7210 *Biochemistry* **2010,** *49,* 7210–7217
DOI: 10.1021/bi100561m

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Coenzyme B_{12}-Dependent Diol Dehydratase Is a Potassium Ion-Requiring Calcium Metalloenzyme: Evidence That the Substrate-Coordinated Metal Ion Is Calcium†

Tetsuo Toraya,*,‡,§ Susumu Honda,§,∥ and Koichi Mori‡

‡*Department of Bioscience and Biotechnology, Graduate School of Natural Science and Technology, Okayama University, Tsushima-naka, Kita-ku, Okayama 700-8530, Japan, and* §*Department of Industrial Biochemistry, Faculty of Engineering, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan* ∥*Present address: Genome Research Laboratories, New Products Development Operations, Laboratory Chemicals Division, Wako Pure Chemical Industries, Ltd., 6-1 Takada-cho, Amagasaki, Hyogo 661-0963, Japan.*

Received April 13, 2010; Revised Manuscript Received July 9, 2010

ABSTRACT: The X-ray analyses of coenzyme B_{12}-dependent diol dehydratase revealed two kinds of electron densities that correspond to metal ions in the active site. One is directly coordinated by substrate [Shibata, N., et al. (1999) *Structure 7*, 997−1008] and the other located near the adenine ring of the coenzyme adenosyl group [Masuda, J., et al. (2000) *Structure 8*, 775−788]. Both have been assigned as potassium ions, although the coordination distances of the former are slightly shorter than expected. We examined the possibility that the enzyme is a metalloenzyme. Apodiol dehydratase was strongly inhibited by incubation with EDTA and EGTA in the absence of substrate. The metal analysis revealed that the enzyme contains ∼2 mol of tightly bound calcium per mole of enzyme. The calcium-deprived, EDTA-free apoenzyme was obtained by the EDTA treatment, followed by ultrafiltration. The activity of the calcium-deprived apoenzyme was dependent on Ca^{2+} when assayed with 1 mM substrate. The K_m for Ca^{2+} evaluated in reconstitution experiments was 0.88 μM. These results indicate that the calcium is essential for catalysis. Ca^{2+} showed a significant stabilizing effect on the calcium-deprived apoenzyme as well. It was thus concluded that the substrate-coordinated metal ion is not potassium but calcium. The potassium ion bound near the adenine ring would be the essential one for the diol dehydratase catalysis. Therefore, this enzyme can be considered to be a metal-activated metalloenzyme.

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, 1,2-ethanediol, and glycerol to propionaldehyde, acetaldehyde, and β-hydroxypropionaldehyde, respectively (*1*, *2*). The enzyme was purified to homogeneity from *Klebsiella oxytoca* ATCC8724 (formerly known as *Aerobacter aerogenes* and *Klebsiella pneumoniae*) (*1*, *3*, *4*) and shown to be composed of two dissimilar protein components designated F and S, both of which are essential for catalysis (*5*). Later, it was demonstrated with the recombinant enzyme that it consists of three kinds of subunits, designated α, β, and γ, and that the whole enzyme and components F and S correspond to $\alpha_2\beta_2\gamma_2$, β, and $\alpha_2\gamma_2$, respectively (*6*, *7*). It is widely accepted that metal-requiring enzymes can be grouped into two categories, so-called metal-activated enzymes and metalloenzymes. Members of the former group are activated by the addition of a certain metal ion, whereas the members of the latter group contain a tightly bound metal ion that is essential for activity. Diol dehydratase absolutely requires K^+ or other monovalent cations with ionic radii similar to that of K^+ for activity (*1*, *8*). It has been shown that such a monovalent cation plays essential roles in the cobalamin binding (*8*) and homolytic cleavage of the coenzyme Co−C bond (*9*). In this sense, diol dehydratase is a metal-activated enzyme.

