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Energetic Feasibility of Hydrogen Abstraction and Recombination in Coenzyme B_{12}-Dependent Diol Dehydratase Reaction[1]

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Coenzyme B_{12} serves as a cofactor for enzymatic radical reactions. The essential steps in all the coenzyme B_{12}-dependent rearrangements are two hydrogen abstraction steps: hydrogen abstraction of the adenosyl radical from substrates, and hydrogen back-abstraction (recombination) of a product-derived radical from 5′-deoxyadenosine. The energetic feasibility of these hydrogen abstraction steps in the diol dehyratase reaction was examined by theoretical calculations with a protein-free, simplified model at the B3LYP/6-311G* level of density functional theory. Activation energies for the hydrogen abstraction and recombination with 1,2-propanediol as substrate are 9.0 and 15.1 kcal/mol, respectively, and essentially not affected by coordination of the substrate and the radical intermediate to K^+. Since these energies can be considered to be supplied by the substrate-binding energy, the computational results with this simplified model indicate that the hydrogen abstraction and recombination in the coenzyme B_{12}-dependent diol dehydratase reaction are energetically feasible.

Key words: adenosylcobalamin, coenzyme B_{12}, density-functional-theory computation, diol dehydratase, enzymatic radical catalysis.

AdoCbl (coenzyme B_{12}) (Fig. 1A) is a naturally occurring organometallic compound that serves as a cofactor for enzymatic radical reactions including carbon skeleton rearrangements, heteroatom eliminations, and intramolecular amino group migrations. In these reactions, a hydrogen atom migrates from one carbon atom of the substrate to an adjacent carbon atom in exchange for a group X that moves in the opposite direction (Fig. 1B) (*1–3*). Diol and glycerol dehydratases catalyze the dehydration of 1,2-diols to the corresponding aldehydes (Eq. 1) (*4–7*).

$$\begin{array}{l} \text{R-CHCH}_2\text{OH} \\ \quad\vert \qquad\qquad \longrightarrow \text{R-CH}_2\text{CHO} + \text{H}_2\text{O} \\ \text{OH} \qquad (\text{R} = \text{CH}_3, \text{H}, \text{HOCH}_2) \end{array} \qquad (1)$$

Figure 1C depicts the minimal mechanism for diol dehydratase that has been established by labeling experiments as well as electron paramagnetic resonance and optical spectroscopy (*1, 8, 9*). This mechanism has later been accepted as a general one for all the AdoCbl-dependent rearrangements (*3, 10*).

AdoCbl-dependent reactions are triggered by the hydrogen abstraction of the adenosyl radical from substrates. This catalytic radical is generated by homolysis of the cobalt-carbon bond of the coenzyme. Recent crystallographic analysis of the diol dehydratase·adeninylpentylcobalamin complex revealed that tight interactions between the enzyme and coenzyme at both the cobalamin moiety and adenine ring of the adenosyl group produce angular strains and tensile force that inevitably break the Co-C bond (*11*). A modeling study based on the X-ray structure revealed that rotation of the ribosyl moiety around the glycosidic linkage makes the 5′-carbon radical accessible to the hydrogen atom of the substrate to be abstracted (Fig. 2) (*11*). Thus, the distance problem (*13, 14*) and the stereospecificity of the hydrogen abstraction (*15*), two generally important problems to be solved for AdoCbl-dependent enzymatic reactions, have been solved by our ribosyl rotation model (*11*). In addition, we have previously reported the direct participation of K^+ in the diol dehydratase catalysis by DFT computations (*16*). Based on the biochemical and computational results as well as the X-ray structure of the enzyme, we have proposed a new mechanism of action of diol dehydratase (Fig. 3) (*11*).

Certain enzymes utilize the high reactivity of radicals to catalyze chemically difficult reactions (*17–19*). We expressed the concept of enzymatic radical catalysis by a simplified potential energy diagram (*20*) and proposed that the enzymatic radical catalysis accelerates the reaction rate by dividing a transition state with a high energy barrier into three or more transition states with lower barriers. To provide energetic feasibility of the proposed concept of enzy-

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Abbreviations: AdoCbl, adenosylcobalamin or coenzyme B_{12}; DFT, density-functional-theory.

