Histidine-α143 Assists 1,2-Hydroxyl Group Migration and Protects Radical Intermediates in Coenzyme B_{12}-Dependent Diol Dehydratase†

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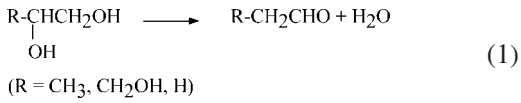
ABSTRACT: Diol dehydratase of *Klebsiella oxytoca* contains an essential histidine residue. Its X-ray structure revealed that the migrating hydroxyl group on C2 of substrate is hydrogen-bonded to Hisα143. Mutant enzymes in which Hisα143 was mutated to another amino acid residue were expressed in *Escherichia coli*, purified, and examined for enzymatic activity. The Hα143Q mutant was 34% as active as the wild-type enzyme. Hα143A and Hα143L showed only a trace of activity. Kinetic analyses indicated that the hydrogen bonding interaction between the hydroxyl group on C2 of substrate and the side chain of residue α143 is important not only for catalysis but also for protecting radical intermediates. Hα143E and Hα143K that did not exist as $(\alpha\beta\gamma)_2$ complexes were inactive. The deuterium kinetic isotope effect on the overall reaction suggested that a hydrogen abstraction step is fully rate-determining for the wild type and Hα143Q and partially rate-determining for Hα143A. The preference for substrate enantiomers was reversed by the Hα143Q mutation in both substrate binding and catalysis. Upon the inactivation of the Hα143A holoenzyme by 1,2-propanediol, cob(II)alamin without an organic radical coupling partner accumulated, 5′-deoxyadenosine was quantitatively formed from the coenzyme adenosyl group, and the apoenzyme itself was not damaged. This inactivation was thus concluded to be a mechanism-based inactivation. The holoenzyme of Hα143Q underwent irreversible inactivation by O_2 in the absence of substrate at a much lower rate than the wild type.

Adenosylcobalamin (AdoCbl,[1] coenzyme B_{12})-dependent diol dehydratase (DL-1,2-propanediol hydrolyase, EC 4.2.1.28) catalyzes the conversion of 1,2-propanediol, glycerol, and 1,2-ethanediol to the corresponding aldehydes (eq 1) (*1, 2*). Previous labeling experiments of Rétey et al. (*3, 4*) and



(1)

Abeles and co-workers (*5*) as well as kinetic (*6–9*) and spectroscopic (*10–15*) studies with this enzyme led to the elucidation of a minimal mechanism (Figure 1A) for AdoCbl-dependent rearrangements; that is, 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, where group X is a generic migrating group (OH group on C2 of 1,2-propanediol for diol dehydratase) (*16–18*). The initial early event is the homolytic Co−C bond cleavage of the enzyme-bound coenzyme forming an adenosyl radical that serves as a catalytic radical in the reactions. The X-ray structures of diol dehydratase have been reported (*19–22*).

Previously, we have reported that diol dehydratase is inactivated by chemical modification with diethyl pyrocarbonate, suggesting that this enzyme has an essential His residue (*23*), although the site of modification was not determined. The X-ray structure of this enzyme revealed that the migrating OH group on C2 and the spectator OH group on C1 of the substrate are hydrogen-bonded to Hisα143 and Aspα335 and to Gluα170 and Glnα296, respectively, in addition to their coordination to K^+ (*19*) (Figure 1B). Theoretical calculations with a simple model of diol dehydratase suggested that the activation barrier heights for the OH group migration could be partly lowered by partial protonation (*24*) or coordination to K^+ (*25–27*) of the migrating OH group. Smith et al. showed by calculation that the barrier height is further lowered by partial deprotonation of the spectator OH group (*28*) and proposed a synergistic retro-push–pull catalysis assisted by the active-site residues. The QM/MM calculation of Kamachi et al. with a whole-enzyme model predicted that the transition state for the OH group migration is markedly stabilized by the interactions with the active-site residues and proposed that Gluα170 and

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[1] Abbreviations: AdoCbl, adenosylcobalamin or coenzyme B_{12}; aqCbl, aquacobalamin; CN-Cbl, cyanocobalamin; EPR, electron paramagnetic resonance; KIE, kinetic isotope effect; OH-Cbl, hydroxocobalamin; MBTH, 3-methyl-2-benzothiazolinone hydrazone; PAGE, polyacrylamide gel electrophoresis; QM/MM, quantum mechanical/molecular mechanical; SDS, sodium dodecyl sulfate.

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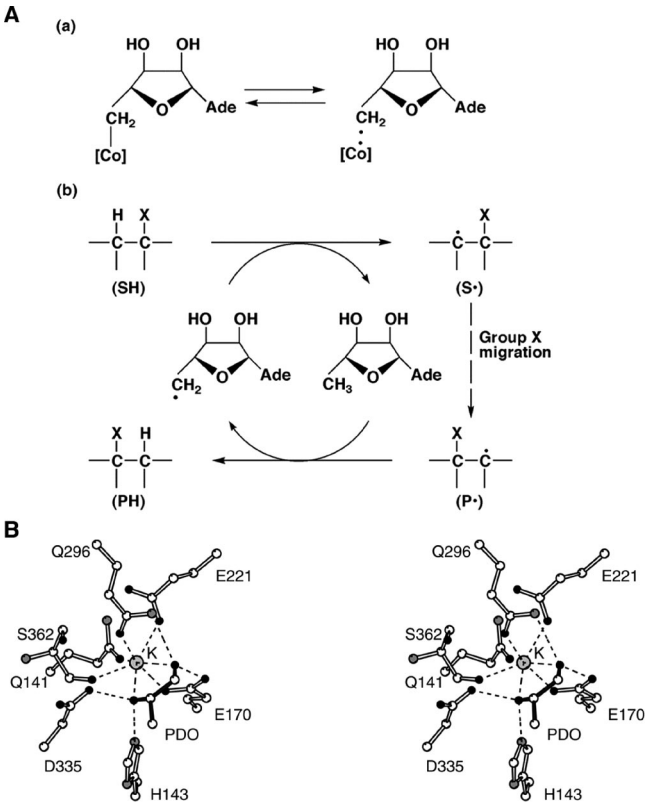


FIGURE 1: Minimal mechanism for AdoCbl-dependent diol dehydratase (A) and active-site structure of the enzyme (B). (a) Homolysis of the Co−C bond of enzyme-bound AdoCbl. (b) Adenosyl radical-catalyzed rearrangements. [Co], cobalamin; Ade, 9-adeninyl; SH, substrate; PH, product; X, a generic migrating group (X = OH on C2 of 1,2-propanediol in the diol dehydratase reaction). Residue numbers in the α subunit. Only the interaction with the (*S*)-1,2-propanediol is shown in panel B. For the interaction with the *R*-enantiomer, see ref *22*. PDO, 1,2-propanediol. The Nε2 atom of the Hisα143 residue is hydrogen-bonded to the OH group on C2 of the substrate.

unprotonated Hisα143 are important for the diol dehydratase catalysis in this order (*29*). Very recently, Sandala et al. suggested a major contribution of the partial deprotonation and a minimum contribution of the partial protonation to lowering the activation barrier for the initial hydrogen abstraction (*30*).

Site-directed mutagenesis is a powerful method of analyzing the functions of the active-site amino acid residues in catalyses. Recently, we surveyed the catalytic residues and reported the essential roles of Gluα170 and Aspα335 in the diol dehydratase catalysis (*31*). Hisα143 is one of the catalytic residues, but detailed mutational analysis of its function has not yet been carried out. To improve our understanding of the action mechanism of this type of enzymes, we studied the functional role of Hisα143 by mutational experiments. We report here the preparation and catalytic properties of mutant diol dehydratases in which Hisα143 is substituted with another amino acid residue. The results of spectroscopic as well as EPR experiments with mutant enzymes are also described here.

MATERIALS AND METHODS

Materials. Crystalline AdoCbl was a gift from Eizai, Co. Ltd. (Tokyo, Japan). [1,1-D_2]-1,2-Propanediol was synthesized by the reduction of ethyl lactate with $LiAlD_4$ and purified by distillation under reduced pressure. (*R*)-1,2-Propanediol was purchased from TCI (Tokyo, Japan). (*S*)-1,2-Propanediol was synthesized by reduction of L-lactide (TCI) with $LiAlH_4$ as described previously (*22*). Alcohol dehydrogenase was obtained from Sigma. All other chemicals were analytical grade reagents and used without further purification.

Site-Directed Mutagenesis of the Gene Encoding the α *Subunit of Klebsiella oxytoca Diol Dehydratase and Construction of Expression Plasmids.* Site-directed mutageneses were generated using a QuikChange site-directed mutagenesis kit (Stratagene). The mutagenic sense primers designed were 5′-ccgtcccagcaggcgcaagtcaccaacgtcaaagataac-3′ for Hα143Q, 5′-ccgtcccagcaggcgaaagtcaccaacgtcaaagataac-3′ for Hα143K, 5′-ccgtcccagcaggcgctcgtcaccaacgtcaaagataac-3′ for Hα143L, and 5′-ccgtcccagcaggcggaagtcaccaacgtcaaagataac-3′ for Hα143E. The oligonucleotides having the complementary sequences in the opposite direction were used as the respective antisense primers. pUC119(DD) (*31*), which contains the *pddABC* genes for the α, *β*, and *γ* subunits of diol dehydratase, was used as a template for the mutant plasmid pUC119(DD-Hα143E). A 0.6 kb *Bam*HI−*Ban*II fragment from pUC119(DD-Hα143E) and a 2.4 kb *Ban*II-*Eco*RI fragment from pUSI2ENd(DD) were then ligated to the *Bam*HI−*Eco*RI region of pUSI2ENd to produce expression plasmid pUSI2ENd(DD-Hα143E) for the Hα143E mutant. The construction of pUSI2ENd(DD-Hα143A) was described in the preceding paper (*31*). For the mutant expression plasmids pUSI2ENd(DD-Hα143Q), pUSI2ENd(DD-Hα143K), and pUSI2ENd(DD-Hα143L), pUSI2ENd(DD) (*32*) was used as a template. The inserts of the mutant plasmids were confirmed by nucleotide sequencing to have intended mutations but not to have unintended mutations.

