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Computational Mutation Analysis of Hydrogen Abstraction and Radical Rearrangement Steps in the Catalysis of Coenzyme B_{12}-Dependent Diol Dehydratase

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Abstract: A mutation analysis of the catalytic functions of active-site residues of coenzyme B_{12}-dependent diol dehydratase in the conversion of 1,2-propanediol to 1,1-propanediol has been carried out by using QM/MM computations. Mutants His143Ala, Glu170Gln, Glu170Ala, and Glu170Ala/Glu221Ala were considered to estimate the impact of the mutations of His143 and Glu170. In the His143Ala mutant the activation energy for OH migration increased to 16.4 from 11.5 kcal mol^{-1} in the wild-type enzyme. The highest activation energy, 19.6 kcal mol^{-1}, was measured for hydrogen back-abstraction in this reaction. The transition state for OH migration is not sufficiently stabilized by the hydrogen-bonding interaction formed between the spectator OH group and Gln170 in the Glu170Gln mutant, which demonstrates that a strong proton acceptor is required to promote OH migration. In the Glu170Ala mutant, a new strong hydrogen bond is formed between the spectator OH group and Glu221. A computed activation energy of 13.6 kcal mol^{-1} for OH migration in the Glu170Ala mutant is only 2.1 kcal mol^{-1} higher than the corresponding barrier in the wild-type enzyme. Despite the low activation barrier, the Glu170Ala mutant is inactive because the subsequent hydrogen back-abstraction is energetically demanding in this mutant. OH migration is not feasible in the Glu170Ala/Glu221Ala mutant because the activation barrier for OH migration is greatly increased by the loss of COO^- groups near the spectator OH group. This result indicates that the effect of partial deprotonation of the spectator OH group is the most important factor in reducing the activation barrier for OH migration in the conversion of 1,2-propanediol to 1,1-propanediol catalyzed by diol dehydratase.

Keywords: computational mutations · density functional calculations · enzymes · QM/MM methods · reaction mechanisms

Introduction

Diol dehydratase is an adenosylcobalamin (AdoCbl)-dependent enzyme that catalyzes the conversion of 1,2-diols into the corresponding aldehydes.[1] It is widely accepted that all AdoCbl-dependent reactions are initiated by homolytic cleavage of the Co–C bond of adenosylcobalamin to form the 5′-deoxy-5′-adenosyl radical ($AdoCH_2^{\bullet}$).[2f–i,3] This adenosyl radical abstracts a hydrogen atom from C1 to form the 1,2-diol radical (substrate-derived radical), and then the OH group on C2 migrates to C1 leading to the formation of the 1,1-diol radical (product-related radical), as shown in Scheme 1.[2–4] The resultant radical species abstracts a hydrogen atom from 5′-deoxyadenosine ($AdoCH_3$), which leads to the formation of the 1,1-diol and the regeneration of the $AdoCH_2$ radical. The rate of radical formation is at least two-to-three times faster than the overall reaction rate, which indicates that homolysis of the Co–C bond is not a rate-determining step.[1i,3a] The stereochemical course of the reaction was established by labeling experiments conducted by the groups of Abeles[2a] and Retey[4] and rationalized recently by X-ray crystallographic analysis of the structures.[5d]

The crystal structures of the complexes of diol dehydratase with cyanocobalamin and adeninylpentylcobalamin (AdePeCbl) were determined by Toraya and co-workers.[5] The K^+ ion is located in the inner part of the active-site

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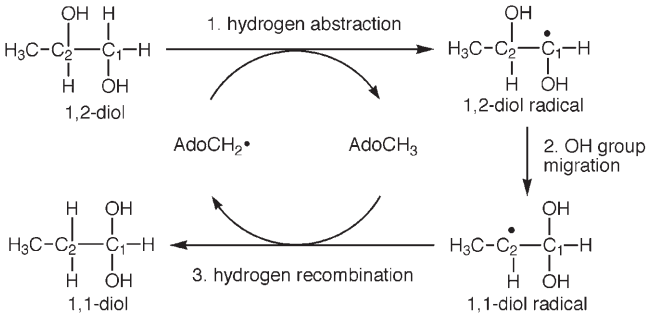

Scheme 1. Mechanism for the formation of 1,1-diols from 1,2-diols catalyzed by diol dehydratase.

cavity, as shown in Figure 1. This K^+ ion is coordinated by five oxygen atoms originating from the side chains of

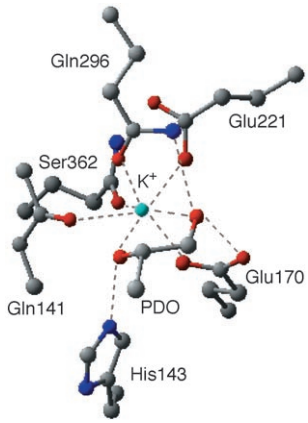

Figure 1. Active site residues, substrate, and the K^+ ion in the crystal structure of diol dehydratase.

Gln141, Glu170, Glu221, Gln296, and the carbonyl group of Ser362. The sixth and seventh coordination positions are occupied by the two hydroxy groups of the substrate. Theoretical calculations have been extensively performed to gain an understanding of the catalytic roles of the K^+ ion and the active-site amino acid residues in the diol dehydratase reaction.[6,7] By using a small model involving a K^+ ion, propanediol, and an ethyl radical, we have shown that the substrate and the radical intermediates are always bound to the K^+ ion during the reaction. The activation energy for OH group migration is lowered by the K^+ ion by only 2.2 kcal mol^{-1}. These results suggest that the most important role of the K^+ ion in the reaction is to fix the substrate and the intermediates in a suitable position to ensure hydrogen abstraction and recombination. Radom and co-workers[7] proposed that the activation energy of the OH group migration is greatly reduced by partial protonation of the migrating OH group (partial proton transfer to the migrating OH group from the protonating species) and partial deprotonation of the spectator OH group (partial proton transfer from the spectator OH group to the deprotonating species). Recently, we built a whole-enzyme model with 13500 atoms based on the crystal structure and investigated the catalytic roles of His143 and Glu170 by using QM/MM calculations.[8,9] The activation energy of the OH migration step is reduced by 1.6 and 5.6 kcal mol^{-1} by partial protonation and deprotonation, respectively.