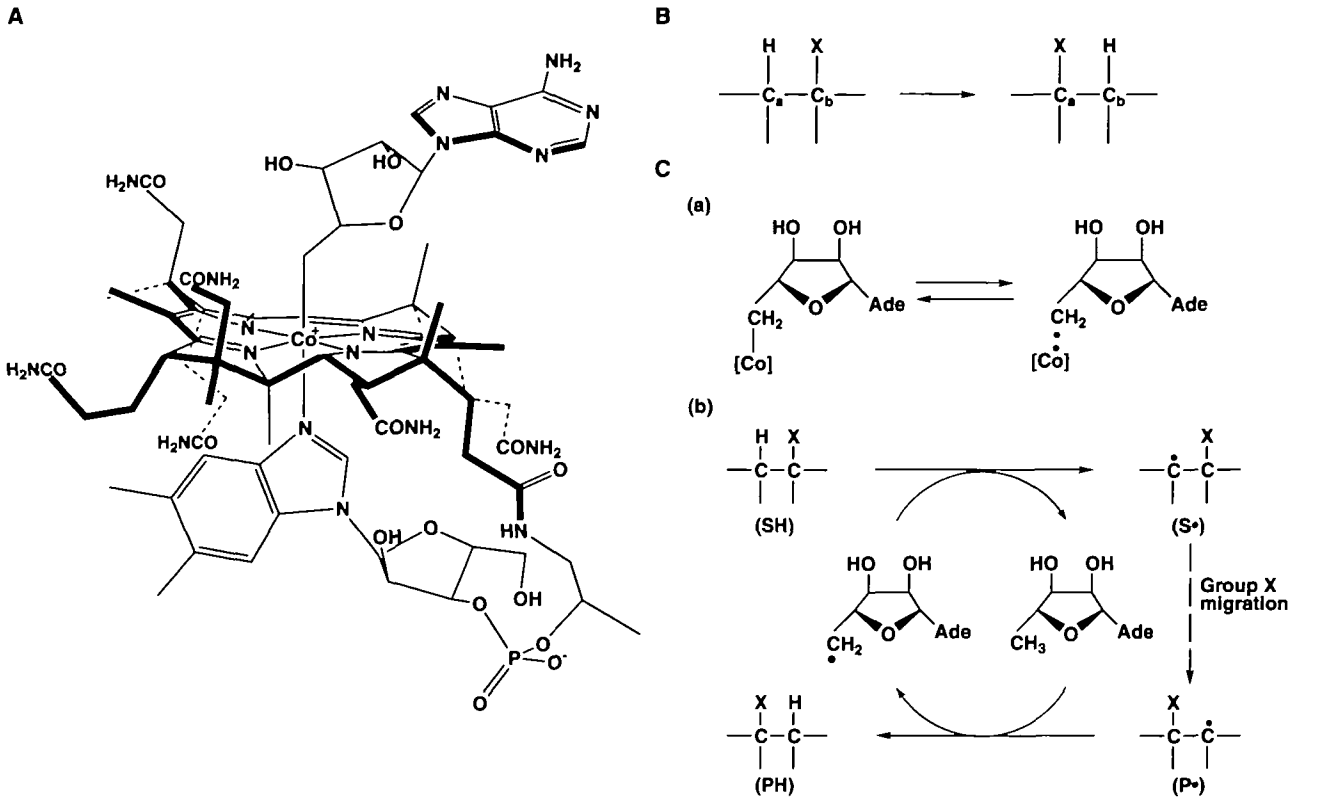

Fig. 1. **AdoCbl and the minimal mechanism of AdoCbl-dependent rearrangements.** A: Structure of AdoCbl (coenzyme B_{12}). B: General representation of AdoCbl-dependent rearrangements. X, a generic migrating group. C: Minimal mechanism for the AdoCbl-dependent rearrangements. (a) Homolysis of the Co-C bond of enzyme-bound AdoCbl. (b) Adenosyl radical-catalyzed enzymatic rearrangements. [Co], cobalamin; X, a generic migrating group.

matic radical catalysis (*20*), we attempted to obtain the entire energy profile of the diol dehydratase reaction by DFT computations on the basis of the crystal structure (*11, 14*).

In this paper, we report the energy profiles of the hydrogen abstraction and recombination steps in the diol dehydratase reaction. These theoretical analyses of structures and energies of intermediates and transition states are expected to provide a deeper insight into the energetic feasibility of the reactions catalyzed by this and other AdoCbl-dependent enzymes. Several papers have reported the mechanism of hydroxyl group migration based on theoretical calculations (*16, 21–27*), but no paper has yet appeared that reports a theoretical approach to the hydrogen abstraction and recombination steps in the AdoCbl-dependent rearrangements.

MATERIALS AND METHODS

DFT Computations—We carried out DFT computations along several possible paths of hydrogen abstraction and recombination in the diol dehydratase reaction using the hybrid Hartree–Fock/density-functional-theory B3LYP method implemented with the Gaussian 94 *ab initio* program package (*28*). This method consists of the Slater exchange, the Hartree–Fock exchange, the exchange functional of Becke (*29, 30*), the correlation functional of Lee, Yang, and Parr (LYP) (*31*), and the correlation functional of Vosko, Wilk, and Nusair (*32*). The triple-zeta 6-311G* basis set of Pople and coworkers (*33*) was used for C, O, and H, and the primitive set of Wachters (*34*) was used for K. We performed structure optimizations on local minima and saddle points (transition state) with models including substrate 1,2-propanediol (or reaction intermediates) and K^+. A vibrational analysis was carried out on each optimized structure to ensure that the structure corresponds to a local minimum that has no imaginary frequency or a saddle point that has only one imaginary frequency. Zero-point vibrational energies were taken into account in calculating the reaction energy profile. At each step of the reaction, some possible isomers (diastereomers) were calculated. The ethyl group was employed as a model of the adenosyl group. The amino acid residues in the active site of the enzyme were excluded from the model for simplicity, because electrostatic effects or hydrogen bonding with substrates would only weakly affect the non-polar process, such as the hydrogen abstraction and recombination by a radical mechanism.