Expression and Purification of Mutant Diol Dehydratases. Escherichia coli JM109 strains harboring expression plasmids were grown aerobically and induced by 1 mM isopropyl-1-thio-*β*-galactopyranoside, essentially as described previously (*33*). The recombinant wild-type enzyme was purified from overexpressing *E. coli* cells, as described previously (*34*). Mutant enzymes were purified similarly but with the following modifications. The amount of buffer used for sonication was 4–10 volumes of cell paste. The debris was washed twice with 1–10 volumes of 50 mM potassium phosphate buffer (pH 8.0) containing 2% 1,2-propanediol, 1 mM phenylmethanesulfonyl fluoride, and 2 mM EDTA and then three times with 4–10 volumes of the same buffer containing 0.2% Brij 35. Mutant enzymes were extracted from crude membrane fractions with the 10 mM potassium phosphate buffer but containing 1% Brij 35. DEAE-cellulose column chromatography was omitted when preparations were found to be almost homogeneous.

Trypsin-solubilized Hα143A apoenzyme used for resolution experiments was prepared as follows (*32*). The crude membrane fraction (ca. 0.1 g) obtained from *E. coli* cells harboring pUSI2ENd(DD-Hα143A) as described above was partially digested at 15 °C with 9 *μ*g of trypsin in 1.8 mL of 50 mM potassium phosphate buffer (pH 8.0) containing 2% 1,2-propanediol. After incubation for 13 h, the mixture was centrifuged, and 0.72 mg of trypsin inhibitor was added to the supernatant that contained solubilized Hα143A mutant apoenzyme.

Enzyme and Protein Assays. Diol dehydratase activity was routinely assayed by the MBTH method (*14*). The standard reaction mixture contained an appropriate amount of apoenzyme, 15 μM AdoCbl, 0.1 M 1,2-propanediol, 50 mM KCl, and 35 mM potassium phosphate buffer (pH 8.0), in a total volume of 1.0 mL. After incubation at 37 °C for 10 min, reactions were terminated by adding 1 mL of 0.1 M potassium citrate buffer (pH 3.5). MBTH·HCl was then added to a final concentration of 0.9 mM, and the mixtures were incubated again at 37 °C for 15 min. The concentrations of aldehyde formed were determined by measuring the absorbance at 305 nm. One unit is defined as the amount of enzyme activity that catalyzes the formation of 1 μmol of propionaldehyde per minute at 37 °C under the standard assay conditions. Time courses of the reaction were measured by the alcohol dehydrogenase−NADH coupled method, as described previously (*15*).

The protein concentration of the purified enzyme was determined by measuring the absorbance at 280 nm. The molar absorption coefficient at 280 nm calculated by the method of Gill and von Hippel (*35*) from the deduced amino acid composition and subunit structure for this enzyme is 120500 M^{-1} cm^{-1} (*36*).

Kinetic Analysis of Mutant Diol Dehydratases. Substrate-free apoenzyme was obtained by dialysis at 4 °C for 24–36 h against 100 volumes of 50 mM potassium phosphate buffer (pH 8.0) containing 1% Brij 35 or 20 mM sucrose monocaprate with two buffer changes. Remaining concentrations of 1,2-propanediol in dialysates were less than 0.2 mM when determined by adding wild-type diol dehydratase and thus negligible when diluted more than 100-fold. For the determination of apparent K_m values for substrates [racemic, (*R*)-, and (*S*)-1,2-propanediols] and AdoCbl, reaction rates with various substrate concentrations and a fixed AdoCbl concentration of 15 μM and with various AdoCbl concentrations and a fixed 1,2-propanediol concentration of 0.10 M, respectively, were measured by the MBTH method. K_m values were determined by Lineweaver–Burk and Eadie−Hofstee plots. For the determination of the deuterium KIE on the rates of overall reaction (k_H/k_D) and inactivation during catalysis ($k_{inact,H}/k_{inact,D}$), time courses of the reaction were measured by the alcohol dehydrogenase−NADH coupled method using unlabeled 1,2-propanediol and [1,1-D_2]-1,2-propanediol. An excess of alcohol dehydrogenase (400 μg/mL) was used in these experiments.

PAGE Analysis of Mutant Diol Dehydratases. PAGE was performed under nondenaturing conditions as described by Davis (*37*) in the presence of 0.1 M 1,2-propandiol or under denaturing conditions as described by Laemmli (*38*). Protein staining was carried out with Coomassie Brilliant Blue R-250.

Spectral Measurements. Optical spectra were recorded on a JASCO V-560 recording spectrophotometer. Experimental details are described in the legend of the corresponding figure. For the measurements under anaerobic conditions, the air in a system was replaced with argon (purity, 99.999%); that is, the AdoCbl solution and a reaction mixture except AdoCbl were degassed separately in a thunberg tubelike quartz cell by repeated evacuation and flushing with argon three times, then mixed, and incubated.

EPR Measurements. Mutant apoenzymes purified by DEAE-cellulose column chromatography (*34*) were used. A substrate-free apoenzyme solution [1.0 mg of protein in 0.6 mL of 50 mM potassium phosphate buffer (pH 8.0) containing 20 mM sucrose monocaprate] was mixed at 0 °C with an AdoCbl solution (50 nmol in 0.05 mL) in a quartz EPR tube (outside diameter of 5 mm) stoppered with a rubber septum. After replacement of the air in the tube with argon by repeated evacuation and flushing with argon three times, holoenzymes were formed, reacted with 1,2-propanediol, and rapidly frozen as described in the figure legend. The frozen sample was transferred to the EPR cavity and cooled with a cold nitrogen gas flow controlled by a JEOL JES-VT 3A or Eurotherm B-VT 2000 temperature controller. EPR spectra were recorded as described previously (*39, 40*) at −130 °C on a JEOL JES-RE 3X or Bruker ESP-380E spectrometer modified with a Gunn diode X-band microwave unit: EPR microwave frequency, 9.171–9.179 (JEOL) or 9.481–9.499 (Bruker) GHz; modulation amplitude, 1 mT; modulation frequency, 100 kHz; microwave power, 10 mW.

Fate of the Adenosyl Group of AdoCbl. To identify adenosyl group-derived product(s) formed from AdoCbl in the inactivation of a mutant holoenzyme by substrate 1,2-propanediol, substrate-free apoenzyme (1.0 mg, 4.6 nmol) was incubated at 37 °C for 30 min in the dark with 15 μM AdoCbl in the presence of 0.1 M 1,2-propanediol. Ethanol was then added to a final concentration of 80% to denature the enzyme protein. After being heated at 90 °C for 10 min, the mixture was centrifuged, and the supernatant was evaporated to dryness and then dissolved in 0.5 mL of 15% methanol containing 1% acetic acid. The nucleoside product from the adenosyl group was identified as 5′-deoxyadenosine by HPLC using a Cosmosil C_{18} column (0.46 cm × 15 cm) (Nacalai Tesque, Kyoto, Japan) with 15% methanol containing 1% acetic acid as a mobile phase. The amount of 5′-deoxyadenosine formed was determined from its peak height when the calibrated column was developed at a flow rate of 0.4 mL/min. The retention time of 5′-deoxyadenosine was ca. 10 min under the conditions employed.

Resolution of the Inactivated Mutant Holoenzyme by Acid Ammonium Treatment. The resolution experiment was not successful with Brij 35-solubilized diol dehydratase, because Brij 35 was salted out in the procedure of acid ammonium sulfate fractionation and no longer effective for solubilizing the enzyme. Instead, trypsin-solubilized enzyme was successfully used in the resolution experiments. The 1,2-propanediol-inactivated Hα143A holoenzyme was obtained by the incubation of trypsin-solubilized apoenzyme (1.5 mg) at 37 °C for 30 min with 27 μM AdoCbl in 1.1 mL of 0.05 M potassium phosphate buffer (pH 8.0) containing 0.08 M 1,2-propanediol, followed by dialysis against 230 volumes of the same buffer with a buffer change. The inactivated complex was then resolved by acid ammonium sulfate treatment, as described previously (*41*). The activity of the apoenzyme used, the inactivated holoenzyme, and the resolved enzyme was measured by the alcohol dehydrogenase−NADH coupled method in the presence of added AdoCbl. The amount of cobalamin bound to the inactivated holoenzyme and the resolved enzyme was determined spectrophotometrically after conversion to aqua and dicyano forms. Enzymes in 1.0 mL of solution were denatured by incubation at 37 °C for 10 min with 6 M guanidine·HCl and 0.06 M citric acid in a total volume of 1.85 mL, followed by neutralization

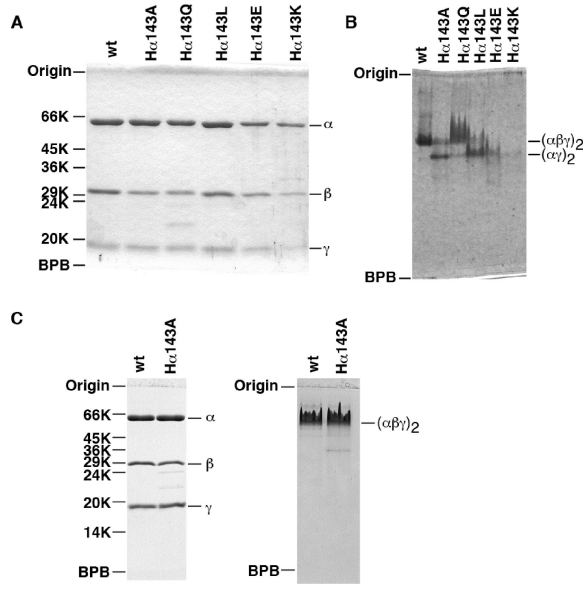


FIGURE 2: Analysis of the purified preparations of mutant diol dehydratases by SDS−PAGE (A) and nondenaturing PAGE (B). Samples were electrophoresed on 11% (A) and 7% (B) polyacrylamide gels. A freshly purified preparation of Hα143A was also electrophoresed in a similar manner (C). The resulting gels were subjected to protein staining with Coomassie Brilliant Blue R-250. Molecular mass markers, SDS-7 (Sigma); BPB, bromophenol blue; wt, wild-type enzyme. The bands of the α, β, and γ subunits are indicated on the right (A and C). The positions of the $(\alpha\beta\gamma)_2$ and $(\alpha\gamma)_2$ complexes are indicated on the right (B and C).

to pH 8.0 by addition of 200 μL of 1 M potassium phosphate buffer (pH 8.0) and 70 μL of 5 N KOH (*31*).

