Survey of Catalytic Residues and Essential Roles of Glutamate-\(\alpha\)170 and Aspartate-\(\alpha\)335 in Coenzyme B\(_{12}\)-dependent Diol Dehydratase*

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The importance of each active-site residue in adenosylcobalamin-dependent diol dehydratase of *Klebsiella oxytoca* was estimated using mutant enzymes in which one of the residues interacting with substrate and/or K\(^+\) was mutated to Ala or another amino acid residue. The E\(\alpha\)170A and D\(\alpha\)335A mutants were totally inactive, and the H\(\alpha\)143A mutant showed only a trace of activity, indicating that Glu-\(\alpha\)170, Asp-\(\alpha\)335, and His-\(\alpha\)143 are catalytic residues. The Q\(\alpha\)141A, Q\(\alpha\)296A, and S\(\alpha\)362A mutants showed partial activity. It was suggested from kinetic parameters that Gln-\(\alpha\)296 is important for substrate binding and Gln-\(\alpha\)296 and Gln-\(\alpha\)141 for preventing the enzyme from mechanism-based inactivation. The E\(\alpha\)221A, E\(\alpha\)170H, and D\(\alpha\)335A did not form the (a\(\beta\)G)\(_2\) complex, suggesting that these mutations indirectly disrupt substrate contacts. Among other Gln-\(\alpha\)170 and Asp-\(\alpha\)335 mutants, E\(\alpha\)170D and E\(\alpha\)170Q were 2.2 \pm 0.3% and 0.02% as active as the wild-type enzyme, respectively, whereas D\(\alpha\)335N was totally inactive. Kinetic analysis indicated that the presence and the position of a carboxyl group in the residue \(\alpha\)170 are essential for catalysis as well as for the continuous progress of catalytic cycles. It was suggested that the roles of Glu-\(\alpha\)170 and Asp-\(\alpha\)335 are to participate in the binding of substrate and intermediates and keep them appropriately oriented and to function as a base in the dehydration of the 1,1-diol intermediate. In addition, Gln-\(\alpha\)170 seems to stabilize the transition state for the hydroxyl group migration from C2 to C1 by accepting the proton of the spectator hydroxyl group on C1.

Diole dehydratase (dl-1,2-propanediol hydro-lyase, EC 4.2.1.28) is a coenzyme B\(_{12}\) (AdoCbl)\(_2\)-dependent enzyme that catalyzes the conversion of 1,2-propanediol, glycerol, and 1,2-ethanediol to the corresponding aldehydes (1–3). Labeling experiments of Rétey et al. (4, 5) and Abeles and co-workers (6) as well as kinetic studies of this enzyme led to the general conclusion that, in the AdoCbl-dependent rearrangements, a hydrogen atom migrates from one carbon atom of substrate to an adjacent carbon atom in exchange for a group X that moves in the opposite direction (Reaction 1) (for reviews, see Refs. 7 and 8). In the case of diole dehydratase,

\[
\begin{align*}
\text{X} + \text{H}_2\text{O} & \rightarrow \text{HX} + \text{OH}_2 \\
\text{HX} & \rightarrow \text{X} + \text{H}_2\text{O}
\end{align*}
\]

\text{REACTION 1}

X is a hydroxyl group on C2 of 1,2-propanediol. A minimal mechanism was established in which the enzyme-bound 5'-deoxyadenosine serves as an intermediate in the hydrogen transfer process (Fig. 1A) (1, 7–11). It was proposed by theoretical calculation that the hydroxyl group on C2 migrates to C1 by a concerted pathway through a three-membered cyclic transition state (12). Other pathways can also be considered for the 1,2-shift of the hydroxyl group from the results of model reactions (13–16). The reaction pathway that we proposed for diol dehydratase is shown in Fig. 1B (1).