Our quantum mechanical calculations include no thermal effects; the relative energies presented in this paper are energies with zero-point energy corrections at 0 K. It should also be noted that our calculations refer to isolated molecules in the gas phase. Since the crystal structure of diol dehydratase (*11, 14*) indicates that the active site is sequestered from water, we applied DFT calculations to this enzyme system to evaluate the energetics of the com-

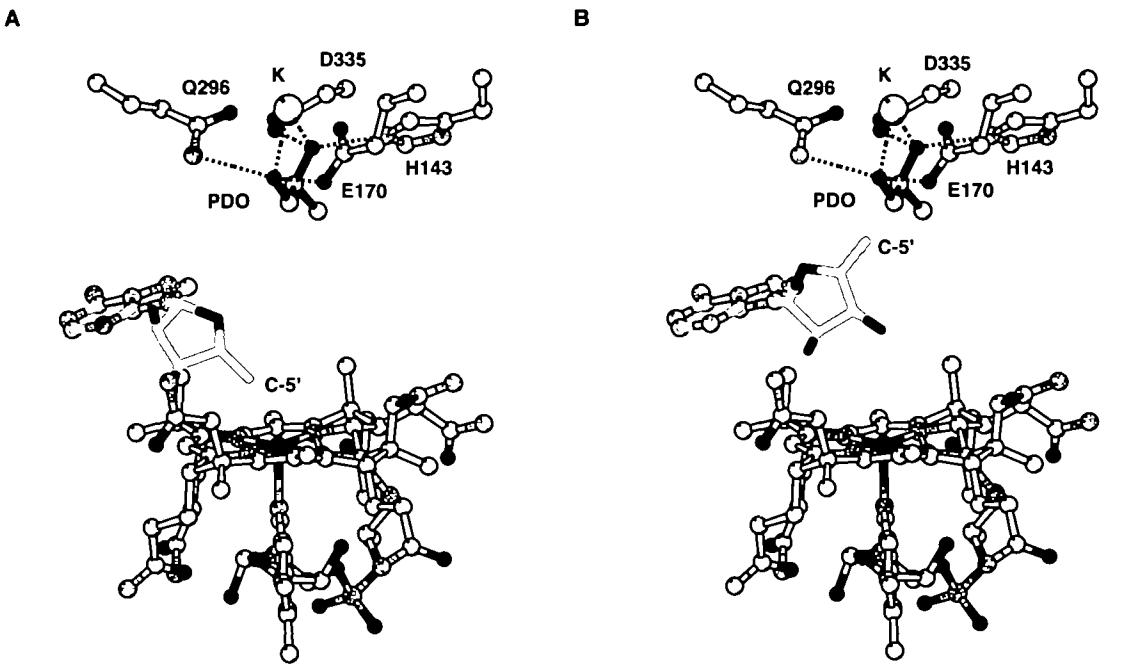

Fig. 2. **Two conformations of the enzyme-bound adenosyl radical.** Drawn with MOLSCRIPT (*12*) using the coordinates obtained by Masuda *et al.* (*11*). The carbon, oxygen, and nitrogen atoms are represented as open, closed, and light gray balls, respectively, and both the cobalt and phosphorus atoms as dark gray balls. Bonds in the substrate are shown as closed sticks. The numbers of amino acid residues represents those in the α subunit. The conformation of the adenosyl radical (gray sticks) were obtained by our ribosyl rotation model (*11*). A: "Proximal" conformation. B: "Distal" conformation.