Site-directed mutagenesis, in which one amino acid residue in a protein is replaced by another, is a powerful tool for revealing catalytic mechanisms and altering the properties of enzymes.[10,11] Several mutants of diol dehydratase were prepared by site-directed mutagenesis to investigate the contributions of the amino acid residues at the active site to the catalysis.[3a,12] The Glu221Ala mutant enzyme does not form an $(\alpha\beta\gamma)_2$ complex, which suggests that this mutation indirectly disrupts subunit contacts. The Glu170Ala, Asp335Ala, and Asp335Asn mutants are totally inactive ($<0.01\,\%$) and the His143Ala mutant shows only 5.1 % of the activity of the wild-type enzyme, which indicates that Glu170, Asp335, and His143 are the catalytic residues. However, it is difficult to clearly and unambiguously interpret the results of such experiments because of the size and complexity of proteins, the extreme sensitivity of catalytic activity brought about by modest structural perturbation, and the complex mechanisms of enzymatic reactions. In this theoretical study we demonstrate that a computational mutation analysis is useful for understanding the catalytic functions of active-site residues. We examine the enzymatic activity of several mutants of diol dehydratase in an effort to understand how the amino acid residues in the active site of diol dehydratase contribute to hydrogen atom abstraction and OH group migration in the dehydration reaction.

Computational Methods

We built a model structure of mutants from the optimized structure of the diol dehydratase–AdoCbl complex obtained in our previous study.[8] One or two amino acid residues were changed in mutants and then initial MM minimization was performed while the QM region was fixed. The completed model with about 13500 atoms was used as an initial structure for two-layer ONIOM (IMOMM) calculations[13] implemented in the Gaussian 03 program.[14] In these calculations, a specified region around the active site was calculated with a QM method, while the rest of the protein was treated at an MM level, as shown in Equation (1). The QM region describes the essential bond-breaking and -forming processes in the enzyme, whereas the MM region can promote interactions with the QM region through partial charges and van der Waals interactions of atoms in the MM region. At the QM/MM border, atoms in the MM region bound to an atom in the QM region were replaced by hydrogen atoms during the QM part of the ONIOM calculation. We applied the mechanical embedding scheme to treat the QM/MM electrostatic interactions.

$$E_{\mathrm{total}} = E_{\mathrm{QM}}(\mathrm{B3LYP}) + E_{\mathrm{all}}(\mathrm{Amber96}) - E_{\mathrm{QM}}(\mathrm{Amber96}) \quad (1)$$

We chose the K^+ ion, the ribose moiety of the adenosyl radical, the side chains of Gln141, His143, Glu170, Glu221, Gln296, the carbonyl group of Ser362, and (*S*)-1,2-propandiol (PDO) as the QM region. The *S* enantiomer is preferred in the binding by diol dehydratase.[15,16] The His143, Glu170, and Glu221 residues were replaced by alanine or glutamine in mutants. QM calculations were performed with the B3LYP method, which consists of the Slater and Hartree–Fock exchanges, the exchange functional of Becke,[17] the correlation functional of Lee, Yang, and Parr

(LYP),[18] and the correlation functional of Vosko, Wilk, and Nusair.[19] We used the 6-31G* basis set[20,21] on all atoms to carry out geometry optimizations. Single-point energies of the optimized structures were computed with the 6-311++G** basis set[22,23] of Pople and co-workers for carbon, oxygen, nitrogen, and hydrogen atoms, and the primitive set of Wachters[24] for the potassium atom. DFT methods have been extensively used in radical reactions and hydrogen-bonded systems.[25–28] The method of choice for MM calculations is the amber force field (Amber96).[29] We used the amber parameters for cobalamin developed by Marques et al.[30] All geometries are fully optimized unless otherwise noted. To estimate the activation energies in this reaction in a similar way to the previous study,[8] we scanned the potential energy surface in fine steps along a selected reaction coordinate by using partial optimization. The highest-energy geometry was used as the optimized structure for the transition state. Detailed data on the transition state search are collected in the Supporting Information (Tables S1–S14). To confirm the reliability of this method, we recalculated the transition state for hydrogen abstraction and back-abstraction of a small model[6] by energy scanning. As summarized in the Supporting Information (Tables S13 and 14), the energy is very similar to the fully optimized structure and the energy scan can thus be reasonably applied to estimate the activation barrier for these reaction steps.

Results and Discussion

Overview of OH group migration in wild-type diol dehydratase: Let us first look at the reaction profile for the hydrogen abstraction and OH group migration steps in the wild-type enzyme before we discuss the catalytic efficiency of several mutants. Because we described the results in detail in a previous paper,[8] herein we refer to the essential aspects of the reaction pathway. Figure 2 shows the key structural features of the calculated geometries of the reactant, intermediates, and transition states for these processes obtained by using a whole-enzyme model. Although we present only the K^+ ion, substrate, adenosyl radical, His143, and Glu170 for clarity, the optimized structure of the reactant complex has the K^+ ion seven-coordinated by five oxygen atoms of amino acid residues and two oxygen atoms of the hydroxy groups of the substrate as the crystal structure. Substrate binding triggers the homolysis of the Co–C bond in this enzyme.[3a,5c] The Co–C bond dissociation energy of coenzyme B_{12} has been estimated to be about 30 kcal mol^{-1} from experimental[31,32] and theoretical studies.[33–38] Toraya and co-workers[5b] have demonstrated through a modeling study of the X-ray structure of the diol dehydratase–AdePeCbl complex that the adenosyl radical produced comes into contact with the *pro-S* hydrogen atom on the C1 atom of the substrate to abstract the hydrogen atom via TS1 in the initial stages of the reaction (Figure 2). The activation barrier calculated for this hydrogen-atom abstraction is 13.6 kcal mol^{-1} when measured relative to the reactant complex. The resultant 1,2-diol radical involves an α-hydroxy radical species that is up to 10^5 times more acidic than the corresponding alcohol.[39–41] This radical species is stabilized in the presence of Glu170 because the anionic carboxy group of Glu170 attracts the partially activated hydroxy proton from the spectator OH group of the radical species. The subsequent OH group migration proceeds in a concerted manner through a triangular transition state (TS2) to form the 1,1-diol radical. This OH group migration has an activation energy of 11.5 kcal mol^{-1}. In this process the COO^- group of Glu170 temporarily accepts a proton from the spectator OH group. The partial deprotonation of the OH group reduces the activation barrier for the OH migration step by 5.6 kcal mol^{-1} as proposed by Radom and co-workers.[7] The His143 residue also contributes to the stabilization of the transition state for OH group migration although the reduction of the barrier caused by the partial protonation of His143 is only 1.6 kcal mol^{-1}. In the following section, we look in detail at how the substitution of these two amino acid residues in mutants changes the activation barrier for OH group migration from energetic, structural, and electronic points of view.