The crystal structures of diol dehydratase in complexes with coenzyme analogs in the substrate-bound (17–19) and substrate-free (20) forms have been determined. The enzyme exists as a dimer of a heterotrimeter (a\(\beta\)G)\(_2\). The (a\(\gamma\)G)\(_2\) complex and the \(\beta\) subunit correspond to components S and F, respectively, and the association of these components forming the (a\(\beta\)G)\(_2\) complex is absolutely necessary for coenzyme binding and, thus, for catalytic activity (21–23). The active site is located in the C-terminal side of a (\(\beta\)/a)\(_8\) barrel (so-called TIM barrel) of the \(\alpha\) subunit above the corrin ring of cobalamin (17). The two hydroxyl groups of 1,2-propanediol are directly coordinated to the K\(^+\) ion that is located in the inner part of the active-site cavity and hydrogen-bonded to respective two-amino acid residues (Fig. 1C). This structure suggests that the K\(^+\) ion and/or the active-site residues might be involved in the transition state stabilization for the hydroxyl group migration from C2 to C1 (24). Theoretical calculations suggested that the K\(^+\) ion is important for substrate binding but stabilizes the transition state rather slightly (25, 26). The long distance between the cobalt atom and C1 (8.37 Å) and C2 (9.03 Å) of the substrate eliminated the possibility that Co(II) of cob(II)alam is directly involved in the hydroxyl group migration through organocobalamin intermediates, such as “substratlycobalamin” and “productlycobalamin” (27). Models also supported the direct rearrangement of the substrate radical to the product radical (12–15).

Smith et al. (28, 29) proposed from the accurate theoretical calculation with a simple model of diol dehydratase that the barrier height for the hydroxyl group migration is lowered by a synergistic retro-push-pull catalysis assisted by partial protonation and partial deprotonation through the active-site imidazolium and carboxylate ions, respectively. In contrast, Kamachi et al. (30) recently predicted from the quantum mechanical/molecular mechanical calculation with a whole-enzyme model that Glu-\(\alpha\)170 and unprotonated His-\(\alpha\)143, rather than the pro-
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A

\[\text{HO} \quad \text{HO} \quad \text{Ade} \quad \text{Co} \quad \text{Co} \]

B

\[\text{CH}_2\text{CH}_2\text{CH}_2\text{OH} \quad \text{C} \quad \text{C} \quad \text{X} \quad \text{X} \]

C

\[\text{Q} \quad \text{S} \quad \text{D} \quad \text{H} \]

FIGURE 1. Mechanism and substrate binding of AdoCbl-dependent diol dehydratase. A, minimal mechanism. a, homolysis of the Co-C bond of enzyme-bound AdoCbl. b, adenosyl radical-mediated rearrangements. (Co), cobalamin; Ade, 9-adeninyl; SH, substrate; PH, product; X, a generic migrating group (X = OH on C2 in diol dehydratase reaction). B, postulated mechanism of hydrogen abstraction and back-abstraction (recombination) in the diol dehydratase reaction. Only the reaction with (S)-1,2-propanediol is shown. For the reaction with (R)-1,2-propanediol, see Shibata et al. (19). C, interactions between active-site residues, substrate, and K⁺ (steric drawing). Residue numbers in the α subunit. PDO, 1,2-propanediol.

tonated His-α143, are important for the diol dehydratase catalysis in that order (30). However, no experimental evidence for the catalytic functions of the active-site residues of diol dehydratase is available yet, although it is essential for our understanding of the action mechanism of this type of enzymes. In this paper catalytic amino acid residues were surveyed to help the understanding of the roles of the active-site residues as well as the pathway of the hydroxyl group migration in the diol dehydratase reaction. Seven mutant enzymes in which one of the active-site residues interacting with substrates and/or K⁺ was mutated to alanine were expressed in Escherichia coli and purified, and their enzymatic functions were examined. The result of so-called “alanine scan” is reported here. In addition, four mutants in which Glu-α170 or Asp-α335 was mutated to Asp, Gln, His, or Asn were also prepared to investigate their catalytic roles in further detail because Glu-α170 and Asp-α335 are hydrogen-bonded to the C1 and C2 hydroxyl groups of substrate, respectively. Glu-α170 is coordinated to K⁺ as well at the active site (17).