O_2 Inactivation of Holoenzymes in the Absence of Substrate. Appropriate amounts of substrate-free wild-type and Hα143Q mutant apoenzymes were incubated aerobically with 15 μM AdoCbl at 37 °C for various time periods in 35 mM potassium phosphate buffer (pH 8.0) containing 50 mM KCl. Substrate (0.1 M 1,2-propanediol) was then added to stop the O_2 inactivation, and remaining activities of enzymes were measured by incubating the mixtures at 37 °C for an additional 10 min.

RESULTS

Expression and Purification of Mutant Diol Dehydratases. Mutant enzymes in which Hisα143 was mutated to another amino acid residue were expressed in *E. coli* cells. As judged from SDS−PAGE analyses of cell homogenates, all the mutants were highly expressed as the wild-type enzyme (to approximately 10–20% of total cellular protein), and the levels of expression of the α, β, and γ subunits were not significantly affected by mutations (data not shown). The wild-type enzyme (*34*) and mutant enzymes purified as described above were subjected to PAGE under denaturing and nondenaturing conditions. As shown in panels A and B of Figure 2, all the preparations were highly purified and contained all the α, β, and γ subunits, but the ratio of the $(\alpha\beta\gamma)_2$ complexes to the $(\alpha\gamma)_2$ complexes varied depending upon mutant enzymes. The wild-type enzyme and the Hα143Q mutant existed mainly as the $(\alpha\beta\gamma)_2$ complexes, although the band of the latter was smeared because a larger quantity of protein with low solubility was applied in the lane. $(\alpha\beta\gamma)_2$ is the subunit structure that is absolutely required for coenzyme binding and thus for catalytic activity (*42, 43*), because the $(\alpha\gamma)_2$ complex and the β subunit correspond to components S and F, respectively (*34*), and neither alone is catalytically active. Hα143A and Hα143L mutants existed as a mixture of $(\alpha\beta\gamma)_2$ and $(\alpha\gamma)_2$. Just after the preparation of Hα143A, the ratio of $(\alpha\beta\gamma)_2$ to $(\alpha\gamma)_2$ was high (~90%) (Figure 2C), but it was lowered to ~40–75% after freezing and thawing. In contrast, Hα143E and Hα143K mutants did not form the $(\alpha\beta\gamma)_2$ complexes, although the α, β, and γ subunits were present in their preparations.

Catalytic Activity and Kinetic Properties of Mutant Diol Dehydratases. Kinetic parameters for mutant diol dehydratases are summarized in Table 1. The Hα143Q mutation did not significantly affect the K_m for substrate (1,2-propanediol) but increased the affinity for the coenzyme (AdoCbl). The affinity for the coenzyme was not affected by the Hα143A mutation. The values of k_{cat} and k_{cat}/K_m decreased to 34 and 25%, respectively, with the Hα143Q mutation. The Hα143A and Hα143L mutants exhibited relative k_{cat} values of 1.4 and 0.01%, respectively, although 40–90% of the former and 30% of the latter exist as the $(\alpha\beta\gamma)_2$ complexes. No activity (<0.01%) was detected with the Hα143E and Hα143K mutants that contain an anionic or cationic amino acid at position α143. This is consistent with the fact that the $(\alpha\beta\gamma)_2$ complexes were not observed with these mutants. These results indicate that the hydrogen bonding capability, but neither the acidity nor the basicity, of side chains of the residue α143 in neutral aqueous solution is important for the catalytic activity of the enzyme.

Inactivation of Mutant Diol Dehydratases during Catalysis. Time courses of 1,2-propanediol dehydration catalyzed by mutant enzymes were measured by the alcohol dehydrogenase−NADH coupled method (data not shown). Unlike the wild-type and Hα143Q enzymes, the Hα143A mutant underwent marked inactivation during catalysis and lost its activity almost completely within 2 min. This inactivation obeyed first-order reaction kinetics (Table 1). The k_{cat}/k_{inact} values, which show the average numbers of catalytic turnovers before inactivation, indicate that the Hα143A and Hα143L mutants are inactivated after only 170 and 10 turnovers on average, respectively. Since these ratios are more suitable parameters than k_{inact} for describing the tendency of enzyme to undergo the mechanism-based inactivation, it was suggested that the hydrogen bonding interaction between the OH group on C2 of the substrate and the side chain of residue α143 is important for preventing the highly reactive radical intermediate(s) from undesirable side reactions as well as for catalysis. When the time course of the reaction with the Hα143A mutant was measured under anaerobic conditions, similar inactivation during catalysis took place. Since k_{inact} values observed under aerobic and anaerobic conditions were essentially the same, it is evident that this inactivation is a kind of mechanism-based inactivation rather than the inactivation by oxygen.

pH Dependence of the Reaction Catalyzed by a Mutant Diol Dehydratase. The pH−catalytic efficiency and pH−V_{max} profiles of the Hα143Q mutant were compared with those of the wild-type enzyme. As shown in panels A and B of Figure 3, both V_{max}/K_m−pH and V_{max}−pH curves of the wild-type enzyme were bell-shaped. V_{max} was less sensitive to pH, which is consistent with the result of Lee and Abeles (*1*). These curves indicate that a minimum of two protona-

Table 1: Kinetic Parameters with Mutant Diol Dehydratases[a]

	k_{cat}[b] (s^{-1})	K_m[c] [1,2-propanediol][d] (mM)	K_m[c] [AdoCbl] (μM)	$k_{cat}/K_m \times 10^{-6}$ (s^{-1} M^{-1})	k_{inact}[b] (min^{-1})	$k_{cat}/k_{inact} \times 10^{-4}$
wild type	354 ± 31	0.22 ± 0.01	0.90[e]	1.6	0.024 ± 0.005	89
Hα143Q	121 ± 25	0.30 ± 0.03	0.26 ± 0.02	0.40	0.13 ± 0.04	5.6
Hα143A[f]	5.1		0.91		1.8	0.017
Hα143L	0.03 ± 0.01				0.19 ± 0.07	0.001
Hα143E	Inactive (<0.03)					
Hα143K	Inactive (<0.03)					

[a] Determined at 37 °C. Average ± standard deviation (n = 3–6). [b] Determined by the alcohol dehydrogenase−NADH coupled method. k_{inact} was calculated from a change in the slope of a tangent to the time course curve of the reaction. [c] Determined by the MBTH method. [d] Racemic 1,2-propanediol was used as the substrate. [e] From ref *14*. [f] From ref *31*.

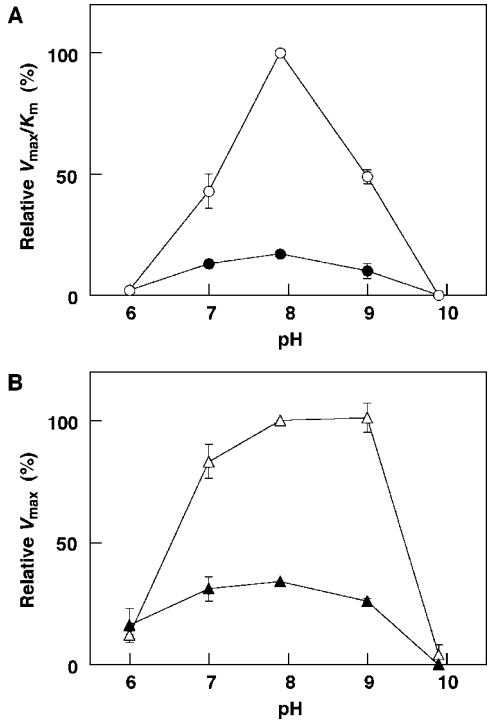


FIGURE 3: pH−relative V_{max}/K_m (A) and pH−relative V_{max} (B) curves of the wild-type enzyme (○ and △) and the Hα143Q mutant (● and ▲). The following buffers containing 0.05 M KCl were used at a concentration of 35 mM: potassium phosphate (pH 6), potassium phosphate (pH 7), potassium phosphate (pH 8), potassium carbonate (pH 9), and potassium carbonate (pH 10). K_m and V_{max} were determined by the MBTH assay method. The V_{max}/K_m value of the wild-type enzyme at pH 8.0 is taken as 100%. Mean ± R/2 (n = 2).

table residues with pK_as of 6.5–7 and 9–9.5 are involved in the pH dependence. The V_{max}/K_m−pH and V_{max}−pH curves for the Hα143Q mutant were still bell-shaped. This suggests that another residue is responsible for the profile, because the carboxamide group of the Gln residue is neither protonated nor deprotonated in this pH range. The pH dependence also indicated that catalytic efficiency declines at higher pH by deprotonation of at least one other group or by denaturation, which was not affected by the Hα143Q mutation.

Deuterium KIE on the Rates of the Overall Reaction and Inactivation during Catalysis. To obtain the information about the rate-determining step with Hisα143 mutants, deuterium KIEs on the rate of the overall reaction (k_H/k_D) were compared between the wild-type and mutant enzymes using [1,1-D_2]-1,2-propanediol as the substrate (Table 2). The k_H/k_D value of the wild type was 10.3, in good agreement with the reported one (*5*). The value of the Hα143Q mutant was in the same range as that of the wild-type enzyme. It was thus concluded that a hydrogen abstraction step is fully rate-determining with the wild-type and Hα143Q enzymes. In contrast, the k_H/k_D value of the Hα143A mutant was 4.0. This indicates that a hydrogen abstraction step is partially rate-determining with this mutant.