Catalysis by the His143Ala mutant: Figure 3 shows the optimized geometries of the intermediates and transition states for the hydrogen abstraction and the OH group migration steps in the His143Ala mutant, in which His143 has mutated to alanine. With this mutant there is no direct interaction between the Ala143 residue and the O2 atom of the substrate throughout the reaction. In the reactant complex, the lengths of the K–O1 and K–O2 bonds were calculated to be 2.85 and 2.89 Å, respectively. These distances are elongated in comparison with the wild-type enzyme, whereas other geometric parameters remain almost unchanged. This result suggests that the His143Ala mutation lowers the affinity of the enzyme for the substrate.

The transition state for hydrogen-atom transfer in the His143Ala mutant was computed to have a C5′–H bond

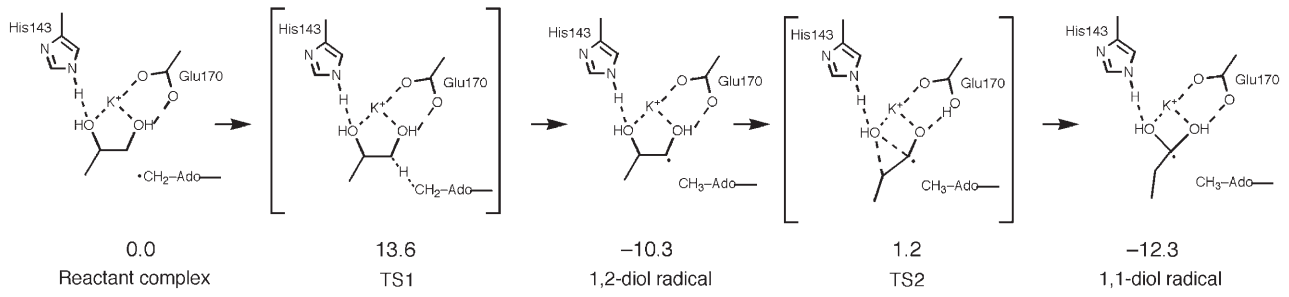

Figure 2. A mechanism for hydrogen abstraction and OH migration in the whole-enzyme model of diol dehydratase. Dotted lines indicate K–O bonds and hydrogen bonds. Energies in kcal mol^{-1}.

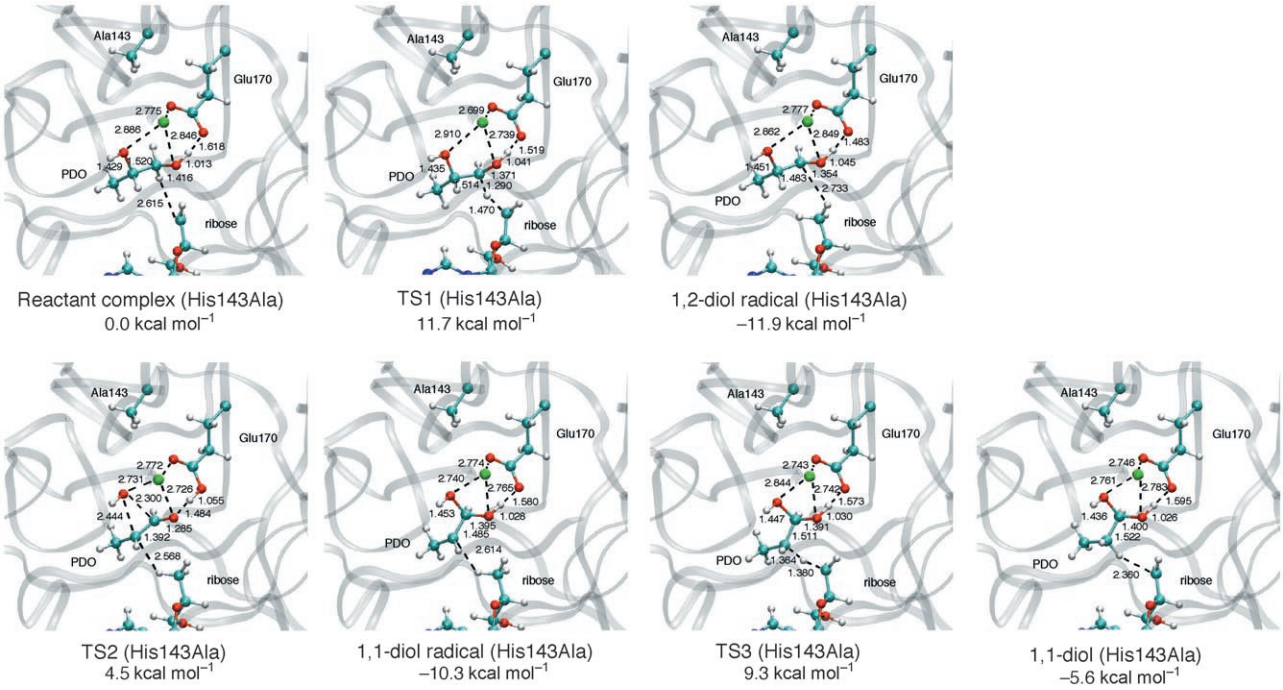

Figure 3. Optimized geometries and computed relative energies for hydrogen abstractions and OH group migration in the His143Ala mutant model.

length of 1.47 Å and a C1–H bond length of 1.29 Å. The activation barrier calculated for hydrogen-atom abstraction is 11.7 kcal mol^{-1} relative to the reactant complex. The O1–H bond is partially activated by the COO^- moiety of Glu170 in the resultant 1,2-diol radical; the O1–H bond length increases by 0.03 Å and the O(Glu170)–H bond length decreases by 0.14 Å. These structural changes are also observed in the wild-type enzyme; the O1–H bond length increases by 0.04 Å and the O(Glu170)–H bond length decreases by 0.13 Å. This result shows that the spectator OH group is activated to a similar extent as in the wild-type enzyme without a hydrogen-bonding interaction between the migrating OH group and His143.