EXPERIMENTAL PROCEDURES

Materials—Crystalline AdoCbl was a gift from Eizai, Co. Ltd. (Tokyo, Japan). Yeast alcohol dehydrogenase was obtained from Sigma. All other chemicals were analytical grade reagents and used without further purification.

Site-directed Mutagenesis of the Gene for the α-Subunit of Diol Dehydratase—Site-directed mutagenesis was generated using a QuikChange™ site-directed mutagenesis kit (Stratagene, CA). The mutagenic sense primers designed were 5′-GCACCCCCGTTCAGGCCGCGACCTCACCAACGTC-3′ for Qα141A, 5′-CCGTCCCAGCAGGCGTTGAG-CAACGTCAAAGATAAC-3′ for Hα143A, 5′-GGATTTGACGAACAGGATACCACCGTTGCGGTAG-3′ for Eα170A, 5′-GGATTTGACGAACAGGCGGCCGTCACCAACGTC-3′ for Qα170A, 5′-GGATTTGACGAACAGGCGGCCGTCACCAACGTC-3′ for Qα296A, 5′-GGATTTGACGAACAGGCGGCCGTCACCAACGTC-3′ for Dα335A, 5′-GGATTTGACGAACAGGCGGCCGTCACCAACGTC-3′ for Qα362A, 5′-GGATTTGACGAACAGGCGGCCGTCACCAACGTC-3′ for Dα335A, and 5′-GGATTTGACGAACAGGCGGCCGTCACCAACGTC-3′ for Sα362A. The oligonucleotides having the complementary sequences in the opposite direction were used as the respective antisense primers. Plasmid pUC(α) (21), which contains the pddA gene encoding the α subunit of Klebsiella oxytoca diol dehydratase, was used as a template for the mutant plasmids designated pUC(αQα141A), pUC(αHα143A), pUC(αEα170A), pUC(αQα296A), pUC(αDα335A), and pUC(αSα362A). A 3.1-kb BamHI-EcoRI fragment from pUS12E(DD)
(31) which contains the pddABC genes encoding all the three subunits of K. oxytoca diol dehydratase, was ligated to the BamHI-EcoRI region of pUC119 to produce pUC119(DD). pUC119(DD) was used as a template for the mutant plasmids designated pUC119(DD-Ea170Q), pUC119(DD-Ea170Q), and pUC119(DD-Ea170Q). pUSI2E(DD) was used as a template for the construction of pUSI2E(DD-Da335N). It was confirmed by nucleotide sequencing that all the mutants do not have unintended mutations.

**Construction of Expression Plasmids**—To avoid undesired mutations, some of mutant plasmids were constructed by substituting the appropriate region of pUC119(DD) with restriction fragments of the corresponding mutated DNAs. A 0.2-kb BanII-Ndel fragment from pUC(α57-Qeα141A), pUC(α57-Haα143A), or pUC(α57-Eaα170A) and a 5.56-kb fragment obtained from pUC119(DD) by digestion partial with BanII and complete with Ndel were ligated, followed by insertion of a 0.36-kb Ndel fragment from pUC119(DD) to construct pUC119(DD-Qeα141A), pUC119(DD-Haα143A), or pUC119(DD-Eaα170A), respectively. A 0.22-kb DraIII-BsmI fragment from pUC(α57-Qeα296A) and a 1.8-kb BsmI fragment from pUC119(DD) were ligated to the 4.1-kb DraIII-BsmI fragment of pUC119(DD) to obtain pUC119(DD-Qeα296A). A 0.18-kb BsmI-AflIII fragment from pUC(α57-Da335A) or pUC(α57-Sa362A) and a 1.6-kb AflIII-BsmI fragment from pUC119(DD) were inserted to the BsmI site of pUC119(DD) to obtain pUC119(DD-Da335A) or pUC119(DD-Sa362A), respectively. A 3.1-kb BamHI-EcoRI fragment from each pUC(DD) mutant plasmid was finally ligated to the corresponding region of vector pUSI2End (32) to obtain an expression plasmid for a mutant enzyme. For the construction of pUSI2End(DD-Ea221A), a 0.08-kb Sphl-DraIII fragment from pUC(α57-Ea221A) was ligated to the Sphl-DraIII region of pUC119(DD). A 2.3-kb Sphl-EcoRI fragment from the resulting plasmid and a 0.66-kb BamHI-Sphl fragment from pUC119(DD) were ligated to the BamHI-EcoRI region of pUSI2End.