Table 2: Deuterium KIEs for Mutant Enzymes on the Rates of the Overall Reaction and Inactivation during Catalysis[a]

	k_H/k_D	$k_{inact,H}/k_{inact,D}$
wild type	10.3 ± 0.6	1.1 ± 0.0
Hα143Q	12.2 ± 0.6	1.7 ± 0.2
Hα143A	4.0 ± 0.4	1.1 ± 0.1

[a] Determined by the alcohol dehydrogenase−NADH coupled method using unlabeled 1,2-propanediol and [1,1-D_2]-1,2-propanediol as substrates. Average ± standard deviation (n = 3).

The deuterium KIEs on the rate of inactivation during catalysis ($k_{inact,H}/k_{inact,D}$) were essentially not observed with the wild-type enzyme and the Hα143A mutant (Table 2). On the other hand, a KIE of 1.7 was observed with the Hα143Q mutant. Thus, it was concluded that a hydrogen abstraction step is not rate-determining for the inactivation during catalysis with the wild-type and Hα143A mutant enzymes, but it might be slightly rate-determining with the Hα143Q mutant.

Stereospecificity of a Mutant Diol Dehydratase for (R)- and (S)-1,2-Propanediols. To determine the preference of the Hα143Q mutant for (*R*)- and (*S*)-1,2-propanediols, kinetic parameters for each enantiomer were determined. As shown in Table 3, the wild-type enzyme exhibited a higher affinity for the *S*-enantiomer, but the *R*-enantiomer reacted 2 times faster than the *S*-enantiomer. These results are in good agreement with the reported data (*5, 44, 45*). In contrast, the preferences of the enzyme in both substrate binding and catalysis were reversed by the Hα143Q mutation; that is, the *R*-enantiomer was preferred in the substrate binding but showed lower activity in the catalysis. It is common in both cases that an enantiomer that shows higher binding affinity undergoes dehydration at a slower rate.

Spectral Change of AdoCbl upon Incubation with Mutant Enzymes in the Presence of Substrate. Upon aerobic incubation of AdoCbl with the wild-type apoenzyme in the presence of substrate, the absorbance at 525 nm decreased and a new peak at 478 nm appeared (Figure 4B), indicating that AdoCbl was converted to cob(II)alamin (B_{12r}). The spectrum obtained after incubation for 3 min suggests that the steady-state concentration of cob(II)alamin during catalysis is approximately 50% of that of added AdoCbl. This is roughly consistent with the previous value (*15*). AdoCbl was partly

Table 3: Stereospecificity of the Hα143Q Mutant for (*R*)- and (*S*)-1,2-Propanediols[a]

	(*R*)-1,2-propanediol			(*S*)-1,2-propanediol				
	K_m (mM)	V_{max} (μmol/min)	V_{max}/K_m (min^{-1})	K_m (mM)	V_{max} (μmol/min)	V_{max}/K_m (min^{-1})	$K_m(R)/K_m(S)$	$V_{max}(R)/V_{max}(S)$
wild type	0.47 ± 0.02	0.010 ± 0.001	0.021	0.11 ± 0.01	0.005 ± 0.001	0.045	4.3	2.0
Hα143Q	0.25 ± 0.02	0.005 ± 0.002	0.020	0.35 ± 0.01	0.008 ± 0.003	0.023	0.71	0.63

[a] K_m and V_{max} were determined by the MBTH method using (*R*)- and (*S*)-1,2-propanediols as substrates. Average ± standard deviation ($n = 3$).

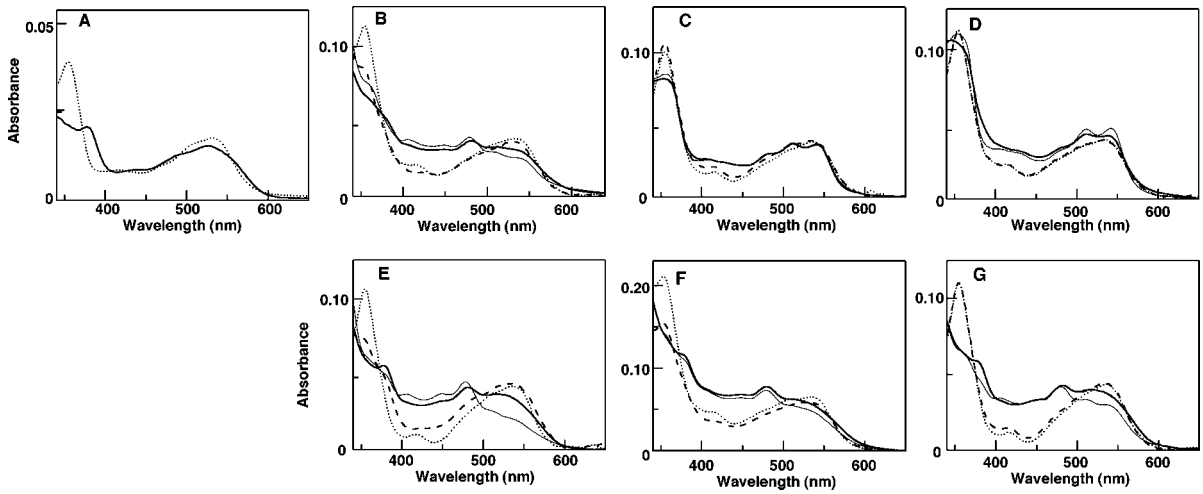


FIGURE 4: Spectral changes of AdoCbl upon aerobic (A−D) and anaerobic (E−G) incubation with mutant diol dehydratases in the presence of 1,2-propanediol. (A) Free AdoCbl (3.5 μM) in 35 mM potassium phosphate buffer (pH 8.0) containing 1 M 1,2-propanediol (—). The spectrum after photolysis was also taken (•••). (B−G) The apoenzyme (5 μM) of the wild-type (B and E), Hα143Q (C and F), or Hα143A (D and G) enzyme was incubated at 30 °C with 4.5 μM AdoCbl in 35 mM potassium phosphate buffer (pH 8.0) containing 1.4 M 1,2-propanediol, 50 mM KCl, and 1% Brij 35 in a volume of 1.0 mL. Spectra were taken after incubation for 3 min (thick solid lines) and 30 min (thin solid lines) after the addition of AdoCbl. Enzymes were then denatured via addition of 6 M guanidine•HCl and 0.06 M citric acid. After incubation at 37 °C for 10 min, the mixture was neutralized to pH 8 via addition of 200 μL of 1 M potassium phosphate buffer (pH 8.0) and 70 μL of 5 N KOH. After the spectral measurement (---), the mixture was photoilluminated in an ice–water bath for 10 min with a 300 W tungsten light bulb from a distance of 20 cm, and the spectrum was recorded (•••). Spectra are corrected for dilution. Anaerobic incubation was carried out under an argon atmosphere, as described in the text.

re-formed upon denaturation of the enzyme after incubation for 30 min and converted to aqCbl upon photoillumination. In contrast, the extents of cob(II)alamin formation were much lower with the Hα143Q and Hα143A mutants under aerobic conditions (Figure 4C,D). Instead, new peaks appeared at 511, 542, and 356 nm with Hα143A. This spectrum is characteristic of OH-Cbl that is bound to diol dehydratase together with 5′-deoxyadenosine (*46*). The spectral change with Hα143A was completed within 10 min of incubation, and until this time, the mutant became completely inactivated. Thus, it is evident that the inactivation of Hα143A during catalysis under aerobic conditions is accompanied by the irreversible conversion of enzyme-bound AdoCbl to OH-Cbl.

Under anaerobic conditions, essentially the same spectral changes of AdoCbl as those under aerobic conditions were observed with the wild-type enzyme (Figure 4E). With the Hα143Q and Hα143A mutants, the accumulation of cob(II)alamin was observed under anaerobic conditions (Figure 4F,G). Since the inactivation of Hα143A during catalysis took place even under anaerobic conditions, the inactivated species under anaerobic conditions likely contains cob(II)alamin. The spectrum was not distinguishable from that of the catalytic intermediate but changed to that of aqCbl upon denaturation under aerobic conditions. It no longer changed upon photoillumination. It is therefore evident that, upon inactivation, AdoCbl is first converted to cob(II)alamin that is then oxidized to OH-Cbl under aerobic conditions (Figure 4D).

EPR Spectra with a Mutant Diol Dehydratase. Upon incubation of the wild-type holoenzyme with 1,2-propanediol at 4 °C for 1 min under anaerobic conditions, the typical EPR spectrum of the reacting holoenzyme was obtained (Figure 5A,C). The high-field doublet signal was identified as 1,2-propanediol-1-yl radical (the substrate-derived radical) (*47*), and the low-field broad signal was assigned to low-spin Co(II) of cob(II)alamin. Such a spectrum arises from weak coupling in the Co(II)−organic radical pair (*48–50*). The signals disappeared within 3 min of incubation at 25 °C, because added substrate had been exhausted by this incubation. The Hα143Q and Hα143A mutant holoenzymes also gave typical EPR spectra of reacting holoenzymes upon incubation with substrate at 4 °C for 1 min (Figure 5B,D). The spin–spin coupling constants (*J* values) for the doublet signals obtained with the wild-type and Hα143A enzymes were 14.3 and 13.9 mT, respectively. Hα143Q gave a doublet signal with an intensity that was 34% of that of the wild type and the same *J* value. These suggest that the EPR-observable intermediates in the reaction with the mutants might be the same as those with the wild-type enzyme. The relative intensity of the organic radical-derived doublet signal with the Hα143Q mutant was 3.0-fold smaller than that with the wild-type enzyme. This was consistent with the catalytic activity of this mutant. In contrast, the relative intensity of the doublet signal with the Hα143A mutant was 1.8-fold larger than that with the wild-type enzyme. Upon further incubation at 25 °C for 3 min, the doublet signal disappeared, and instead, a new singlet signal with a *g* value of ~2.0