The X-ray structure of diol dehydratase in complex with CN-Cbl, an inactive analogue of AdoCbl, and the substrate 1,2-propanediol was determined in 1998, and the electron density near the two OH groups of substrate was assigned to K^+ (*10*). The structure revealed that, in addition to these OH groups, the five oxygen atoms from five amino acid residues are coordinated to the metal ion. When the X-ray structure of the enzyme−AdePeCbl complex was analyzed (Figure 1), it turned out that another metal ion is bound near the adenine moiety of the coenzyme adenosyl group (*11*). This metal ion was also identified as K^+ (*11*). From the structural, mutational, biochemical, and theoretical studies, we have proposed a detailed mechanism of action of diol dehydratase, based on the assumption that the substrate and all the radical intermediates remain coordinated to K^+ throughout the course of the reaction (*12*, *13*).

This assumption seems reasonable for explaining the mechanism of strict stereochemical control (*14*−*16*) during the diol dehydratase reaction (*17*). Furthermore, additional energy would

†This work was supported in part by Grants-in-Aid for Scientific Research [(B) 13480195 and (B) 17370038 and Priority Areas 753 and 513 to T.T.] from the Japan Society for Promotion of Science and the Ministry of Education, Culture, Sports, Science and Technology, Japan, and a Grant in Aid for Natural Sciences Research from the Asahi Glass Foundation (Tokyo, Japan).

*To whom correspondence should be addressed. Telephone: 81-86-251-8194. Fax: 81-86-251-8264. E-mail: toraya@cc.okayama-u.ac.jp.

[1]Abbreviations: AdePeCbl, adeninylpentylcobalamin; AdoCbl, adenosylcobalamin or coenzyme B_{12}; CN-Cbl, cyanocobalamin; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; ESEEM, electron spin-echo envelope modulation; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonate; MBTH, 3-methyl-2-benzothiazolinone hydrazone; PAGE, polyacrylamide gel electrophoresis; QM/MM, quantum mechanical/molecular mechanical; SDS, sodium dodecyl sulfate.