We previously reported that OH group migration proceeds in a concerted manner via a transition state with a triangular structure and that the activation barrier for this step is lowered mainly by deprotonation of the spectator OH group induced by Glu170 in the whole-enzyme model of the wild-type enzyme.[6,8] The OH group migration occurs in a similar concerted manner in the His143Ala mutant. As depicted in Figure 3, the COO^- group of Glu170 temporarily accepts a proton from the spectator OH group to promote O2–C2 bond cleavage. The transition state for this step also has a triangular structure; the C–C bond length is 1.39 Å and the C–O1 and C–O2 bond lengths are 2.30 and 2.44 Å, respectively. Although these optimized bond lengths are similar to those in the wild-type enzyme, the computed activation barrier of 16.4 kcal mol^{-1} for OH group migration in the His143Ala mutant is 4.9 kcal mol^{-1} higher than the corresponding energy in the wild-type enzyme. This result shows that replacing His143 with an alanine residue leads to an increase in the activation energy for this step because the hydrogen-bonding interaction between His143 and the migrating OH group energetically stabilizes the transition state in the wild-type enzyme.

Previously we considered two His143 models of the wild-type enzyme: the HIE model that has a hydrogen atom at the ε-nitrogen (Nε2) and the HID model that has a hydrogen atom at the δ-nitrogen (Nδ1).[8] Because the HID model has no hydrogen-bonding interaction between the O2 atom of the substrate and His143, we can predict the reduction of the barrier caused by partial protonation of the migrating OH group from the difference in the activation energies of the HIE and HID models. The reduction of the barrier was evaluated to be 1.6 kcal mol^{-1}, which is 3.3 kcal mol^{-1} lower than that in the His143Ala mutant. This result implies that the mutation of His143 affects the activation barrier for OH group migration as a result of significant structural changes induced by the replacement of the bulky side chain in addition to the effect of partial protonation.

Radom and co-workers[42,43] and our group[6b] independently reported that the activation barrier for the hydrogen back-abstraction (hydrogen recombination) reactions are slightly larger than those of the initial abstraction reactions and proposed that hydrogen back-abstraction is the rate-limiting step in the wild-type enzyme. We considered the hydrogen back-abstraction process in the His143Ala mutant to assess the impact of the mutation on the activation barrier in this process. For the purpose of comparison, the optimized geometries of the transition state for hydrogen back-abstraction and the 1,1-diol in the wild-type enzyme are shown in the Supporting Information (Figure S1). The C2 atom of the 1,1-diol radical is close to the CH_3 group of 5′-deoxyadenosine and abstracts a hydrogen atom from it to

produce the 1,1-diol and reform the adenosyl radical. To find the transition state for hydrogen back-abstraction, we scanned the potential energy surface of this process along the C2–H and C5′–H coordinates. The relative energy of the system increases with a shortening of the C2–H bond and the total profile reaches a maximum at a C5′–H bond length of 1.38 Å. The hydrogen back-abstraction was found to be the most energetically demanding step in this reaction, the activation energy from the 1,1-diol radical was calculated to be 19.6 and 17.9 kcal mol^{-1} in the His143Ala mutant and the wild-type enzyme, respectively. Inclusion of the free energy of the entire protein would lead to a reduction of the barriers for hydrogen-atom abstraction because of the loss of a high-frequency C–H mode that becomes imaginary in the transition states (2–4 kcal mol^{-1}).[44] This result suggests that the structural changes at the active site induced by the mutation raises the activation barrier for this C–H bond cleavage by 1.7 kcal mol^{-1}, which corresponds to a 16-fold rate deceleration. The reported k_{cat} value for this mutant is approximately 2% of that of the wild-type enzyme,[12] which is in reasonable agreement with this prediction. In fact, significant structural differences are observed between the His143Ala mutant and the wild-type enzyme; the C2–H distance increases by 0.13 Å in the 1,1-diol radical and the C5′–H distance decreases by 0.45 Å in the 1,1-diol. These structural changes would block the access of the adenosyl radical to the hydrogen atom and promote a backward reaction in the His143Ala mutant. Thus, the His143 residue plays an important role, not only in OH group migration, but also in the hydrogen back-abstraction reaction, which has no direct relevance to His143. The deuterium kinetic isotope effect (KIE) on the rate of the overall reaction is 4.0 for the His143Ala mutant when determined with [1,1-D_2]1,2-propanediol as the substrate,[3a] which is much less than that for the wild-type enzyme (KIE = 10). This result indicates that breaking of the C–H bond is not fully, but partially, rate-determining in the His143Ala mutant. This finding is reasonable because the barrier heights for OH group migration (16.4 kcal mol^{-1}) and hydrogen back-abstraction (19.6 kcal mol^{-1}) with the mutant are closer than those with the wild-type enzyme.

Mutational analysis of the catalytic functions of the Glu170 residue: In a previous study we showed that the effect of partial deprotonation is more important for transition-state stabilization than that of partial protonation.[8] To gain a better understanding of the catalytic functions of Glu170, we consider three types of mutants of diol dehydratase, the Glu170Gln, Glu170Ala, and Glu170Ala/Glu221Ala mutants. The activation energy for OH group migration should be raised in these mutants because glutamine and alanine cannot serve as good proton acceptors.