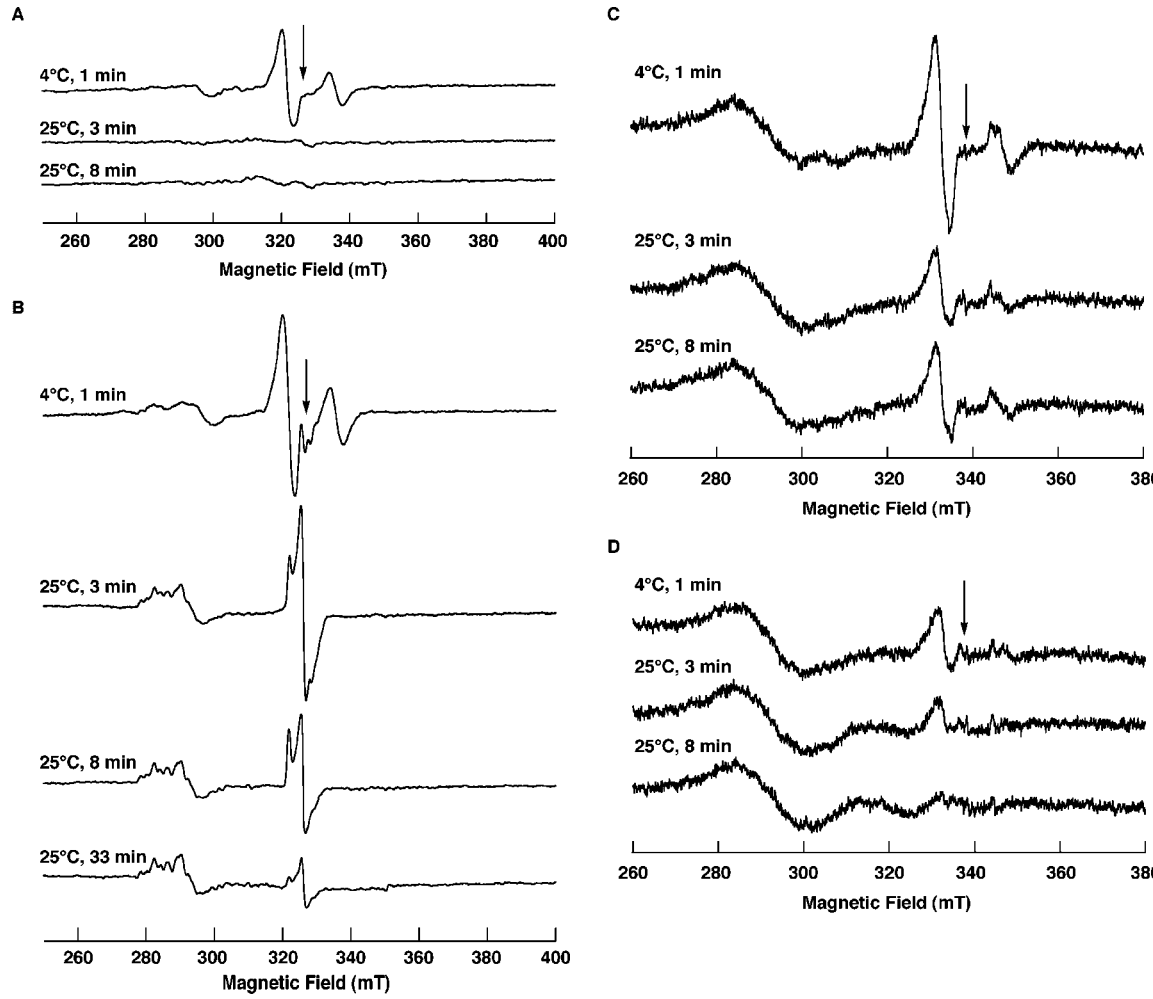


FIGURE 5: EPR spectra observed upon incubation of holodiol dehydratases of the wild-type enzyme (A and C) and the Hα143A (B) and Hα143Q (D) mutants with 1,2-propanediol. The arrows correspond to $g = 2.0$. Holoenzymes were formed under an argon atmosphere by incubating 1.0 mg (4.6 nmol) of substrate-free wild-type, Hα143A, and Hα143Q apoenzymes at 25 °C for 3 min with 50 nmol of AdoCbl in 0.65 mL of 0.05 M potassium phosphate buffer (pH 8.0) containing 18 mM sucrose monocaprate. The enzyme reaction was started by adding 50 μmol of 1,2-propanediol in 0.05 mL. After 1 min at 4 °C, the reaction mixture was rapidly frozen in an isopentane bath that had been preliminarily cooled to approximately −160 °C and then in a liquid nitrogen bath. EPR spectra were recorded at –130 °C on the JEOL (A, B) or the Bruker (C, D) spectrometer. After the first measurement, the mixture was incubated at 25 °C for 3 min and frozen again, as described above for the second measurement. The mixture was then incubated at 25 °C for an additional 5 and 25 min for the third and fourth measurements, respectively.

appeared. The latter signal is often observed upon inactivation of the wild-type holoenzyme as well, although the radical species giving this signal has not yet been identified. The relative intensity of the latter signal decreased with time of incubation, and the typical signal of cob(II)alamin without an organic radical coupling partner (*51*) became predominant. This change is consistent with the spectral change under anaerobic conditions (Figure 4G). These results support the observation that the Hα143A mutant retains enzyme activity but undergoes rapid inactivation during catalysis.

Fate of the Adenosyl Group of AdoCbl in Inactivation of a Mutant Diol Dehydratase during Catalysis. To investigate the fate of the upper axial ligand of AdoCbl in the inactivation of a mutant holoenzyme, the adenosyl group-derived product(s) from AdoCbl was identified. After the mechanism-based inactivation of the Hα143A mutant during catalysis, the inactivated holoenzyme was denatured, and products formed from the coenzyme in the inactivation were extracted and analyzed by HPLC on a reversed phase column. The nucleoside product formed from the adenosyl group was identified as 5′-deoxyadenosine, and the formation of adenine, adenosine, 4′,5′-anhydroadenosine, 5′,8-cyclic adenosine, or adenosine 5′-aldehyde was not observed at all (data not shown). The amounts of 5′-deoxyadenosine formed in the inactivation during catalysis with 1,2-propanediol as the substrate corresponded to approximately 2.3 mol/mol of enzyme. Since diol dehydratase exists as a dimer of the $\alpha\beta\gamma$ heterotrimer, it is likely that both heterotrimeric units are involved in the formation of 5′-deoxyadenosine. It is thus evident with the Hα143A mutant that the hydrogen abstraction from substrate by coenzyme adenosyl radical takes place as the initial event of catalysis and that the inactivation is a mechanism-based, suicidal one.

Recovery of Apoenzyme by Resolution of an Inactivated Mutant Holodiol Dehydratase. As shown in Table 4, the remaining specific activity of the 1,2-propanediol-inactivated Hα143A holoenzyme was 3% of that of the apoenzyme used. After the resolution by acid ammonium sulfate treatment, the specific activity increased to 42%. The spectrum of the inactivated holoenzyme indicated that the cobalamin bound was a mixture of cob(II)alamin and OH-Cbl, the former being predominant. The cobalamin recovered in the supernatant

Table 4: Resolution of the 1,2-Propanediol-Inactivated Hα143A Mutant Holoenzyme by Acid Ammonium Sulfate Treatment

	specific activity		B_{12} bound	
	units/mg	%	μM	%
apoenzyme used	0.59	(100)	0	0
inactivated holoenzyme	0.02	3	2.7	(100)
resolved enzyme	0.25	42	1.4	52

upon ammonium sulfate treatment was aqCbl, but the spectrum of the resolved enzyme showed that the resolution was not complete and that the resolved enzyme still contains a mixture of cob(II)alamin and OH-Cbl, the former being predominant. aqCbl was recovered by the denaturation of resolved enzyme, and the amount of cobalamin bound to the resolved enzyme was 52% of that of the inactivated holoenzyme, indicating that the extent of resolution was 48%. This value is in reasonable agreement with the recovery of enzyme activity. It was therefore concluded that resolved apoenzyme recovered from the inactivated holoenzyme could be reconstitutable to fully active holoenzyme; that is, the Hα143A apoenzyme itself was not damaged in the mechanism-based inactivation by substrate 1,2-propanediol.

Inactivation of a Mutant Holoenzyme by O_2 in the Absence of Substrate. The holoenzyme of diol dehydratase undergoes irreversible inactivation by O_2 in the absence of substrate (*52*). This inactivation is accompanied by the formation of enzyme-bound OH-Cbl. It is thus believed that the inactivation is caused by the reaction of the activated Co−C bond of the coenzyme with O_2. The inactivation followed the first-order reaction kinetics with a rate constant (k_{inact,O_2}) of 0.223 ± 0.004 s^{-1} for the wild-type holoenzyme. The Hα143Q holoenzyme also underwent O_2 inactivation that obeyed first-order kinetics, but the k_{inact,O_2} value of this mutant was much smaller (0.037 ± 0.002 s^{-1}). This indicated that the degree of Co−C bond activation upon binding to the apoenzyme in the absence of substrate is greatly lowered by the Hα143Q mutation.

DISCUSSION

The X-ray structure of diol dehydratase revealed that the OH group on C2 of substrates is hydrogen-bonded to the Hisα143 and Aspα335 residues (*19*). These residues are conserved in glycerol dehyratase as well (*53*). A mutational study indicated that these two and Gluα170 are catalytic residues of diol dehydratase (*31*). Biochemical evidence for the presence of an essential His residue had also been obtained by chemical modification with diethyl pyrocarbonate (*23*). Theoretical calculations by a QM/MM method predicted that unprotonated Hisα143 stabilizes the transition state for the OH group migration by hydrogen bonding with the migrating OH group (*29*). To study the functional role of Hisα143 by mutational analysis, five Hisα143 mutants were constructed, expressed, purified, and examined for catalytic activity. The wild-type enzyme exists as the $(\alpha\beta\gamma)_2$ complex (*34, 43*) that is responsible for coenzyme binding and thus for catalytic activity (*42, 43*). The Hα143Q mutant was also electrophoresed mainly as the $(\alpha\beta\gamma)_2$ complex upon nondenaturing PAGE. Hα143A and Hα143L existed as a mixture of $(\alpha\beta\gamma)_2$ and $(\alpha\gamma)_2$, the ratio for the former being ~90% just after purification. Hα143E and Hα143K did not form the $(\alpha\beta\gamma)_2$ complexes, although the α, β, and γ subunits were present in their preparations. These results suggest that subunit contacts are disrupted completely when Hisα143 is substituted with a residue with an ionic side chain and partially with a residue with a hydrophobic side chain. Because the Hisα143 residue does not exist in the subunit interface (*19*), the effects of these mutations seem to be indirect, and probably due to nonlocalized changes in the protein structure of the α subunit outside of the Hisα143 region. The roles of global alterations in the subunit structure must be negligible for the Hα143Q and Hα143A mutants, because they exist mainly as the $(\alpha\beta\gamma)_2$ complexes. The degree of global changes in the α subunit structure would be small for Hα143L but large for Hα143E and Hα143K.