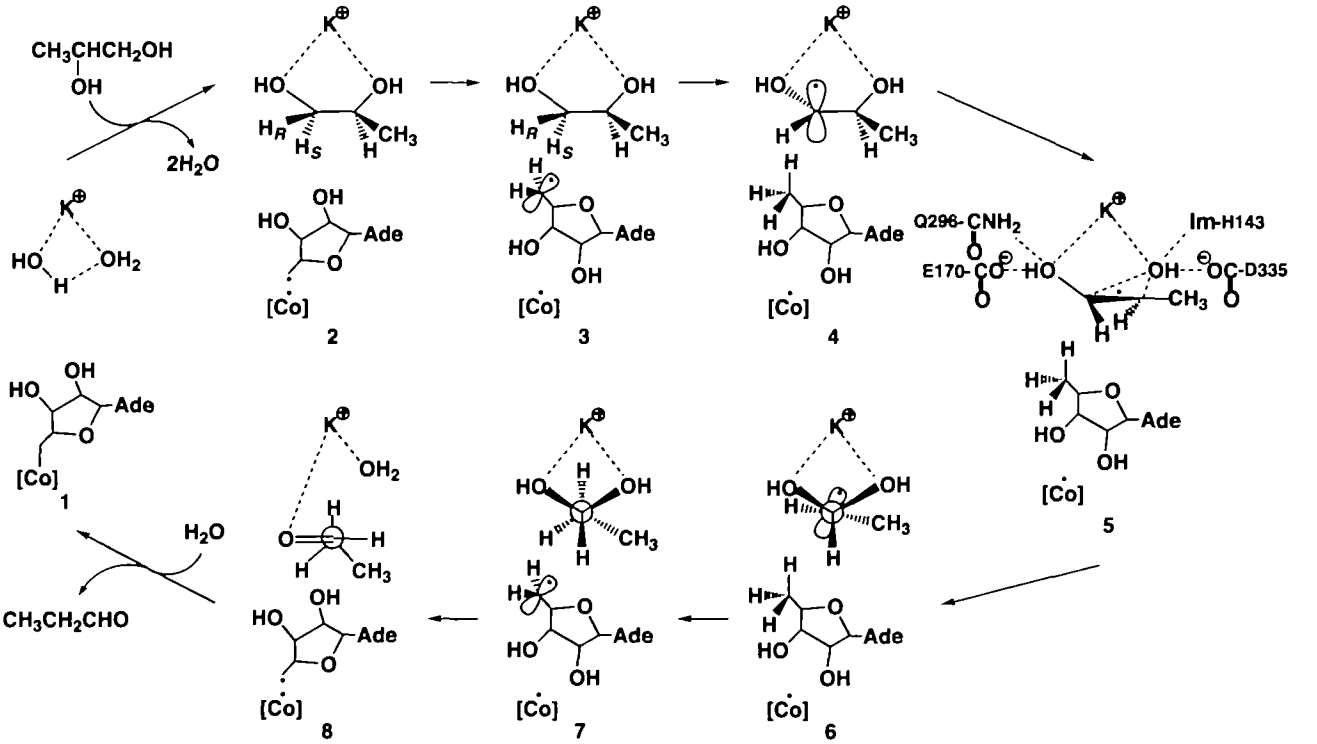

Fig. 3. **The proposed mechanism for AdoCbl-dependent diol dehydratase.** The reaction with (*S*)-1,2-propanediol is illustrated. [Co], cobalamin; Ade, 9-adeninyl group; Im, imidazole group of Hisα143.

plete reaction pathway.

RESULTS AND DISCUSSION

Hydrogen Abstraction—The whole reaction catalyzed by diol dehydratase was divided into three electronic processes: hydrogen abstraction (binding and hydrogen abstraction), OH group migration, and aldehyde production (hydrogen recombination, dehydration, and displacement). First, we optimized the structure of an adenosyl radical and compared its spin density on C(5) with that on C(1) of the ethyl radical. It was confirmed that the ethyl radical is suitable as a model of the adenosyl radical except for the steric hindrance of the adenosyl radical.

K^+ in the active site is seven-coordinated by five oxygen atoms from amino acid residues and two oxygen atoms of hydroxyl groups of substrate (Fig. 2) (*11*). This is in line with the general trend of K^+, which is usually six- to eight-coordinated in living organisms (*35*). According to the recently determined X-ray structure (*36*), K^+ in the substrate-free enzyme is also seven-coordinated: two oxygen atoms from two hydrogen-bonded water molecules coordinate to K^+ in the absence of substrate. The addition of substrate to the holoenzyme brings about a ligand exchange reaction with water molecules on K^+, which triggers a conformational change of the protein and advances the reaction to the next step. The relative energy of **1** [+ $K^+(OH_2)_2$ + ethyl radical + 1,2-propanediol] was taken as a standard (0.0 kcal/mol) to represent relative energies of the other states in the figures and in the following discussion. $K^+(OH_2)_2$ represents the potassium ion coordinated by only two hydrogen-bonded water molecules.