**Expression and Purification of Mutant Diol Dehydratases**—All the mutants were expressed as described before (21). The Qeα141A, Haα143A, Qeα296A, Eaα170A, and Sa362A mutant enzymes were purified essentially as described previously for the recombinant wild-type enzyme (21). The Eaα170Q and Eaα170H mutants were purified similarly but with the following modifications; the amount of buffer used for sonication and washing was decreased to 2 or 3 volumes of precipitate, and the precipitate was washed only twice with 50 mM potassium phosphate buffer (pH 8.0) containing 2% 1,2-propanediol and 0.2% Brij35, because the solubility of these mutants is higher than the wild-type enzyme. Furthermore, the DEAE-cellulose column chromatography was omitted, since the mutants extracted from crude membrane fractions with the same buffer but containing 1% Brij35 were found to be almost homogeneous. In some preparations of Eaα170A mutant enzyme, (αβγ)2 and (αγ)2 complexes corresponding to the apoenzyme and component S, respectively (21), coexisted. The Eaα170D, Ea221A, Da335A, and Da335N mutants were purified simply by washing the precipitate with 50 mM potassium phosphate buffer (pH 8.0) containing 2% 1,2-propanediol but without Brij35.

**Enzyme and Protein Assays**—Diol dehydratase activity was determined by the 3-methyl-2-benzothiazolinone hydrazone method (33). The standard reaction mixture contained an appropriate amount of apoenzyme, 15 μM AdoCbl, 0.1 mM 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% potassium citrate buffer (pH 3.6). 3-Methyl-2-benzothiazolinone hydrazone 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/min at 37 °C under the standard assay conditions. Time courses of diol dehydratase reaction were measured by the alcohol dehydrogenase-NADH-coupled method (34).

Protein concentration of 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 120,500 M−1 cm−1 (36).

**Kinetic Analysis of Mutant Diol Dehydratases**—K+ free wild-type and mutant enzymes were obtained by gel filtration on a Sephadex G-25 (fine) column that had been previously equilibrated and eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M 1,2-propanediol and 1% Brij35. The wild-type enzyme, thus prepared, did not show any observable activity without added monovalent cation, such as K+, and was, therefore, assumed to be K+ free, although metal analysis was not made. Apparent Km values for K+ were determined by assaying the enzyme activity at fixed concentrations of 1,2-propanediol (100 mM) and AdoCbl (15 μM) with varying concentrations of KCl.

Substrate-free apoenzyme used for the measurement of Km values for 1,2-propanediol 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% Brij35 or 20 mM sucrose mononacrose with two buffer changes. The 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. Apparent Km values for 1,2-propanediol and AdoCbl were determined by Lineweaver-Burk plots at a fixed AdoCbl concentration of 15 μM and at a fixed 1,2-propanediol concentration of 100 mM.

**Other Analytical Procedures**—PAGE analysis of mutant diol dehydratases was performed under non-denaturing conditions as described by Davis (37) in the presence of 0.1 M 1,2-propanediol or under denaturing conditions as described by Laemmli (38). Protein staining was carried out with Coomassie Brilliant Blue R-250.

Optical spectra were obtained on a Jasco V-560 recording spectrophotometer. Experimental details are described in the legends to Figs. 3 and 4. For the measurements under anaerobic conditions, the air in the system was replaced by argon (purity, 99.999%); that is, a AdoCbl solution and a reaction mixture without AdoCbl were degassed separately in a Thunberg tube-like quartz cell by repeating evacuation/argon-introduction 3 times, then mixed, and incubated.