Catalysis by the Glu170Gln mutant: In the Glu170Gln mutant there may be a weak hydrogen bond between the amido group of Gln170 and the spectator OH group of the substrate, in addition to the coordination of the carbonyl oxygen atom to the K^+ ion. One possible mode for the hydrogen-bonding interaction is shown in Figure 4, with the amido nitrogen atom serving as the hydrogen-bond acceptor. An alternative would be for the amido group of Gln170 to act as the donor and the OH group to act as the acceptor. Because the Gln296 residue is also hydrogen bonded to the spectator OH group, its amido group may serve as the

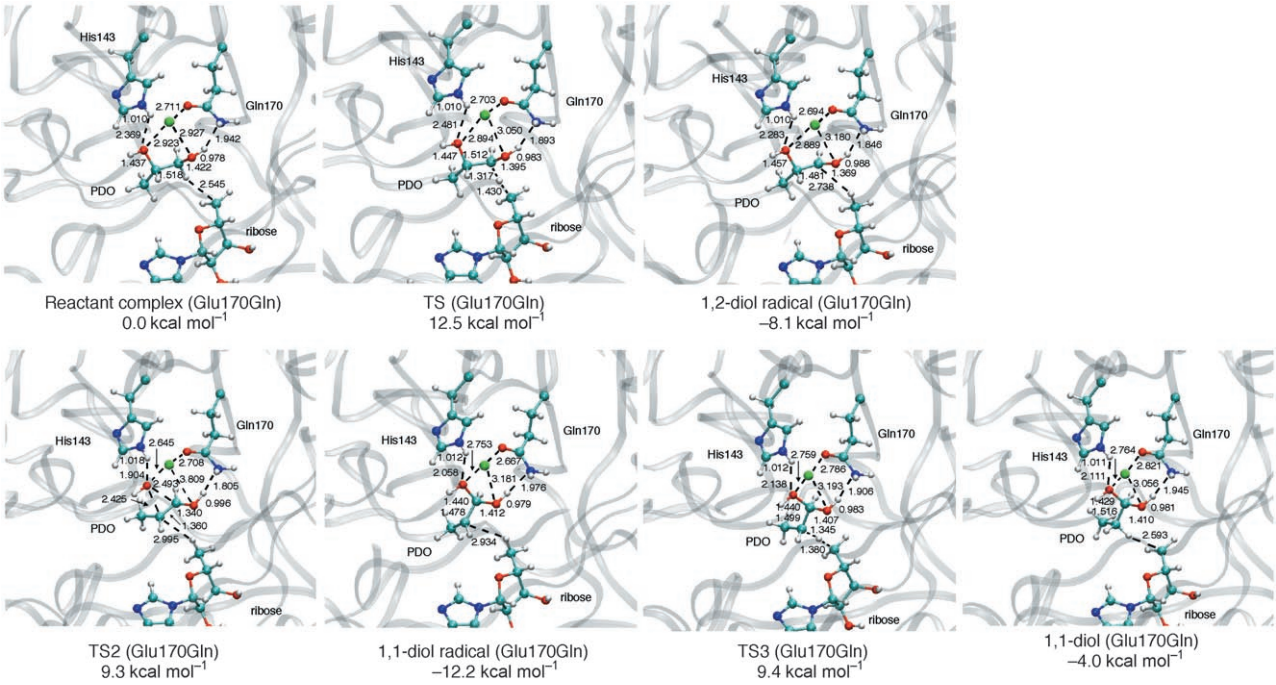

Figure 4. Optimized geometries and computed relative energies for hydrogen abstractions and OH group migration in the Glu170Gln mutant model.

donor in the former and the acceptor in the latter. As the energies of the two structures would not be so very different, we calculated the energy on the basis of the former. The activation energy for hydrogen-atom abstraction was computed to be 12.5 kcal mol^{-1} and the resultant 1,2-diol radical species is 8.1 kcal mol^{-1} more stable than the reactant complex; therefore, we expect that this process can take place in the Glu170Gln mutant. In contrast to the wild-type enzyme and the His143Ala mutant, the O–H bond length of the spectator OH group in the 1,2-diol radical of the Glu170Gln mutant remains essentially unchanged from the reactant complex; the O–H and N–H bond lengths are 0.99 and 1.85 Å, respectively. The energy of the 1,2-diol radical measured relative to the reactant complex is 2.2 and 3.8 kcal mol^{-1} higher than the corresponding energies in the wild-type enzyme and the His143Ala mutant, respectively. This difference is a direct consequence of the mutation of Glu170. These results indicate that the 1,2-diol radical species is not as well stabilized in this mutant by the weak hydrogen-bonding interaction between Gln170 and the spectator OH group.

The loss of the strong hydrogen-bonding interaction greatly destabilized the transition state for OH group migration, as we expected. The O–H bond length of the spectator OH group was calculated to be 1.00 Å, which demonstrates that Gln170 cannot activate the OH group in this transition state of the Glu170Gln mutant. The activation barrier of 17.4 kcal mol^{-1} computed for this step is 5.9 and 1.0 kcal mol^{-1} higher than that of the wild-type enzyme and the His143Ala mutant, respectively. This result supports our proposal[8] that Glu170 is the main catalytic residue in the catalysis by diol dehydratase. The transition state has a C1–O bond length of 2.49 Å, a C2–O bond length of 2.43 Å, and a C–C bond length of 1.36 Å in the triangular structure. Interestingly, the C–C bond length is a typical value for C=C double bonds in contrast to that in the wild-type enzyme and the His143Ala mutant; 1.41 Å in the wild-type enzyme and 1.39 Å in the His143Ala mutant. A similar short C–C bond is seen in the transition state for OH group migration in previous small-model calculations[6] that only involve the K^+ ion and (*S*)-1,2-propanediol, the C–C bond length of which is 1.36 Å. This result indicates that partial deprotonation of the OH group leads to better electron donation to the C–C bond, which would be essential for the reduction of the activation barrier of OH group migration in the wild-type enzyme and the His143Ala mutant.

In the 1,1-diol radical species produced, the distance between the C2 atom of the 1,1-diol radical and the CH_3 group of 5′-deoxyadenosine is significantly changed relative to the wild-type enzyme; 2.93 Å for the Glu170Gln mutant and 2.49 Å for the wild-type enzyme. This result indicates that the Glu170Gln mutant cannot control the position of the 1,1-diol radical species through the weak interaction between the spectator OH group and Gln170, which would increase the activation energy for the following hydrogen back-abstraction. The estimated C2–H and C5′–H bond lengths are 1.35 and 1.38 Å in the transition state for hydrogen back-abstraction, respectively. The activation barrier for this step is increased to 21.6 kcal mol^{-1}, which is 3.7 kcal mol^{-1} higher than that for the wild-type enzyme. Judging from these high activation barriers for OH group migration and hydrogen back-abstraction, the activity of the Glu170Gln mutant is likely to be low, which is consistent with the report by Kawata et al.[12] that the Glu170Gln mutant is essentially inactive (k_{cat}, 0.02% that of the wild-type enzyme).