 Published on Web 07/12/2010

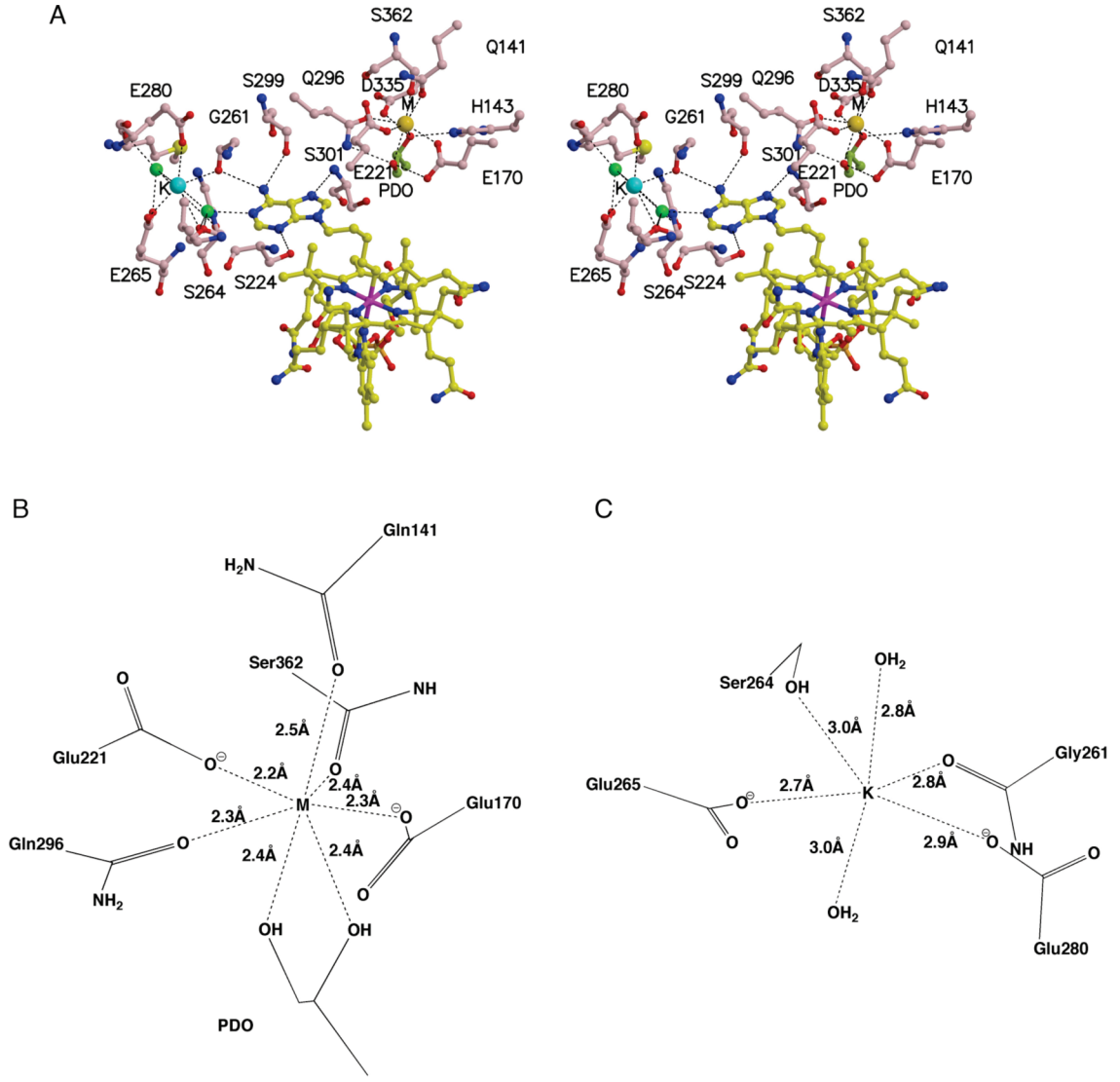

FIGURE 1: Two metal ions bound in the active site of diol dehydratase. (A) Metal ions located near the substrate OH groups and near the adenine moiety of the coenzyme adenosyl group. M represents the substrate-coordinated metal ion and K the potassium ion. (B) Coordination distances between M and ligands from amino acid residues and substrate 1,2-propanediol (PDO). (C) Coordination distances between K and ligands from amino acid residues and water molecules.

be required if the initial coordinating interaction between substrate OH groups and K^+ were broken during catalysis. There might be 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 (*18*)]. However, there is a strong argument against the assumption. 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 (*19*). Very recently, they found 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 (*20*). Therefore, one could point out that the roles of metal ions in diol dehydratase remain to be established unambiguously.

To address this problem, we have experimentally and theoretically examined the possibility that the substrate-coordinated metal ion might not be K^+ but another metal ion. The first experimental support for this possibility was our finding that diol dehydratase is almost completely inhibited by the preincubation with EDTA in the absence of substrate. This led us to examine the possibility that this enzyme is a metalloenzyme. In this paper, we report that diol dehydratase is a calcium metalloenzyme and that the substrate-coordinated metal ion is not K^+ but calcium. The theoretical support for this conclusion has also been published elsewhere (*21*). The mechanism of inhibition with EDTA as well as the role of calcium in the catalysis is also described here.

MATERIALS AND METHODS

Materials. Crystalline AdoCbl was a gift from Eisai, Co. Ltd. (Tokyo, Japan). All the standard solutions for metal analyses were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Centriplus YM-10 and Centricon YM-10 for ultrafiltration were obtained from Millipore Corp. (Bedford, MA). Dialysis tubing was purchased from Viskase Co. Inc. (Darien, IL). Sephadex G-25 (fine) and Bio-Gel P-200 for gel filtration were the products of GE Healthcare UK Ltd. (Little Chalfont, U.K.) and Bio-Rad Laboratories Inc. (Hercules, CA), respectively. All other chemicals were analytical grade reagents from commercial sources.

Preparations of Diol Dehydratase. For comparison, both the recombinant enzyme and the enzyme from *K. oxytoca* were used. The former was purified from overexpressing *Escherichia coli* JM109 cells harboring expression plasmid pUSI2E(DD) or pUSI2ENd(DD), essentially as described previously (*7*). The latter was purified from *K. oxytoca* ATCC8724 cells grown without aeration in glycerol-1,2-propanediol medium, as described previously (*3*). Both enzyme preparations were established to be homogeneous by the criteria of nondenaturing and denaturing PAGEs. Enzymes were purified as apoenzymes in the presence of substrate (1,2-propanediol), but substrate-free apoenzymes were obtained by either dialysis against substrate-free buffers or ultrafiltration through Centriplus YM-10 using substrate-free buffers.