The crystal structure of diol dehydratase (*14*) indicates that 1,2-propanediol coordinates to K^+ before the hydroxyl group migration from C(2) to C(1) in the substrate-derived radical. We considered the following reaction path for K^+·1,2-propanediol radical complex (**4**): that is, substrate coordinates to K^+ before the hydrogen abstraction (Fig. 4). The relative energies and the optimized structures of the possible intermediates and transition states are shown in Fig. 4. Starting from $K^+(OH_2)_2$ and 1,2-propanediol including an intramolecular hydrogen bond (**1** in Fig. 4), substrate replaces the K^+-bound $(H_2O)_2$ to form an unstable complex with a net binding energy of –4.9 kcal/mol (from **1** to **2**) in the initial stage of the reaction. Of course, the actual substrate-binding energy must be positive, because the two hydroxyl groups of 1,2-propanediol form four hydrogen bonds with the four active-site residues in addition to their coordination to K^+. After the substrate binding, abstraction of the *pro-S* hydrogen atom from (*S*)-1,2-propanediol requires the activation energy of 9.0 kcal/mol (from **2** to **3**). The activation energy for the hydrogen abstraction from C(2) is smaller (7.5 kcal/mol), and the energy of the generated C(2) radical species is 2.4 kcal/mol lower than that of **4**. The activation energy for abstraction of the *pro-R* hydrogen atom from C(1) of the *S*-enantiomer would be nearly equal to that for the *pro-S* hydrogen abstraction. Despite these results of calculation, the adenosyl radical abstracts the *pro-S* hydrogen atom from C(1) of the *S*-enantiomer (*15*). Such strict stereospecificity is quite reasonable, because the catalytic radical is not a free radical but a protein-bound radical. The stereospecificity of the hydrogen abstraction has recently been well accounted for by our modeling study based on the X-ray structure (*11*). The barrier height for the hydrogen abstraction from C(1) of 1,2-propanediol is 9.6 kcal/mol without K^+, suggesting that this activation energy is almost not affected by coordination of the substrate to K^+.

The most notable geometric feature in **2** is the dihedral angle of O(1)–C(1)–C(2)–O(2). Because of the steric repulsion between O(1) and O(2), these four atoms and K^+ ion cannot form a plane. It is noteworthy that **2** is a local minimum structure, but it is not exactly the most stable structure on the potential energy surface. However, we chose **2** to illustrate the energetics in Fig. 4, because it is the conformation actually seen in the crystal structure (*11, 14*). The interactions of the substrate hydroxyl groups with active-site residues and the methyl group with surrounding protein residues would determine the conformation of the enzyme-bound substrate. This is likely to cause the difference of binding affinity between (*S*)- and (*R*)-1,2-propanediol. This is actually the case for diol dehydratase ($K_{M(R)}/K_{M(S)}$ = 3.2) (*37, 38*).

In structure **4**, as compared with **2**, slight changes in bond lengths of O(1)–C(1) (decrease by 0.06 Å) and K–O(1) (increase by 0.08 Å) were observed. These arise from the

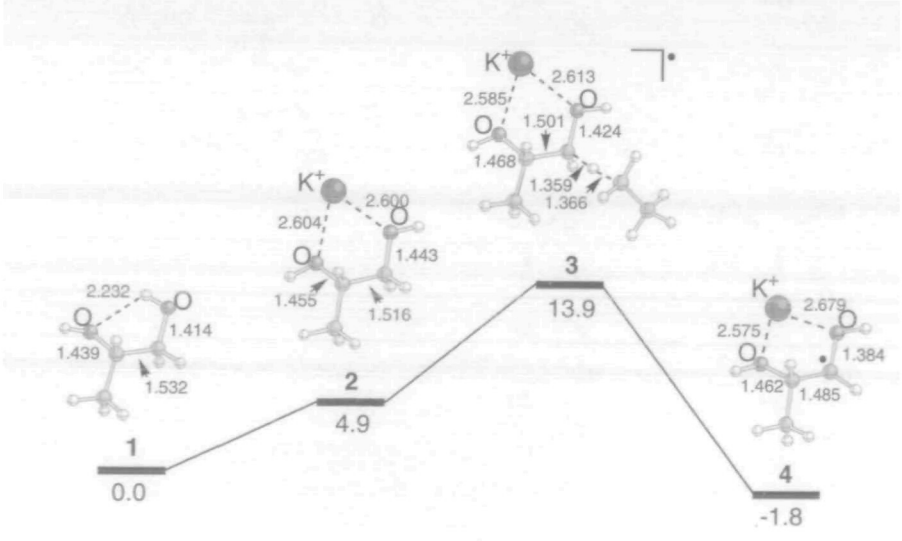

Fig. 4. **Energy diagrams for the hydrogen abstraction process (binding and hydrogen abstraction) (1 to 4) obtained by DFT computations.** Relative energies are in kcal/mol. The relative energy of **1** [+ $K^+(OH_2)_2$ + ethyl radical + 1,2-propanediol] was taken as a standard (0.0 kcal/mol).

slight contribution of a resonance structure possessing an O-centered radical and a concomitant C(1)–O(1) double bond, because the spin density is mostly localized on C(1) (0.90) and a little distributed on O(1) (0.10). Atom O(1) becomes electron-deficient in **4**, which causes an increase in the K–O(1) bond. Although the K–O bond lengths in the crystal structure are 2.38 Å [K–O(1)] and 2.40 Å [K–O(2)] (*14*), they are about 2.60 Å in the computed structure **2**. Almost all the K–O bond lengths in each step are about 2.6 Å in our model, except those in the structure of the transition state for the hydroxyl group migration. Therefore, the oxygen atoms of the enzyme-bound substrate may be a little more negatively charged by hydrogen bonding with Glu170 and Asp335 (*14*).