For EPR measurements, mutant apoenzymes purified by DEAE-cellulose column chromatography (21) were used. Substrate-free apoenzyme solution (~1–2 mg of protein in 0.6 ml of 50 mM potassium phosphate buffer (pH 8.0) containing 20 mM sucrose mononacrose) was mixed at 0 °C with AdoCbl solution (50 nmol in 0.05 ml) in a quartz EPR tube (5 mm in diameter) stoppered with a rubber septum. After placement of the air in the tube with nitrogen by repeating evacuation/nitrogen-introduction three times, holoenzymes were formed, and EPR spectra were taken as described in the legend to Fig. 5. The sample prepared was transferred to the EPR cavity and cooled with a cold N2 gas flow controlled by a JEOL JES-VT3A temperature controller. EPR spectra were measured as described previously (39, 40) at ~130 °C on JEOL JES-RE3X spectrometer modified with a Gunn diode X-band microwave unit. EPR microwave frequency, 9.170–9.179 GHz; modulation amplitude, 1 millitesla; modulation frequency, 100 kHz; microwave power, 10 milliwatt.
Coomassie Brilliant Blue R-250. Molecular mass markers were SDS-7 (Sigma). Bands of the enzymes in which one of the active-site residues was mutated to alanine propanediol but without the detergent. Among them, D and D densitometric determination (Fig. 2, same procedure as that for the wild-type enzyme (21); that is, by extraction from the initial velocity (kcat) and kcat/Km values for 1,2-propanediol, K+, and AdoCbl were determined with the wild-type and catalytically active mutant enzymes (Table 1). The Qa296A mutation increased Km for substrate by a factor of 250. The Qa141A and Sa362A mutations also lowered the affinity for the substrate but to much lesser degrees. The values of catalytic efficiency (kcat/Km) calculated from the initial velocity (kcat) and Km for 1,2-propanediol are also given in Table 1. It is reasonable that Gln-a296 plays an important role in the substrate binding and catalysis, because the x-ray structure revealed its ligation to both K+ and substrate (Fig. 1C). It is also reasonable that the Sa362A mutation did not lower the affinity of enzyme for K+, because Ser-a362 coordinates to K+ by its amide oxygen. It is rather surprising that the loss of the side-chain amide oxygen of Gln-a141 and Gln-a296 did not result in the lowering of binding affinity for K+, although they coordinate to K+ by the side-chain amide oxygen. Although these residues do not directly interact with the coenzyme, the binding affinity for AdoCbl slightly increased upon the Qa141A and Qa296A mutations.

Inactivation of Mutant Diol Dehydratases during Catalysis—Although the Sa362A mutant did not show significant inactivation during catalysis at least for 15 min (k1/2 = 0.018 min⁻¹), the Qa296A and Qa141A mutants underwent inactivation during catalysis under aerobic conditions and lost their activities almost completely within 5–6 and 10–12 min, respectively (data not shown). Their inactivation rates obeyed the first-order reaction kinetics with k1/2 values of 0.56 and 0.26 min⁻¹, respectively. kcat/k1/2 values, which show the average numbers of catalytic turnovers before inactivation, indicated that Gln-a296 and Gln-a141 are important for the continuous progress of catalytic cycles (Table 1). When time courses of the reaction with mutant enzymes were measured under anaerobic conditions (under an argon atmosphere), the inactivation during catalysis took place similarly, although the inactivation rates were slower than those under aerobic by a factor of about two. It was, therefore, concluded that the inactivation is mainly due to the mechanism-based inactivation (suicide inactivation) rather than the inactivation by oxygen. The Ea170D and Ea170Q mutants also have tendencies to undergo inactivation during catalysis. The kcat/k1/2 values of Ea170Q and Ha143A indicated that these mutant enzymes undergo inactivation after only 90 and 170 turnovers on average, respectively (Table 1). These ratios are more suitable parameters than k1/2 values.
for comparing the tendencies of enzymes to the mechanism-based inactivation. Thus, it is evident that Glu-α170 and His-α143 play important roles not only in the catalysis but also in the prevention of highly reactive radical intermediate(s) from undesirable side reactions.