The k_{cat} and k_{cat}/K_m values of the enzyme were lowered partially by the Hα143Q mutation and drastically by the Hα143A and Hα143L mutations. These results suggest that the hydrogen bonding interaction between the OH group on C2 of the substrate and the side chain of the α143 residue is important for diol dehydratase catalysis. The pH−catalytic efficiency and pH−V_{max} profiles of both the wild-type enzyme and the Hα143Q mutant were bell-shaped, although V_{max} was less sensitive to pH. Since the carboxamide group of Gln is neither acidic nor basic in this pH range, the hydrogen bond with the migrating OH group of substrate, rather than the acidity or the basicity, at the side chain of the residue α143 is essential for catalysis. This conclusion is consistent with the prediction from the recent QM/MM calculations of Yoshizawa and co-workers (*29*) which showed that the reaction pathway with unprotonated Hisα143 is kinetically more favorable than that with protonated Hisα143 for 1,2-propanediol dehydration. The rates of O_2 inactivation of the wild-type and mutant holoenzymes in the absence of substrate indicated that the extent of Co−C bond activation greatly decreased upon the Hα143Q mutation. This might be one of the reasons why the relative activity of this mutant was lowered to 34% of that of the wild-type enzyme, because the Co−C bond cleavage is kinetically coupled with the abstraction of hydrogen from substrate (*17*).

The k_{cat}/k_{inact} values of the mutants decrease markedly as their catalytic activities decrease. The mutants that are unable to form a hydrogen bond between the OH group on C2 of the substrate and residue α143 became inactivated after less than 200 turnovers on average. It is therefore suggested that Hisα143 is important not only for catalytic activity but also for protection of the reactive radical intermediates against undesirable side reactions (so-called negative catalysis (*54*)) or escape out of the active site. The deuterium KIE on the inactivation during catalysis was essentially not observed. A similar result was obtained in the case of mechanism-based inactivation by a coenzyme analogue (*41*). Spectral changes indicated that the Co−C bond of AdoCbl is irreversibly homolyzed by the Hα143A mutant, and cob(II)alamin formed is stable under anaerobic conditions but oxidized to OH-Cbl under aerobic conditions. EPR spectra suggested that the doublet signal assigned to the 1,2-propanediol-1-yl radical (*47*), which is weakly coupled with cob(II)alamin, was initially observed with the Hα143A mutant as with the wild-type enzyme in the presence of 1,2-propanediol. The relative intensity of the doublet signal with the Hα143A mutant was 1.8-fold larger than that with the wild-type enzyme. The steady-state concentrations of organic radical and cob(II)alamin observed with the wild-type

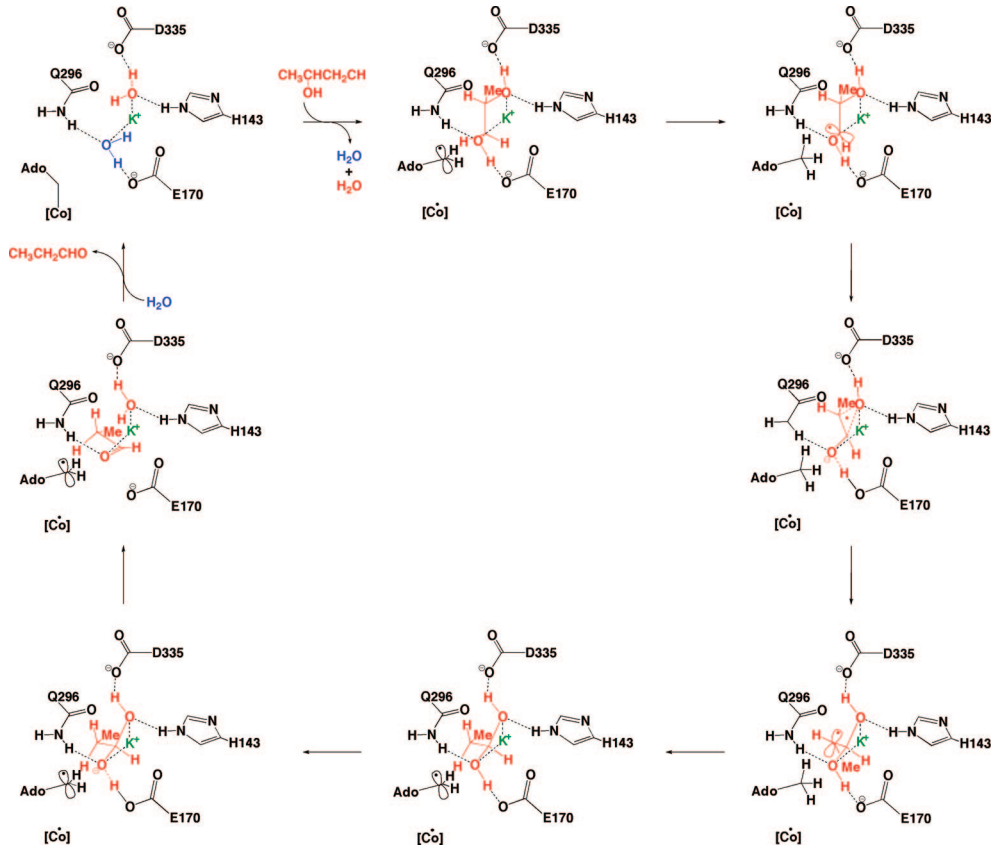
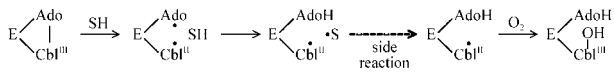


FIGURE 6: Possible roles of Hisα143 and other active-site residues in the diol dehydratase reaction. Ado-CH_2•, adenosyl radical; [Co], cobalamin. Residue numbers in the α subunit. Hisα143, Gluα170, and Aspα335 are catalytic residues.

enzyme are 50–60% of that of the diol dehydratase-bound coenzyme (*15*). It is thus evident that the concentration of substrate-derived radical with the Hα143A mutant is almost ~100% of that of the enzyme-bound conenzyme in the initial phase. Upon prolonged incubation, the doublet signal was rapidly replaced by a singlet signal. These observations reflect the inactivation of Hα143A during catalysis with 1,2-propanediol. Upon this inactivation, a quantitative amount of 5′-deoxyadenosine was formed from the enzyme-bound coenzyme, but apoenzyme itself was not damaged. Thus, it is likely that the inactivation of Hα143A is a mechanism-based inactivation as shown below:

The suggestion that hydrogen bonding interaction between



(E, enzyme; SH, substrate; Cbl, cobalamin)

the OH group on C2 of the substrate and residue α143 is important for both catalytic activity and preventing the enzyme from mechanism-based inactivation is consistent with the idea (*17, 19, 29, 31*) that the Hisα143 residue stabilizes the transition state for the OH group migration and maintains the proper orientation of the reactive radical intermediates.

The deuterium KIE on the rate of inactivation ($k_{inact,H}/k_{inact,D}$) suggested that a hydrogen abstraction step is not rate-determining for the inactivation during catalysis with Hα143A, but slightly rate-determining with Hα143Q. This ratio would be determined by a relative barrier height for the hypothetical inactivating side reaction to that of a hydrogen abstraction step. The rate of inactivation (k_{inact}) with Hα143Q is 14 times slower than that with Hα143A, but the reason for this small $k_{inact,H}/k_{inact,D}$ value is not clear, because the barrier height for the hydrogen abstraction with the mutant is not available at present.

The preferences for (*R*)- and (*S*)-1,2-propanediols were reversed between the wild-type and Hα143Q enzymes; that is, the former exhibited a higher affinity for the *S*-enantiomer in the substrate binding and higher activity for the *R*-enantiomer in the catalysis, but the reverse is the case for the latter. In both cases, an enantiomer that showed higher affinity underwent dehydration at a slower rate. This is reasonable because a larger activation energy would be required for an enantiomer that is bound more tightly in the ground state.

The deuterium KIE on the overall reaction with the Hα143Q mutants was in the same range as that with the wild-type enzyme. This value is much higher than the usual and might be explained in terms of hydrogen tunneling, although definitive evidence of hydrogen tunneling has not yet been obtained. It was thus indicated that breaking of the C−H bond is the rate-determining step in the diol dehydratase reaction (*5, 9*). Interestingly, this effect obtained with Hα143A was 4.0, indicating that a hydrogen abstraction step is partially rate-determining with this mutant. This might suggest that the barrier height for the 1,2-migration of the OH group increases and thus becomes comparable to that for the hydrogen abstraction with the Hα143A mutant. On the basis of the assumption that the activation energies for the hydrogen abstraction from a substrate by the adenosyl radical and hydrogen back-abstraction (recombination) from the methyl group of 5′-deoxyadenosine by a product-derived

radical are not so much affected by this mutation, it is likely that the activation energy for the OH group migration increased with the Hα143A mutation. In other words, Hisα143 may stabilize the transition state for the OH group migration. This conclusion was supported by our computational mutation analysis (*55*). According to the results of QM/MM calculations by Yoshizawa and co-workers with a whole-enzyme model of the Hisα143 mutant, the activation energy for the OH group migration increases to 16.4 from 11.5 kcal/mol in the wild-type enzyme. The activation barriers for the hydrogen abstraction and back-abstraction are estimated to be 13.6 and 17.9 kcal/mol, respectively, for the wild-type enzyme (*29, 55*) and 11.7 and 19.6 kcal/mol, respectively, for the Hisα143 mutant (*55*). It was therefore predicted that the hydrogen back-abstraction is the rate-determining step for the diol dehydratase reaction catalyzed by the wild-type enzyme, in accordance with the earlier proposals by ourselves (*26*) and Radom and co-workers (*30, 56*), and that the difference between the activation energies for the OH group migration and the hydrogen back-abstraction in the Hisα143 mutant is smaller than in the wild-type enzyme.