Catalysis by the Glu170Ala mutant: We assumed that a water molecule coordinates to the K^+ ion in place of Glu170 in the Glu170Ala mutant because a five-coordinate K^+ ion is highly unlikely. There is no hydrogen-bonding interaction between the spectator OH group of the substrate and Ala170 in the Glu170Ala mutant. Thus, this alanine residue cannot keep the position of the spectator OH group in the protein environment of the Glu170Ala mutant, as shown in Figure 5. In the reactant complex of this mutant, the spectator OH group is strongly attracted electrostatically by Glu221, which brings about a significant change in the binding of the substrate. This interaction shortens the K–O2 bond from 2.79 to 2.64 Å and elongates the K–O1 bond from 2.81 to 3.06 Å to form a hydrogen bond of 1.70 Å between the spectator OH group and Glu221. The newly formed hydrogen bond has a structural feature similar to the important hydrogen-bonding network between the spectator OH group and Glu170 in the wild-type enzyme. Thus, the Glu221 residue is likely to act as a proton acceptor to reduce the activation barrier for OH group migration in the Glu170Ala mutant. The distance between the C5′ atom and the hydrogen atom to be cleaved is 2.47 Å in the reactant complex, which shows that the adenosyl radical is still in close contact with the substrate despite the serious structural changes in this mutant. The activation energy for hydrogen abstraction was calculated to be 9.1 kcal mol^{-1}, this value being 4.5 kcal mol^{-1} lower than that in the wild-type enzyme. In the 1,2-diol radical of this mutant, the H–O (Glu221) bond lengths are shortened to 1.60 Å. Moreover, the relative energy of the 1,2-diol radical was calculated to be −9.7 kcal mol^{-1}, which is comparable to the corresponding energy in the wild-type enzyme. The 1,2-diol radical species is stabilized in this mutant by the hydrogen-bonding interaction between Glu221 and the spectator OH group in a similar manner to the wild-type enzyme.

The subsequent OH group migration proceeds in a concerted manner through a triangular transition state. The C–C bond length is 1.37 Å, and the C–O1 and C–O2 bond lengths are 2.32 and 2.41 Å, respectively. The His143 and Glu221 residues are hydrogen bonded to the migrating and spectator OH groups, respectively. The activation barrier of 13.6 kcal mol^{-1} for OH group migration is only 2.1 kcal mol^{-1} higher than the corresponding barrier in the wild-type enzyme. These results indicate that partial protonation and deprotonation would take place with Glu221 instead of Glu170 and with the His143 residue in the catalysis with the Glu170Ala mutant. We expected that this mecha-

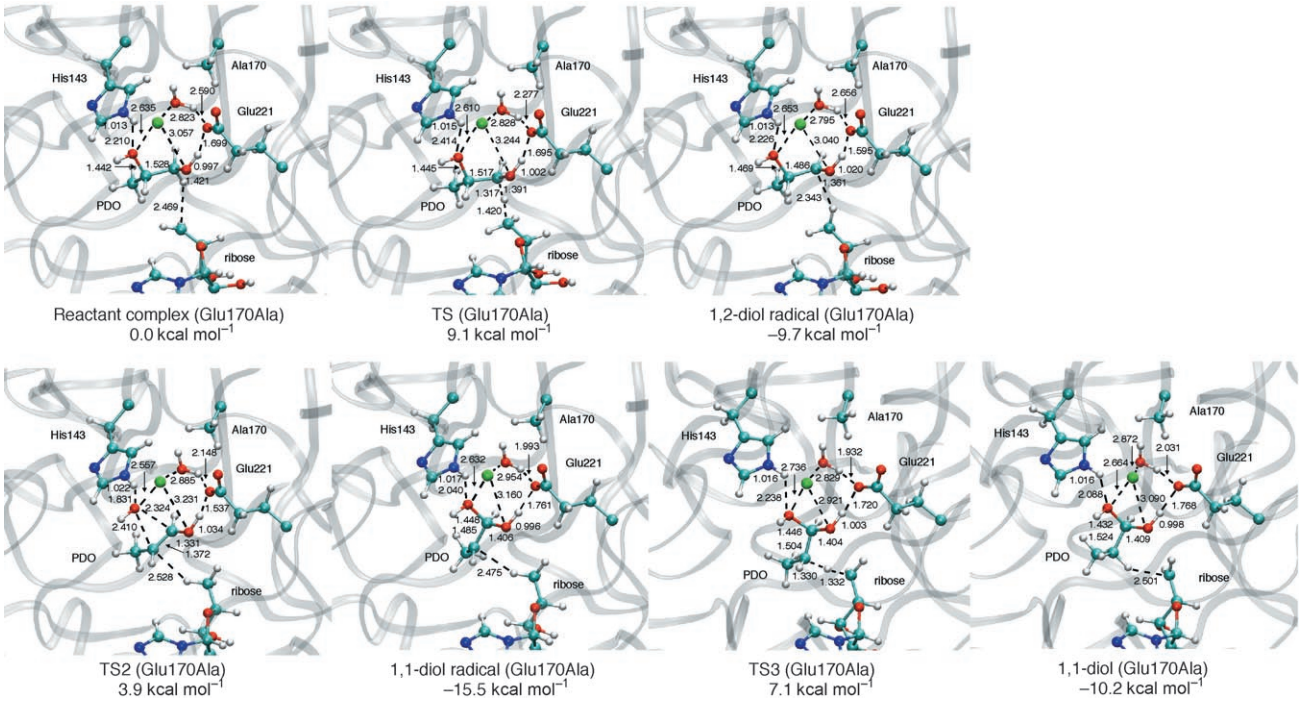

Figure 5. Optimized geometries and computed relative energies for hydrogen abstractions and OH group migration in the Glu170Ala mutant model.

nism might not work in this mutant because the side chain of Ala170 cannot abstract a proton from the migrating OH group, but this expectation turned out to be invalid because the Glu221 residue substitutes for Glu170 in this effect. QM/MM calculations suggest that the substrate is converted into the 1,1-diol radical more easily with the His143Ala and Glu170Ala mutants because of the low activation barriers in the hydrogen abstraction and the OH group migration processes. However, the activity (k_{cat}) of the Glu170Ala mutant is less than 0.01 % of the wild-type enzyme.[12] This inactivity of the Glu170Ala mutant has been ascribed to a high barrier for hydrogen back-abstraction. The activation energy for hydrogen back-abstraction was calculated to be 22.6 kcal mol^{-1}, which is 4.7 kcal mol^{-1} higher than that in the wild-type enzyme.