Spectral Changes of AdoCbl upon Incubation with Mutant Diol Dehydratases in the Presence of Substrate—Even under aerobic conditions AdoCbl (Fig. 3A) bound to the wild-type enzyme undergoes the spectral change to that of the enzyme-bound cob(II)alamin (B12a); the absorbance at 525 nm decreases and a new peak at 478 nm appears (Fig. 3B). This spectrum shows the steady-state concentration of cob(II)alamin during catalysis of wild-type enzyme was ~50% that of the enzyme-bound AdoCbl, which was roughly consistent with the previous data (34). AdoCbl was partly re-formed upon denaturation of the enzyme and then converted to aquacobalamin upon photolllumination.

When a similar experiment was carried out with the E170A mutant, cob(II)alamin was similarly formed but was converted to aquacobalamin upon denaturation without photolllumination (Fig. 3C). This indicated that AdoCbl underwent the irreversible cleavage of the Co-C bond upon binding to E170A in the presence of substrate, although this mutant was catalytically inactive (<0.01%). In contrast, with the Qa296A and Qe141A mutants, partially active enzymes, the gradual accumulation of OH-Cbl was observed (Fig. 3, D and E). The rate of conversion of AdoCbl to OH-Cbl seems to be correlated with their rates of mechanism-based inactivation. With the Sa362A mutant, cob(II)alamin reached a low steady-state concentration quickly and was then converted to OH-Cbl very slowly (Fig. 3F). The concentrations of cob(II)alamin formed by these mutants were ~5, ~15, and ~30% that of the enzyme-bound AdoCbl, respectively, when calculated from the absorbance at 478 nm.

Under anaerobic conditions, the accumulation of cob(II)alamin was observed with wild-type (Fig. 4B) and all the mutant enzymes (Fig. 4, C–F). Because the mechanism-based inactivation took place even under anaerobic conditions, it is likely that inactivated products also show a cob(II)alamin-like spectrum under anaerobic conditions. It is, therefore, evident that one of the机制化 active products is cob(II)alamin, which cannot be spectroscopically distinguishable from the catalytic intermediate and oxidized to OH-Cbl under aerobic conditions (Fig. 3, D–F). It should be noted that the cob(II)alamin-like species formed by E170A is resistant to oxidation to OH-Cbl even under aerobic conditions (Fig. 3C).

EPR Spectra with Mutant Diol Dehydratases—When the wild-type enzyme was incubated with AdoCbl in the presence of 1,2-propanediol, the same EPR spectrum as that previously reported (39, 40) was obtained (Fig. 5A). The low-field broad signal was assigned to low-spin Co(II) of cob(II)alamin, and the high-field doublet signal has recently been identified as 1,2-propanediol-1-yl radical (41). The spectrum was interpreted to arise from weak coupling in this radical pair (42–44). The signals disappeared within 3 min of incubation at 25 °C because of the exhaustion of substrate added (Fig. 5A). The holoenzyme of Qa296A mutant gave an EPR spectrum similar to that with the wild-type enzyme, although the signal intensity was lower (Fig. 5C). The relative intensity of the doublet signal decreased with time of incubation at 25 °C, and the finally obtained spectrum resembled that of cob(II)alamin without an organic radical coupling partner (45). This result is consistent with the spectral change with this mutant under anaerobic conditions (Fig. 4E) and supports the observation that the mutant is partially active but undergoes rapid inactivation during catalysis.

In contrast, a very low concentration of organic radical was observed with the holoenzyme of E170A mutant (Fig. 5B). The shape and g value of the signal were obviously different from those with the wild-type enzyme, suggesting that this unidentified organic radical is not the usual intermediate (1,2-propanediol-1-yl radical) in the catalytic cycle. The doublet signal disappeared upon further incubation at 25 °C, and upon prolonged incubation, the EPR spectrum of the enzyme-bound cob(II)alamin without an organic radical coupling partner was obtained. This result with E170A is also consistent with the spectroscopic observation that O2-resistant cob(II)alamin species was formed with this mutant. It is, thus, evident that Glu-α170 is absolutely required for catalysis but not essential for the homolysis of the coenzyme Co-C bond.