Figure 6 depicts the possible roles of Hisα143 and other active-site residues. This conclusion is drawn from structural, mutational, biochemical, and theoretical studies and based on the assumptions that (i) Gluα170 is ionized at the start of the reaction and (ii) K^+ remains coordinated to the substrate and all radical intermediates throughout the course of the reaction. There is an argument against the latter assumption. Diol dehydratase absolutely requires K^+ or another monovalent cation with an ionic radius similar to that of K^+ for activity (*1*). Such a monovalent cation plays essential roles in catalysis (*1*), cobalamin binding (*57*), and the homolytic cleavage of the coenzyme Co−C bond (*58*) for the wild-type diol dehydratase. On the basis of the recent X-ray structures of this enzyme (*19–22*), we have proposed a mechanism of action of diol dehydratase on the assumption that the substrate and intermediates remain coordinated to K^+ during the course of the reaction (*17, 22*). Strict stereochemical courses of the diol dehydratase reaction (*3–5*) have also been explained on the basis of this assumption (*22*). If the coordinating interaction observed in the resting state between substrate vicinal OH groups and K^+ is broken during catalysis, additional energy would be required. Frey and co-workers reported that pulsed EPR experiments with Tl^+-holodiol dehydratase fail to detect the magnetic interaction between the Tl^+ nucleus and the inhibitor-derived *cis*-ethanesemidione radical (*59*). Very recently, they described that the unpaired electron on the substrate-derived radical interacts with solvent-exchangeable protons and that no spectroscopic evidence of a direct coordination of the substrate radical to Tl^+ was obtained using EPR, ENDOR, and ESEEM spectroscopy (*60*). This might be due to a very low or negligible spin density on K^+ in the substrate radical intermediate [0.0 according to the theoretical calculation by the QM/MM method (*29*)]. One could point out that, at present, there are only negative spectroscopic data on the location of monovalent cation and that its role as a Lewis acid or in orienting the substrate (or for both) remains to be established unambiguously. Mutant enzymes also require K^+ for activity (*31*), and enzyme activity was thus measured in the presence of >85 mM K^+. However, there is no direct evidence for the binding of K^+ to their active sites, and we have to await the crystallographic analyses of them.

The X-ray structure of methylmalonyl-CoA mutase revealed that the His244 residue is hydrogen-bonded to the carbonyl oxygen of the migrating thioester group (*61*). Thomä and Leadlay reported that this hydrogen bond is crucial for the stability and reactivity of substrate-derived radical (*62*). They concluded from substrate-dependent tritium partitioning that His244 assists radical rearrangement (*63*). Banerjee and co-workers proposed that partial protonation by this residue may facilitate the rearrangement reaction (*64*) in support of the prediction by theoretical calculations of Wetmore et al. (*56*). Therefore, the roles of His244 in methylmalonyl-CoA mutase and Hisα143 in diol dehydratase may be functionally 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.

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REFERENCES

1. Lee, H. A., Jr., and Abeles, R. H. (1963) Purification and properties of dioldehydrase, an enzyme requiring a cobamide coenzyme. *J. Biol. Chem. 238*, 2367–2373.
2. Toraya, T., Shirakashi, T., Kosuga, T., and Fukui, S. (1976) Coenzyme B_{12}-dependent diol dehydrase: Glycerol as both a good substrate and a potent inactivator. *Biochem. Biophys. Res. Commun. 69*, 475–480.
3. Rétey, J., Umani-Ronchi, A., Seibl, J., and Arigoni, D. (1966) On the mechanism of the propanediol dehydrase reaction. *Experientia 22*, 502–503.
4. Rétey, J., Umani-Ronchi, A., and Arigoni, D. (1966) On the stereochemistry of the propanediol dehydrase reaction. *Experientia 22*, 72–73.
5. Zagalak, B., Frey, P. A., Karabatsos, G. L., and Abeles, R. H. (1966) The stereochemistry of the conversion of D and L 1,2-propanediols to propionaldehyde. *J. Biol. Chem. 24*, 3028–3035.
6. Frey, P. A., Essenberg, M. K., and Abeles, R. H. (1967) Studies on the mechanism of hydrogen transfer in the cobamide coenzyme-dependent dioldehydrase reaction. *J. Biol. Chem. 242*, 5369–5377.
7. Frey, P. A., and Abeles, R. H. (1966) Studies on the mechanism of hydrogen transfer in the cobamide coenzyme-dependent diol-dehydrase reaction. *J. Biol. Chem. 241*, 2732–2733.
8. Frey, P. A., Kerwar, S. S., and Abeles, R. H. (1967) The participation of the two non-equivalent C-5′ hydrogens of B_{12}-coenzyme in the catalytic process. *Biochem. Biophys. Res. Commun. 29*, 873–879.
9. Essenberg, M. K., Frey, P. A., and Abeles, R. H. (1971) Studies on the mechanism of hydrogen transfer in the coenzyme B_{12} dependent dioldehydrase reaction II. *J. Am. Chem. Soc. 93*, 1242–1251.
10. Abeles, R. H., and Lee, H. A., Jr. (1964) Studies of the mechanism of action of cobamide coenzymes. *Ann. N.Y. Acad. Sci. 112*, 695–702.
11. Cockle, S. A., Hill, H. A. O., Williams, R. J. P., Davies, S. P., and Foster, M. A. (1972) The detection of intermediates during the conversion of propane-1,2-diol to propionaldehyde by glycerol-dehydrase, a coenzyme B_{12} dependent reaction. *J. Am. Chem. Soc. 94*, 275–277.
12. Finlay, T. H., Valinsky, J., Mildvan, A. S., and Abeles, R. H. (1973) Electron spin resonance studies with dioldehydrase. Evidence for radical intermediates in reactions catalyzed by coenzyme B_{12}. *J. Biol. Chem. 248*, 1285–1290.
13. Valinsky, J. E., Abeles, R. H., and Fee, J. A. (1974) Electron spin resonance studies on diol dehydrase 3. Rapid kinetic studies on the rate of formation of radicals in the reaction with propanediol. *J. Am. Chem. Soc. 96*, 4709–4710.
14. Toraya, T., Ushio, K., Fukui, S., and Hogenkamp, H. P. C. (1977) Studies on the mechanism of the adenosylcobalamin-dependent diol