Enzyme and Protein Assays. Diol dehydratase activity was routinely assayed by the MBTH method (*22*). The standard assay 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 0.2−1.0 mL. After incubation at 37 °C for 10 min, the reaction was terminated by addition of potassium citrate buffer (pH 3.6) to a concentration of 0.05 M. MBTH-HCl was then added to a final concentration of 0.9 mM, and the mixture was incubated again at 37 °C for 15 min. The concentration of propionaldehyde formed was 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.

The protein concentration of the purified enzyme was determined by measuring the absorbance at 280 nm. The molar absorption coefficient at 280 nm for this enzyme, calculated by the method of Gill and von Hippel (*23*) from the deduced amino acid composition and subunit structure, is 120500 M^{-1} cm^{-1} (*24*).

Preparation of Calcium-Deprived Apoenzyme. Apoenzyme was deprived of calcium by incubation at 37 °C for 10 min with 0.2 mM EDTA (pH 8.0) in the absence of substrate, followed by ultrafiltration through a YM-10 membrane.

Metal Analysis. All solutions were prepared form glass-distilled deionized water and were stored in polyethylene containers. All the buffers were freed from metal ions by extraction with freshly dissolved 0.01% dithizone in CCl_4. Whenever possible, plastic containers were cleaned by being soaked in a 1:1 solution of nitric and sulfuric acids for several hours and rinsed thoroughly with metal-free water. Polyethylene ware was washed successively with diluted detergent, 0.1 N NaOH, 0.1 N HCl, 1 mM EDTA, and metal-free water. Cellulose dialysis tubing was boiled in 0.1 M EDTA (pH 8.0) for 10 min. It was then transferred to a plastic container and stored at 4 °C in 0.01 M EDTA (pH 8.0). All dialysis tubing, magnetic stirbars, etc., were handled with disposable polyethylene gloves to prevent the contamination of metal ions.

The zinc, calcium, copper, iron, magnesium, manganese, molybdenum, cobalt, and chromium contents of the purified enzyme from *K. oxytoca* and the calcium content of the recombinant enzyme were determined with Nippon-Jarrell Ash and Hitachi Z-2000 Polarized Zeeman atomic absorption spectrophotometers, respectively. To remove unbound metal ions, purified diol dehydratase (0.3−2.6 mg) was either extensively dialyzed against 2 L of 0.05 M potassium phosphate buffer (pH 8.0) containing 0.26 M 1,2-propanediol or ultrafiltered through a YM-10 membrane and diluted in 0.01 M HEPES buffer (pH 8.0) containing 10 mM KCl, 0.26 M 1,2-propanediol, and 0.2% Brij35. Buffer blanks were run for all the metal analyses. $LaCl_3$ was used as the internal standard in the calcium analysis. To check the sensitivity and accuracy of the calcium analyses, the following measurements were taken as controls. Bovine serum albumin (approximately 1.0 mg/mL) was confirmed to be essentially free of calcium and not to affect the measured value of calcium concentration when added to a standard calcium solution. The calcium analysis was thus not appreciably affected by the presence of protein. The calcium content of α-amylase from *Bacillus subtilis* (Sigma) was 3 mol/mol of enzyme, in good agreement with the published value (*25*).