Hydroxyl Group Migration—Labeling experiments demonstrated that the oxygen atom of the product aldehyde is derived from O(1) and O(2) of (*S*)- and (*R*)-1,2-propanediol, respectively (*39*). These results indicate that 1,1-*gem*-diol is involved as an intermediate and undergoes stereospecific dehydration on the enzyme. The predissociation mechanism (*25*) seems inconsistent with these results. It was also reported that the unhydrated aldehyde is released from the enzyme (*40*). Based on the assumption that the 1,1-diol radical is formed bound to K^+, two possible pathways were considered for its conversion to the product aldehyde.

Figure 5 illustrates the energy diagram obtained with the protein-free, simplified model for the hydroxyl group migration from C(2) to C(1), that is, for the conversion of a 1,2-diol radical (substrate radical) to a 1,1-diol radical (product radical). Between a stepwise abstraction/recombination pathway *via* a direct hydroxyl group abstraction by K^+ and a concerted pathway assisted by K^+, both of which are consistent with the X-ray structure of the enzyme (*11, 14*), only a transition state for the concerted pathway was found by our computations. This is in accordance with our previous results (*16*). The barrier height for the transition state

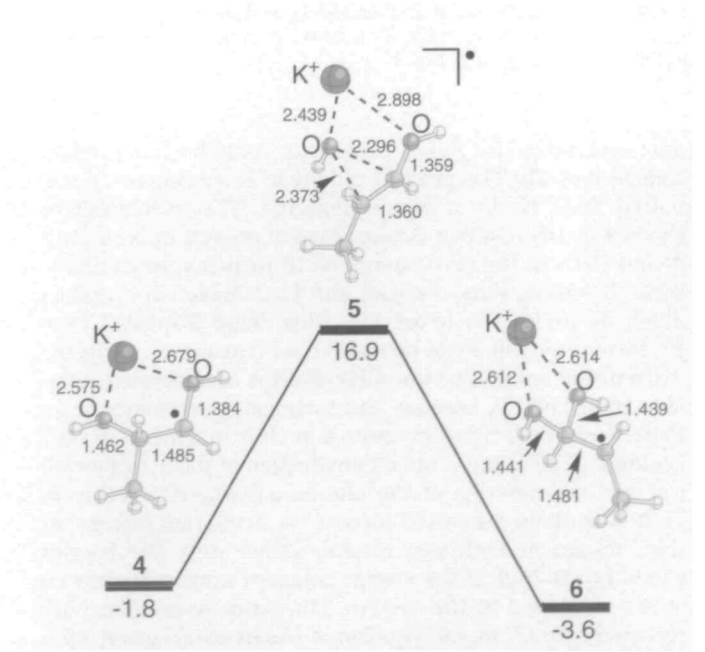

Fig. 5. **Energy diagrams for the hydroxyl group migration (4 to 6) obtained by DFT computations.** Relative energies are expressed as described in the legend to Fig. 4.

is 23.5 kcal/mol in the absence of K^+. The presence of K^+ lowers the activation energy of the hydroxyl group migration, but only by 4.8 kcal/mol (Fig. 5). This value of stabilization is not so large as we expected, and the activation energy for the hydroxyl group migration is larger than that for the hydrogen abstraction and hydrogen recombination. This is obviously contradictory to the earlier conclusion (*15, 41*) that the hydrogen abstraction is the rate-determining step for the overall diol dehydratase reaction. However, it should be noted that our computations are based on the simplified model without amino acid residues. If the contributions of active-site residues are taken into consideration, the energy of the transition state in the hydroxyl group migration may be lowered.

According to our test calculations at the B3LYP/3-21G level of theory, the presence of imidazolium is effective for transition state stabilization in the absence of K^+, but not effective in its presence (data not shown). In proteins, it is likely that Lewis acidity of K^+ is lowered by cancellation of its positive charge with negative charges of nearby amino acid residues. X-ray structure of diol dehydratase revealed that the active site is inside the negatively-charged $(\beta/\alpha)_8$ barrel (*14*). Hence, we concluded that the contribution of the electrostatic effect of K^+ to the 1,2-shift of the hydroxyl group is rather limited, and that a further mechanism of transition state stabilization is needed. We believe that the most important role of K^+ in the diol dehydratase catalysis is to fix substrates and intermediates in proper positions in order to ensure the stereospecific hydrogen abstraction and stereoselective hydrogen recombination between them and the adenosyl group. As described before (*16*), another important role of K^+ would be to increase the substrate-binding energy, at least part of which is utilized for the Co-C bond homolysis through the substrate-induced conformational change of the enzyme.