Catalysis by the Glu170Ala/Glu221Ala double mutant: As discussed in the previous section, the Glu221 residue is hydrogen bonded to the spectator OH group in place of Glu170 in the Glu170Ala mutant. Consequently, we cannot estimate the activation barrier for OH group migration in the absence of the COO^- group that is in the vicinity of the spectator OH group. Here we re-evaluate the effect of Glu170 by considering the Glu170Ala/Glu221Ala double mutant in which no COO^- groups are hydrogen bonded to the spectator OH group. This mutant has not been successfully constructed so far because the Glu221Ala mutation indirectly disrupts subunit contacts.[12] The computational mutagenesis approach described herein is a useful strategy for exploring the catalytic behavior of such an unknown mutant.

Figure 6 shows the optimized geometries of the intermediates and transition states for the hydrogen abstraction and the OH group migration steps in the Glu170Ala/Glu221Ala mutant. The distance between the O1 atom and the Cβ atom of Ala170 (Ala221) was calculated to be 5.54 (4.18) Å in the reactant complex, and therefore, these alanine residues would have a negligible effect on the spectator OH group. There is some argument as to the effect of Glu170 on the activation barrier for the hydrogen abstraction process. Semialjac and Schwarz[45] investigated hydrogen abstraction from 2-aminoethanol by the 1,5-dideoxyribos-5-yl radical and reported that the activation barrier for this hydrogen abstraction is lowered by an interaction of the substrate with only a protonating group in the catalysis by ethanolamine ammonia lyase. On the other hand, Sandala et al.[43] recently reported that the partial protonation of the migrating OH group has a minimal effect on the activation barrier, whereas partial deprotonation of the spectator OH group can substantially lower the barrier for the initial hydrogen abstraction. Our calculations show that an activation energy of 14.9 kcal mol^{-1} is required for this mutant to cleave the C1–H bond, which is the highest of the four mutants of diol dehydratase. The barrier height is raised as a result of the mutation of Glu170 and Glu221, which is consistent with the report of Sandala et al.[43]

To examine the role of substrate deprotonation on the activation energy for C–H bond cleavage, we computed the bond dissociation energy of the C–H bond of the substrate by using a simplified model in which formate is used as a model for Glu170, as shown in Figure 7. This formate molecule is hydrogen bonded to the OH group that corresponds

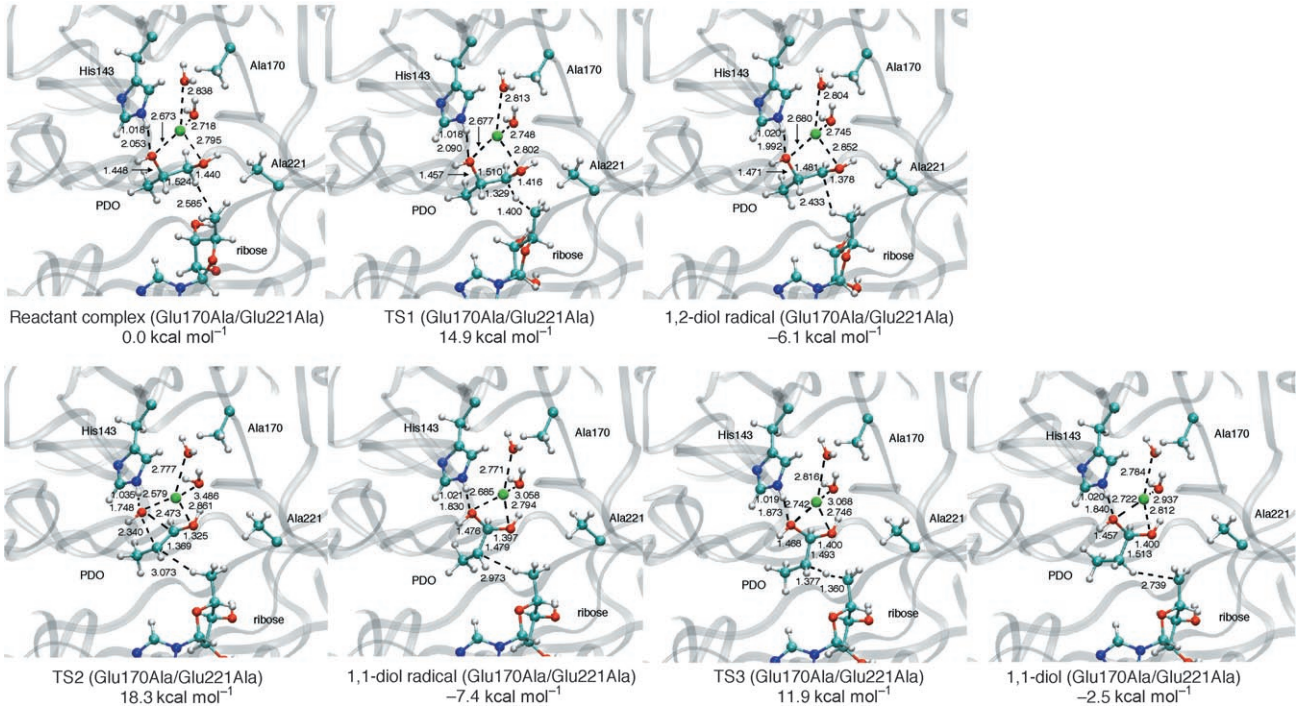

Figure 6. Optimized geometries and computed relative energies for hydrogen abstractions and OH group migration in the Glu170Ala/Glu221Ala mutant model.