**DISCUSSION**

Catalytic functions of the active-site residues in the diol dehydratase reaction remained open questions because no mutational studies have been reported yet. In the present paper seven mutant diol dehydratases in which one of the amino acid residues interacting with substrate and/or K+ was substituted by alanine were expressed in E. coli cells in high levels, purified, and examined for activity. Among the active-site residues, Glu-α170, Asp-α335, and His-α143 turned out to be catalytic residues of diol dehydratase.

Catalytic functions of Glu-α170 and Asp-α335 were further investigated in details. Because Glu-α170 and Asp-α335 interact with the hydroxyl groups on C1 and C2 of the substrate, respectively, one role of these residues would be to participate in the binding of substrate and intermediates and keep them appropriately oriented for the reaction. Another role of them may be to function as bases in the dehydration of the 1,1-diol intermediate. Analysis of stereocchemical courses of the reaction suggested that the Glu-α170 and Asp-α335 residues are involved in the dehydration reactions with (S)- and (R)-1,2-propanediol as substrate, respectively (19). The other important role of Glu-α170 may be to stabilize the transition state for the hydroxyl group migration from C2 to C1 by accepting the proton of the spectator hydroxyl group on C1. The catalytic roles of Glu-α170 are summarized in Fig. 6, which is consistent with the following studies; model reactions of diol dehydratase suggested that 1,2-dihydroxyethyl radical undergoes a proton-catalyzed (13, 14) and OH−-catalyzed (14) dehydration to the formylmethyl radical. Buckel and co-workers (16) proposed an elimination/readdition reaction.
mechanism for the radical rearrangement in the diol dehydratase reaction in which the hydroxyl group on C1 undergoes deprotonation to a ketyl. Radom and co-workers (29) predicted from theoretical calculations that retro-push-pull effects of His-H9251143 and Glu-H9251170 synergistically lower the barrier height for the 1,2-shift of the hydroxyl group. Based on the quantum mechanical/molecular mechanical calculation on a whole-enzyme model, Kamachi et al. (30) predicted recently that unprotonated His-H9251143 is involved in the reaction and that the pull effect of Glu-H9251170 is more important for the transition state stabilization.

Optical and EPR spectra indicated that the coenzyme undergoes the Co-C bond homolysis when it is incubated with the Ea170A mutant in the presence of substrate and that the cob(II)alamin species formed is O2-insensitive. This might be an extreme case of the mechanism-based inactivation. The undesirable side reaction leading to the inactivation might occur with this mutant at much higher probability because the transition state for the hydroxyl group migration is not stabilized by Glu-H9251170. Alternatively, the inactivation of this mutant at an extremely high probability may be due to the inappropriate orientation of bound intermediate(s) without COO- in the residue H170. The apparent O2 insensitivity of cob(II)alamin in the inactivated complex might be explained in terms of inaccessibility of O2 molecules to cob(II)alamin in the presence of tightly bound 1,2-diol or other intermediates without turnovers.
It is also reasonable that His-α143 serves as a catalytic residue in the reaction, because it is hydrogen-bonded to the hydroxyl group on C2 of 1,2-propanediol (17). Kinetic analyses indicated that this residue is important for both stabilizing the transition states and protecting reactive radical intermediates against side reactions. However, there are arguments on its protonation state in the diol dehydratase catalysis (29, 30). Mutational analysis of the catalytic function of His-α143 in further detail is in progress, and the results will be reported elsewhere.