These conclusions are consistent with the finding from our mutational experiments that His143 and Glu170 are catalytically important residues (M. Kawata, T. Tobimatsu, and T. Toraya, to be published). Smith *et al.* (*23*) reported that the activation energy for the hydroxyl group migration is lowered by partial protonation of the migrating hydroxyl group. Recently, they have demonstrated by calculations that the barrier height for the transition state can be further lowered by partial deprotonation as well (*24*). According to their calculations, a Lewis acid, such as Li^+ or Na^+, alone is rather anticatalytic but facilitates the hydroxyl group migration in the presence of $HCOO^-$ (*24*). They also described that the effectiveness of K^+ is likely to be diminished by the surrounding oxygens in the active site. By taking the X-ray structure of diol dehydratase (*14*) into consideration, they proposed the retro-push-pull catalysis assisted by His143 and Glu170, although they have not calculated with a model including K^+ or imidazole.

Aldehyde Production—In Path A, parent 1,1-propanediol radical back-abstracts (recombines with) a hydrogen atom from 5′-deoxyadenosine prior to dehydration (Fig. 6). In Path B, dehydration of the 1,1-propanediol radical occurs first and is followed by hydrogen recombination. The contributions of amino acid residues in the protein were completely neglected in our model for simplicity. The equilibrium between 1,1-diol and the corresponding aldehyde in solution is well known, and the activation energy for this dehydration is quite small, especially in the presence of

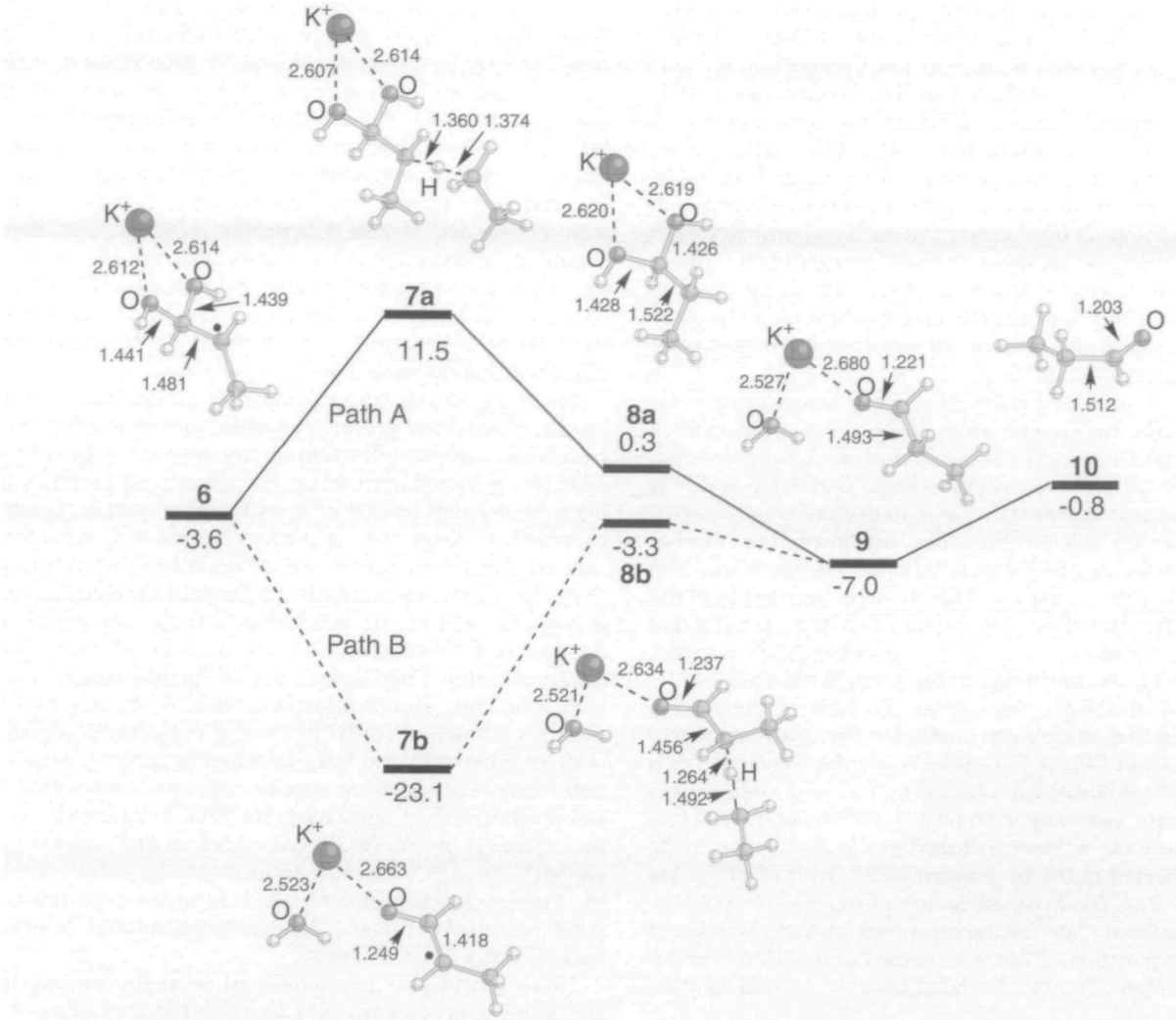

Fig. 6. **Energy diagrams for the aldehyde production process (hydrogen recombination, dehydration, and displacement) (6 to 10) obtained by DFT computations.** The path from **9** to **10** represents the displacement of product aldehyde from K^+ by water. Path A, solid lines; Path B, dotted lines. Relative energies are expressed as described in the legend to Fig. 4.

