribosyl rotation model may be common to the AdoCbl-dependent enzymes catalyzing isomerizations (eliminating), and the ribose pseudorotation model may be applicable to the enzymes catalyzing carbon-skeleton rearrangements. These two groups of enzymes are different not only in the mode of cobalamin binding but also in the distances between the cobalt atom and substrates and might use the different strategy. Third, results of the site-directed mutagenesis as well as theoretical calculations have made it possible to postulate the pathway of the radical rearrangements. In the 1,2-shift of the OH group catalyzed by diol dehydratase, the results of mutational experiments are consistent with the predictions by theoretical computations. These conclusions led us to propose the refined mechanism for diol dehyratase that is consistent with all the results of biochemical, structural, mutational, and theoretical investigations so far reported.

Enzymatic radical catalysis can be defined as the mechanism of catalysis by which enzymes catalyze chemically difficult reactions by utilizing the high reactivity of radicals. A simplified potential energy diagram was proposed as the concept of enzymatic radical catalysis by extending the mechanism of catalysis of AdoCbl-dependent rearrangements. In these reactions, substrates become activated to substrate radicals in exchange for stabilization of catalytic radicals by hydrogen abstraction from substrate. It is likely that these enzymes accelerate reaction rates by dividing a single transition state with a high barrier height into three or more transition states with lower barrier heights. In this respect, strategy of substrate activation in radical catalysis is similar

to that reported recently in covalent catalysis.[283,284] In these cases, enzymes create a high-energy state (ground-state destabilization) by introducing steric strains in the absence of substrate. When the substrate is bound by the enzyme, it becomes activated in coupling with the cancellation of the energized state. Unlike covalent catalysis, no covalent bond is formed between substrate and enzyme in radical catalysis. Another marked difference is that the radical enzymes can catalyze chemically more difficult reactions that require larger activation energy. Catalytic radical formation is usually triggered by the substrate binding. This is quite reasonable because catalytic radicals are highly reactive to oxygen. DFT computations suggested that the proposed concept of enzymatic radical catalysis seems to be energetically feasible, but computations with a realistic model that includes the active-site residues are awaited to solve the remaining discrepancy with experimental results.

One of the most interesting aspects obtained is that dynamic changes of the structures of the enzyme and the coenzyme are important. In diol dehydratase, AdoCbl undergoes substantial activation of its Co−C bond upon its binding to apoenzyme in the absence of substrate. Small but distinct conformational changes of the enzyme are induced by substrate binding which triggers the Co−C bond homolysis. The radical center formed comes closest to the substrate by the rotation of the ribosyl moiety. When the product dissociates from the active site, the ribosyl moiety undergoes back-rotation to regenerate the Co−C bond. The other possible movement is the oscillation of the DBI moiety with the axial Co−N bond length between 2.2 and 2.5 Å accompanying with the Co−C bond homolysis and reformation. This oscillation may be synchronized with the ribosyl rotation at a frequency of approximately 300−400 times/sec during catalysis. In methylmalonyl-CoA mutase, opening and closure of the active-site barrel are postulated to occur associating with the substrate uptake.[136] In glutamate mutase, ribose pseudorotation is postulated. Thus, it has been revealed that such dynamic structural changes of both enzyme and coenzyme are generally important for the catalysis of AdoCbl-dependent enzymes.

Most of AdoCbl-dependent enzymes tend to undergo inactivation during catalysis or by oxygen in the absence of substrate. In the case of diol and glycerol dehydratases, it has been an enigma for a long time that the enzymes undergo mechanism-based inactivation by glycerol, a physiological growth substrate for the bacteria that produce these enzymes. The respective reactivating factors for three AdoCbl-dependent enzymes were characterized so far. They reactivate the inactivated holoenzymes by releasing the tightly bound, modified coenzyme from the enzymes, forming apoenzymes that are reconstitutable to active holoenzymes with intact coenzyme. Their action mechanisms are molecular chaperone-like, and they actually show fragmentary sequence similarities with Hsp70 family molecular chaperones. The regions that showed similarity constitute the ADP-binding site of Hsp70. The recently reported structure of glycerol dehydratase-reactivating factor supports this prediction.[280] It was thus suggested that these reactivating factors and Hsp70 molecular chaperones are evolved from a common ancestor protein, and that the ADP/ATP-switching mechanisms may be conserved between them in the molecular evolution.

X. Acknowledgments

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