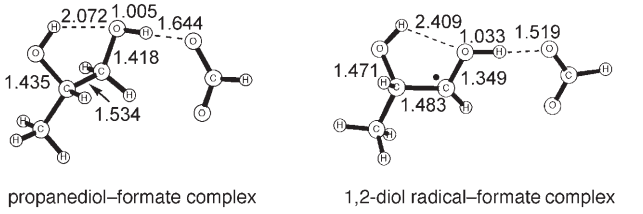

Figure 7. Optimized geometries of the propanediol– and 1,2-diol radical–formate complexes. Distances in Å.

to the spectator OH group in the wild-type enzyme and its mutants. The 1,2-diol radical species is partially deprotonated by the formate molecule, and the O–H bond length is elongated by 0.03 Å. The bond dissociation energy is computed to be 86.8 kcal mol^{-1} at the B3LYP/6-311++G** level of theory, which is 7.0 kcal mol^{-1} lower than that of free 1,2-propanediol. Full deprotonation further weakens the C–H bond; the bond dissociation energy is 75.2 kcal mol^{-1} at the same level of theory. These results suggest that partial deprotonation of the substrate induced by Glu170 contributes to the reduction of the barrier for hydrogen transfer.

As mentioned above, the transition state for OH group migration is stabilized only by His143 in this mutant; the distance between the O1 atom and the Cβ atom of Ala170 (Ala221) is 5.44 (5.18) Å, and the length of the hydrogen bond between the migrating OH group and His143 is 1.75 Å. This transition state has a short C1–C2 bond of 1.37 Å, which is essentially identical to that in the Glu170Gln mutant and in the simplified model we used previously.[6] The OH group migration is predicted to be very difficult with an activation barrier of 24.4 kcal mol^{-1} in this double mutant, which demonstrates that the effect of partial protonation of the migrating OH group does not sufficiently reduce the activation barrier for this process. Although the activation energy of 19.3 kcal mol^{-1} for hydrogen back-abstraction is low, the Glu170Ala/Glu221Ala double mutant would exhibit no reactivity towards 1,2-propanediol as a result of the high activation barrier for OH group migration.

Conclusions

In this study we have evaluated the reactivity of four kinds of mutants of diol dehydratase, His143Ala, Glu170Gln, Glu170Ala, and Glu170Ala/Glu221Ala, to understand the enzymatic functions of important amino acid residues in the active site by using ONIOM calculations. The whole-enzyme model of the mutants with about 13500 atoms was constructed by replacing one or two amino acid residues of diol dehydratase with other amino acid residues. The activation barriers for the His143Ala mutant, in which the migrating OH group is not activated, were computed to be 11.7 and 16.4 kcal mol^{-1} for the initial hydrogen abstraction and the OH group migration, respectively. The mutation of His143 raises the activation barrier for OH group migration by 4.9 kcal mol^{-1}, mainly as a result of the significant structural changes induced by the mutation of the His143 residue. We found that hydrogen back-abstraction is the most energetically demanding step in this reaction, which is consistent with our proposals[6b] and those by Radom and co-work-

ers[42,43] that the back-abstraction barrier is slightly larger than the initial abstraction barrier. In the Glu170Gln mutant, the Gln170 residue is hydrogen bonded to the spectator OH group of the substrate, but the hydrogen-bonding interaction is not sufficient to stabilize the 1,2-diol radical intermediate and the transition state for OH group migration. This weak hydrogen bond cannot fix the 1,1-diol radical in the proper position at the active site, which leads to a significant increase in the activation barrier for hydrogen back-abstraction. These results indicate that a strong proton acceptor at residue 170 is essential for catalysis by diol dehydratase. The Glu170Ala mutant has no hydrogen bond between the spectator OH group and Ala170, and thus, the spectator OH group is attracted to the nearest COO^- group of Glu221 at the active site, which lead to serious structural changes at the binding site. In this mutant, the Glu221 residue can serve as a good proton acceptor in place of Glu170. The activation barriers were computed to be 9.1 and 13.6 kcal mol^{-1} for the initial hydrogen abstraction and OH group migration, respectively, which indicates that the substrate is readily converted into the 1,1-diol radical in this mutant. However, the Glu170Ala mutant is totally inactive because a high energy of 22.6 kcal mol^{-1} is required to overcome the hydrogen back-abstraction barrier. We evaluated the reactivity of the Glu170Ala/Glu221Ala mutant in which there is no COO^- group in the vicinity of the spectator OH group. The His143 residue cannot stabilize the transition states for the initial hydrogen abstraction and OH group migration processes sufficiently in this mutant. This result is in good agreement with our previous conclusion that the effect of partial deprotonation of the spectator OH group is the most important factor that reduces the activation barriers for the diol dehydratase catalyzed dehydration reaction. Table 1 summarizes the calculated activation barriers and the estimated relative activities of the mutants. The experimentally determined low or negligible activities of the mutants tested were roughly predictable from their highest activation barriers. These results will encourage the application of this computational mutation approach to mechanistic studies of enzymatic reactions, to functional analysis of active-site residues, and to the rational design of enzymes with new catalytic functions.

Table 1. Activation barriers calculated for catalysis by diol dehydratase and its mutants.

	Activation barrier [kcal mol^{-1}]			
	TS1	TS2	TS3	Relative activity (exp[a])
wild type	13.6	11.5	17.9[b]	1.0 (1.0)
His143Ala	11.7	16.4	19.6[b]	6.3×10^{-2} (1.6×10^{-2})
Glu170Gln	12.5	17.4	21.6[b]	2.5×10^{-3} (2×10^{-4})
Glu170Ala	9.1	13.6	22.6[b]	4.9×10^{-4} ($<1\times10^{-4}$)
Glu170Ala/Glu221Ala	14.9	24.4[b]	19.3	6.4×10^{-18} (N.A.)

[a] Experimental data from ref. [12]. [b] We estimated the relative activities of the mutants by using these values.

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