The Qa296A, Qα141A, and Sa362A mutants were partially active enzymes with $k_{\text{cat}}$ decreasing in this order. The catalytic efficiency ($k_{\text{cat}}/K_m$) of the Qa296A mutant was much lower than the other two due to its lower affinity for substrate. It should be noted that $k_{\text{cat}}/k_{\text{inact}}$ values of Qa296A and Qα141A were 20–40 times lower than that of the wild-type enzyme. The disappearance of an organic radical intermediate in the inactivation was demonstrated by EPR with the Qa296A mutant. These findings suggest that Gln-α296 and Gln-α141 are not essential for activity but important for continuous progress of catalytic cycles, probably by keeping the radical intermediates properly oriented through direct binding of substrate (Gln-α296) or indirect interaction via the coordination to K$^+$. According to the Rétey’s concept (46), enzymes prevent highly reactive intermediates from undesired side reactions by negative catalysis. It is intriguing that $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ values of the Sa362A mutant were lowered to some extent as compared with the wild-type enzyme, although this residue coordinates to K$^+$ with the main-chain amide oxygen. However, the $k_{\text{cat}}/k_{\text{inact}}$ value was close to that of the wild-type enzyme, indicating that Ser-α362 does not play an important part in controlling the radical intermediates.

The $k_{\text{cat}}/k_{\text{inact}}$ values of mutant enzymes under anaerobic conditions decreased to their halves. It is well known that O$_2$ causes the inactivation of holodiol dehydratase in the absence of substrate (47). This inactivation is believed to be due to the reaction of activated Co-C bond of holoenzyme with oxygen. Therefore, it is likely that holoenzymes of mutant enzymes undergo not only mechanism-based inactivation but also inactivation by O$_2$ even during catalysis. Substrate and O$_2$ may compete for the adenosyl radical-Co(II) radical pair in the catalytic site.

As judged from $K_m$ for K$^+$, the affinity of the enzyme for K$^+$ was not much affected by either of the Qa296A, Qα141A, and Sa362A mutations. This may be partly because K$^+$ is coordinated by multiple ligands and partly because each coordination to K$^+$ through an amide oxygen atom is rather weak. In contrast, COO$^-$ of Glu-α221 and Glu-α170 is coordinated to K$^+$ much more strongly.
Catalytic Residues in Diol Dehydratase

The Ez221A, E170H, and D335A mutants neither existed as the (αβγ), complexes, although they were expressed in high levels, nor showed any detectable activity. Because neither of Glu-α221, Glu-α170, and Asp-α335 exists in the subunit interface, these mutations seem to disrupt the subunit contacts in an indirect manner. As reported previously (17), the active site of diol dehydratase in the (βα)6 barrel is negatively charged due to many acidic amino acid residues including Glu-α221 and Glu-α170. These residues may contribute to the proper folding of the α subunit for the formation of functional (αβγ)2 complex through the coordination to K+. This speculation is supported by the fact that K+ or another monovalent cation having a similar ionic radius is absolutely required for the cobalamin binding by this enzyme (48).

In class I and II ribonucleotide reductases, enzymes that use similar chemistry as diol dehydratase, the glutamate residue hydrogen-bonded to the non-eliminating hydroxyl group of substrates is conserved (49, 50). The essential early event of ribonucleotide reduction is the hydrogen abstraction from C3’ of the vicinaldiol moiety of ribonucleotide substrates (51). The roles of this glutamate were considered to participate in the binding of substrates and to facilitate the leaving of the C2’-hydroxyl (49–52), both of which are similar in diol dehydratase. It was reported by mutational experiments that the carboxylic functionality at residue 441 is absolutely essential (53–55) and that the reduction of the 3’-ketodeoxynucleotide by a sulfide radical anion is the rate-determining step in the ribonucleotide reduction and facilitated by the protonated form of this glutamate residue (55). In contrast, no suitably oriented acidic side chain is found in class III ribonucleotide reductase (56). In this case, it was postulated that formate serves as both an overall or another monovalent cation having a similar ionic radius is absolutely required for the cobalamin binding by this enzyme (48).

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Survey of Catalytic Residues and Essential Roles of Glutamate-α170 and Aspartate-α335 in Coenzyme B12-dependent Diol Dehydratase
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