dehydrase reaction by the use of analogs of the coenzyme. *J. Biol. Chem. 252*, 963–970.
15. Toraya, T., Krodel, E., Mildvan, A. S., and Abeles, R. H. (1979) Role of peripheral side chains of vitamin B_{12} coenzymes in the reaction catalyzed by dioldehydrase. *Biochemistry 18*, 417–426.
16. Abeles, R. H. (1979) Current status of the mechanism of action of B_{12}-coenzyme, in *Vitamin B12* (Zagalak, B., and Friedrich, W., Eds.) pp 373–388, Walter de Gruyter, Berlin.
17. Toraya, T. (2003) Radical catalysis in coenzyme B_{12}-dependent isomerization (eliminating) reactions. *Chem. Rev. 103*, 2095–2127.
18. Banerjee, R. (2003) Radical carbon skeleton rearrangements: Catalysis by coenzyme B_{12}-dependent mutases. *Chem. Rev. 103*, 2083–2094.
19. Shibata, N., Masuda, J., Tobimatsu, T., Toraya, T., Suto, K., Morimoto, Y., and Yasuoka, N. (1999) A new mode of B_{12} binding and the direct participation of a potassium ion in enzyme catalysis: X-ray structure of diol dehydratase. *Structure 7*, 997–1008.
20. Masuda, J., Shibata, N., Morimoto, Y., Toraya, T., and Yasuoka, N. (2000) How a protein generates a catalytic radical from coenzyme B_{12}: X-ray structure of a diol-dehydratase-adeninylpentylcobalamin complex. *Structure 8*, 775–788.
21. Shibata, N., Masuda, J., Morimoto, Y., Yasuoka, N., and Toraya, T. (2002) Substrate-induced conformational change of a coenzyme B_{12}-dependent enzyme: Crystal structure of the substrate-free form of diol dehydratase. *Biochemistry 41*, 12607–12617.
22. Shibata, N., Nakanishi, Y., Fukuoka, M., Yamanishi, M., Yasuoka, N., and Toraya, T. (2003) Structural rationalization for the lack of stereospecificity in coenzyme B_{12}-dependent diol dehydratase. *J. Biol. Chem. 278*, 22717–22725.
23. Kuno, S., Fukui, S., and Toraya, T. (1990) Essential histidine residues in coenzyme B_{12}-dependent diol dehydrase: Dye-sensitized photooxidation and ethoxycarbonylation. *Arch. Biochem. Biophys. 277*, 211–217.
24. Smith, D. M., Golding, B. T., and Radom, L. (1999) Toward a consistent mechanism for diol-dehydratase-catalyzed reactions: An application of the partial-proton-transfer concept. *J. Am. Chem. Soc. 121*, 5700–5704.
25. Toraya, T., Yoshizawa, K., Eda, M., and Yamabe, T. (1999) Direct participation of potassium ion in the catalysis of coenzyme B_{12}-dependent diol dehydratase. *J. Biochem. 126*, 650–654.
26. Toraya, T., Eda, M., Kamachi, T., and Yoshizawa, K. (2001) Energetic feasibility of hydrogen abstraction and recombination in coenzyme B_{12}-dependent diol dehydratase reaction. *J. Biochem. 130*, 865–872.
27. Eda, M., Kamachi, T., Yoshizawa, K., and Toraya, T. (2002) Theoretical study on the mechanism of catalysis of coenzyme B_{12}-dependent diol dehydratase. *Bull. Chem. Soc. Jpn. 75*, 1469–1481.
28. Smith, D. M., Golding, B. T., and Radom, L. (2001) Understanding the mechanism of B_{12}-dependent diol dehydratase: A synergistic retro-push-pull proposal. *J. Am. Chem. Soc. 123*, 1664–1675.
29. Kamachi, T., Toraya, T., and Yoshizawa, K. (2004) Catalytic roles of active-site amino acid residues of coenzyme B_{12}-dependent diol dehydratase: Protonation state of histidine and pull effect of glutamate. *J. Am. Chem. Soc. 126*, 16207–16216.
30. Sandala, G. M., Smith, D. M., Coote, M. L., Golding, B. T., and Radom, L. (2006) Insights into the hydrogen-abstraction reactions of diol dehydratase: Relevance to the catalytic mechanism and suicide inactivation. *J. Am. Chem. Soc. 128*, 3433–3444.
31. Kawata, M., Kinoshita, K., Takahashi, S., Ogura, K., Komoto, N., Yamanishi, M., Tobimatsu, T., and Toraya, T. (2006) Survey of catalytic residues and essential roles of glutamate-α170 and aspartate-α335 in coenzyme B_{12}-dependent diol dehydratase. *J. Biol. Chem. 281*, 18327–18334.
32. Tobimatsu, T., Kawata, M., and Toraya, T. (2005) The N-terminal regions of β and γ subunits lower the solubility of adenosylcobalamin-dependent diol dehydratase. *Biosci., Biotechnol., Biochem. 69*, 455–462.
33. Tobimatsu, T., Hara, T., Sakaguchi, M., Kishimoto, Y., Wada, Y., Isoda, M., Sakai, T., and Toraya, T. (1995) Molecular cloning, sequencing and expression of the genes encoding adenosylcobalamin-dependent diol dehydrase of *Klebsiella oxytoca*. *J. Biol. Chem. 270*, 7142–7148.
34. Tobimatsu, T., Sakai, T., Hashida, Y., Mizoguchi, N., Miyoshi, S., and Toraya, T. (1997) Heterologous expression, purification, and properties of diol dehydratase, an adenosylcobalamin-dependent enzyme of *Klebsiella oxytoca*. *Arch. Biochem. Biophys. 347*, 132–140.
35. Gill, S. C., and Von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem. 182*, 319–326.
36. Toraya, T., and Mori, K. (1999) A reactivating factor for coenzyme B_{12}-dependent diol dehydratase. *J. Biol. Chem. 274*, 3372–3377.
37. Davis, B. J. (1964) Disc electrophoresis-II: Method and application to human serum proteins. *Ann. N.Y. Acad. Sci. 121*, 404–427.
38. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680–685.
39. Toraya, T., Yamanishi, M., Muguruma, H., Ushio, K., Yamauchi, J., and Kawamura, T. (1997) An electron paramagnetic resonance study on the mechanism-based inactivation of adenosylcobalamin-dependent diol dehydrase by glycerol and other substrates. *Biochim. Biophys. Acta 1337*, 11–16.
40. Finlay, T. H., Valinsky, J., Mildvan, A. S., and Abeles, R. H. (1973) Electron spin resonance studies with dioldehydrase. Evidence for radical intermediates in reactions catalyzed by coenzyme B_{12}. *J. Biol. Chem. 248*, 1285–1290.
41. Toraya, T., and Abeles, R. H. (1980) Inactivation of dioldehydrase in the presence of a coenzyme-B_{12} analog. *Arch. Biochem. Biophys. 203*, 174–180.
42. Toraya, T., Uesaka, M., and Fukui, S. (1974) Coenzyme B_{12} dependent diol dehydrase system. Dissociation of the enzyme into two different protein components and some properties of the components. *Biochemistry 13*, 3895–3899.
43. Poznanskaja, A. A., Tanizawa, K., Soda, K., Toraya, T., and Fukui, S. (1979) Coenzyme B_{12}-dependent diol dehydrase: Purification, subunit heterogeneity, and reversible association. *Arch. Biochem. Biophys. 194*, 379–386.
44. Jensen, F. R., and Neese, R. A. (1975) Relative enantiomer binding and reaction rates with propanediol dehydrase. *Biochem. Biophys. Res. Commun. 62*, 816–821.
45. Bachovchin, W. W., Eagar, R. G., Jr., Moore, K. W., and Richards, J. H. (1977) Mechanism of action of adenosylcobalamin: Glycerol and other substrate analogues as substrates and inactivators for propanediol dehydratase-kinetics, stereospecificity, and mechanism. *Biochemistry 16*, 1082–1092.
46. Toraya, T., Watanabe, N., Ushio, K., Matsumoto, T., and Fukui, S. (1983) Ligand exchange reactions of diol dehydrase-bound cobalamins and the effect of the nucleoside binding. *J. Biol. Chem. 258*, 9296–9301.
47. Yamanishi, M., Ide, H., Murakami, Y., and Toraya, T. (2005) Identification of the 1,2-propanediol-1-yl radical as an intermediate in adenosylcobalamin-dependent diol dehydratase reaction. *Biochemistry 44*, 2113–2118.
48. Boas, J. F., Hicks, P. R., Pilbrow, J. R., and Smith, T. D. (1978) Interpretation of electron spin resonance spectra due to some B_{12}-dependent enzyme reactions. *J. Chem. Soc., Faraday Trans. 2 74*, 417–431.
49. Pilbrow, J. R. (1982) EPR of B_{12}-dependent enzyme reactions and related systems, in *B_{12}* (Dolphin, D., Ed.) Vol. 1, pp 431–462, John Wiley & Sons, New York.
50. Schepler, K. L., Dunham, W. R., Sands, R. H., Fee, J. A., and Abeles, R. H. (1975) A physical explanation of the EPR spectrum observed during catalysis by enzymes utilizing coenzyme B_{12}. *Biochim. Biophys. Acta 397*, 510–518.
51. Yamanishi, M., Yamada, S., Muguruma, H., Murakani, Y., Tobimatsu, T., Ishida, A., Yamauchi, J., and Toraya, T. (1998) Evidence for axial coordination of 5,6-dimethylbenzimidazole to the cobalt atom of adenosylcobalamin bound to diol dehydratase. *Biochemistry 37*, 4799–4803.
52. Wagner, O. W., Lee, H. A., Jr., Frey, P. A., and Abeles, R. H. (1966) Studies on the mechanism of action of cobamide coenzymes. Chemical properties of the enzyme-coenzyme complex. *J. Biol. Chem. 249*, 1751–1762.
53. Yamanishi, M., Yunoki, M., Tobimatsu, T., Sato, H., Matsui, J., Dokiya, A., Iuchi, Y., Oe, K., Suto, K., Shibata, N., Morimoto, Y., Yasuoka, N., and Toraya, T. (2002) The crystal structure of coenzyme B_{12}-dependent glycerol dehydratase in complex with cobalamin and 1,2-propanediol. *Eur. J. Biochem. 269*, 4484–4494.
54. Rétey, J. (1990) Enzymic reaction selectivity by negative catalysis or how do enzymes deal with highly reactive intermediates? *Angew. Chem., Int. Ed. 29*, 355–361.
55. Kamachi, T., Toraya, T., and Yoshizawa, K. (2007) Computational mutation analysis of hydrogen abstraction and radical rearrangement steps in the catalysis of coenzyme B_{12}-dependent diol dehydratase. *Chem.—Eur. J. 13*, 7864–7873.

56. Wetmore, S. D., Smith, D. M., and Radom, L. (2001) Catalysis by mutants of methylmalonyl-CoA mutase: A theoretical rationalization for a change in the rate-determining step. *ChemBioChem* 919–922.
57. Toraya, T., Sugimoto, Y., Tamao, Y., Shimizu, S., and Fukui, S. (1971) Propanediol dehydratase system. Role of monovalent cations in binding of vitamin B_{12} coenzyme or its analogs to apoenzyme. *Biochemistry 10*, 3475–3484.
58. Schwartz, P. A., and Frey, P. A. (2007) Dioldehydrase: An essential role for potassium ion in the homolytic cleavage of the cobalt-carbon bond in adenosylcobalamin. *Biochemistry 46*, 7293–7301.
59. Schwartz, P., LoBrutto, R., Reed, G. H., and Frey, P. A. (2003) Suicide inactivation of dioldehydratase by 2-chloroacetaldehyde: Formation of the '*cis*-ethanesemidione' radical, and the role of a monovalent cation. *Helv. Chim. Acta 86*, 3764–3775.
60. Schwartz, P. A., Lobrutto, R., Reed, G. H., and Frey, P. A. (2007) Probing interactions from solvent-exchangeable protons and monovalent cations with the 1,2-propanediol-1-yl radical intermediate in the reaction of dioldehydrase. *Protein Sci. 16*, 1157–1164.
61. Mancia, F., Keep, N. H., Nakagawa, A., Leadlay, P. F., McSweeney, S., Rasmussen, B., Bösecke, P., Diat, O., and Evans, P. R. (1996) How coenzyme B_{12} radicals are generated: The crystal structure of methylmalonyl-coenzyme A mutase at 2 Å resolution. *Structure 4*, 339–350.
62. Thomä, N. H., and Leadlay, P. F. (1998) Mechanistic and structural studies on methylmalonyl-CoA mutase. *Biochem. Soc. Trans. 26*, 293–298.
63. Thomä, N. H., Evans, P. R., and Leadlay, P. F. (2000) Protection of radical intermediates at the active site of adenosylcobalamin-dependent methylmalonyl-CoA mutase. *Biochemistry 39*, 9213–9221.
64. Maiti, N., Widjaja, L., and Banerjee, R. (1999) Proton transfer from histidine 244 may facilitate the 1,2 rearrangement reaction in coenzyme B(12)-dependent methylmalonyl-CoA mutase. *J. Biol. Chem. 274*, 32733–32737.

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