acid or base catalyst. By analogy, it can be assumed that there would be no step requiring large activation energy for the dehydration of 1,1-diol in the diol dehydratase reaction. Side chains of active-site residues, such as His143 and/or Glu170, could catalyze the dehydration by serving as proton donor and acceptor, respectively (M. Kawata, T. Tobimatsu, and T. Toraya, to be published).

The energy diagrams of these pathways and the optimized structures for the relevant intermediates and transition states are shown in Fig. 6. In Path A, the hydrogen recombination from **6** to **8a** requires activation energy of 15.1 kcal/mol. The activation energy for the hydrogen recombination from 1,1-*gem*-diol radical to 1,1-*gem*-diol is 14.5 kcal/mol without K^+ and is thus considered not to be affected by coordination to K^+. By the following dehydration, the energy of the system decreases by 7.3 kcal/mol when the reacting system moves from **8a** to **9**. On the other hand, in Path B, the dehydration from **6** to **7b** brings about stabilization by 19.5 kcal/mol. The activation energy for the subsequent hydrogen recombination from **7b** to **9** is 19.8 kcal/mol. In either pathway, 5′-deoxyadenosine reforms the adenosyl radical by giving a hydrogen atom back to product radical **6** or **7b**. The product aldehyde is subsequently displaced from K^+ by a water molecule. This displacement (from **9** to **10**) requires 6.2 kcal/mol of energy in both Path A and Path B. Relative energy of **10** includes propionaldehyde, $K^+(OH_2)_2$, ethyl radical, and H_2O. When the product aldehyde undergoes hydration after being displaced from K^+, its energy (aldehyde hydrate) is –7.0 kcal/mol in Fig. 6.

From the energetic viewpoint, Path A seems more favorable than Path B, because the hydrogen recombination in Path B requires more energy than that in Path A by 4.7 kcal/mol. Stabilization upon dehydration of the 1,1-diol radical and coordination of the eliminated water molecule in Path B leads to a marked increase of activation energy for the subsequent hydrogen recombination step. The barrier would be too high if the energy released upon dehydration is not conserved in the system. The strict stereoselectivity reported (*15, 42*) in substitution of the hydroxyl group by a hydrogen atom on C(2) also suggests that Path A is more likely, because the 1,1-diol radical can be more tightly fixed to K^+ and active-site residues and more easily controllable

than the aldehyde radical. It should be noted again that the interactions between the active-site residues and intermediates or transition states are completely neglected in our model. If they are taken into consideration, all the diol intermediates and transition states in Figs. 4–6 will be stabilized by hydrogen bonding with the amino acid residues in the active site, whereas the aldehyde radical **7b** and the transition state **8b** will not. This would be why the reaction intermediate is not trapped in **7b**.

The release of the product aldehyde from the active site results in conformational change of the enzyme to the substrate-free form. Upon this conformational change, the adenosyl radical would take a "proximal" conformation (Fig. 2) and recombine with Co(II) of cob(II)alamin to regenerate AdoCbl (*11*). Although the theoretical calculation of this step has not yet been done, release of the energy upon reformation of the Co–C bond would ensure the final steps of the reaction. When the next substrate comes into the active site, it replaces the K^+-bound $(H_2O)_2$, and a new catalytic cycle starts.

The activation energies for hydrogen abstraction and recombination in enzymatic radical catalysis (*20*) were obtained here for the first time by computations with a protein-free, simplified model of the AdoCbl-dependent diol dehydratase system. The activation energies for the hydrogen abstraction and recombination are 9.0 and 15.1 kcal/mol, respectively. These two steps correspond to the hydrogen abstraction from a CH_2OH group by a primary carbon radical (adenosyl radical) and the hydrogen abstraction from a CH_3 group by a secondary carbon radical (product-derived radical), respectively. Thus, the difference between these values of activation energy seems quite reasonable. It was also demonstrated here that the activation energies for the hydrogen abstraction and recombination are essentially not affected by coordination of the substrate and the radical intermediate to K^+. Although the activation energy for the hydroxyl group migration obtained here may be altered in the future by computations with a model including the active site residues ("realistic" model), the computational results reported in this paper with the simplified model are highly suggestive of the energetic feasibility of the hydrogen abstraction and recombination steps in the radical catalysis of diol dehydratase.

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