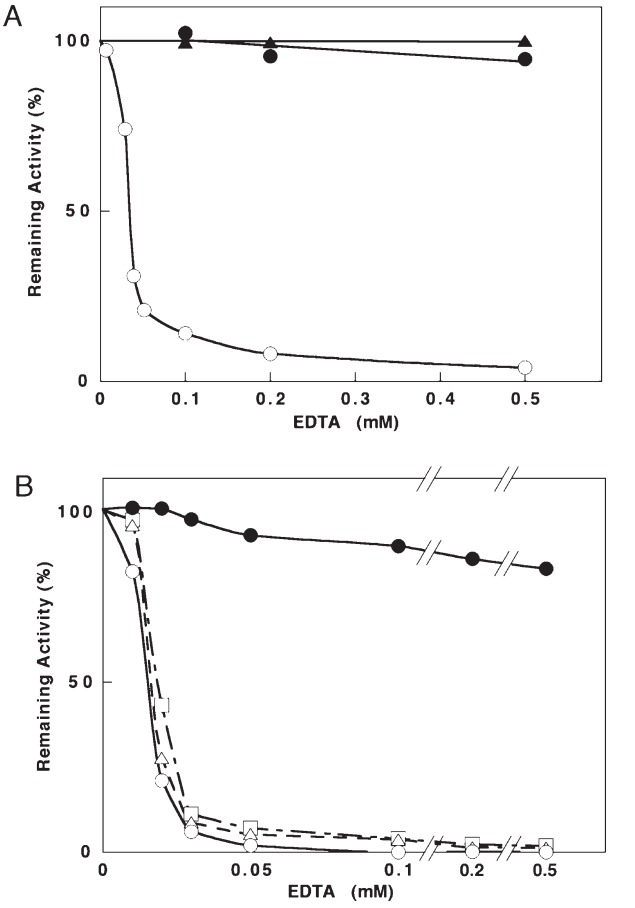

FIGURE 2: Inhibition of diol dehydratase by preincubation with EDTA. (A) Purified apoenzyme (0.1 unit) from *K. oxytoca* was preincubated with the indicated concentrations of EDTA in 1 mL of 0.03 M potassium phosphate buffer (pH 8.0) containing 0.05 M KCl in the absence (○) and presence of 10 mM 1,2-propanediol (●) or 15 μM AdoCbl (▲). (B) Purified apoenzyme (0.1 unit) from *K. oxytoca* was preincubated with EDTA in 1 mL of 0.03 M Tris-HCl buffer (pH 8.0) containing no additions (△), 0.05 M KCl (○), 10 mM 1,2-propanediol (□), or 0.05 M KCl with 10 mM 1,2-propanediol (●). After preincubation at 37 °C for 5 min, the remaining reactants of the usual assay mixture were added to small aliquots, and the remaining activity was measured under standard assay conditions. The activity of each enzyme incubated without EDTA was taken as the control (100%).

PAGE Analysis. Nondenaturing PAGE was performed as described by Davis (*26*) and denaturing PAGE (SDS−PAGE) as described by Laemmli (*27*). Proteins were stained with Coomassie Brilliant Blue R-250.

RESULTS

Inhibition of Diol Dehydratase with EDTA or EGTA. The effect of EDTA treatment on enzyme activity was investigated.

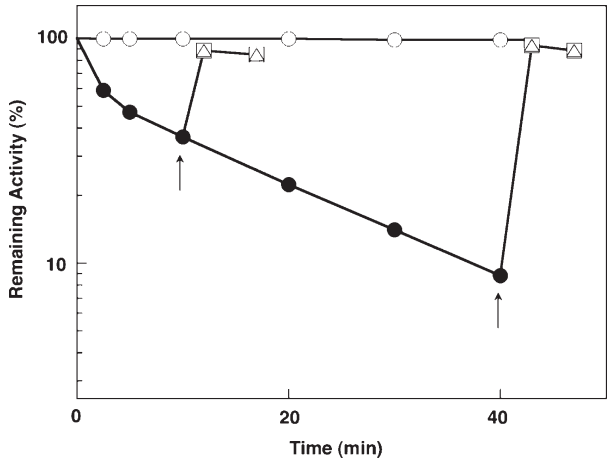

FIGURE 3: Time dependence of EDTA inhibition of diol dehydratase. Purified apoenzyme (0.076 unit) from *K. oxytoca* was incubated without (○) and with (●) 0.5 mM EDTA in 1 mL of 0.036 M potassium phosphate buffer (pH 8.0) at 0 °C. After incubation for 10 and 40 min, $CaCl_2$ (□) or $MgCl_2$ (△) was added to a final concentration of 2 mM. After standing at 0 °C for the indicated time periods, the remaining reactants of the usual assay mixture were added, and the remaining activity was measured.

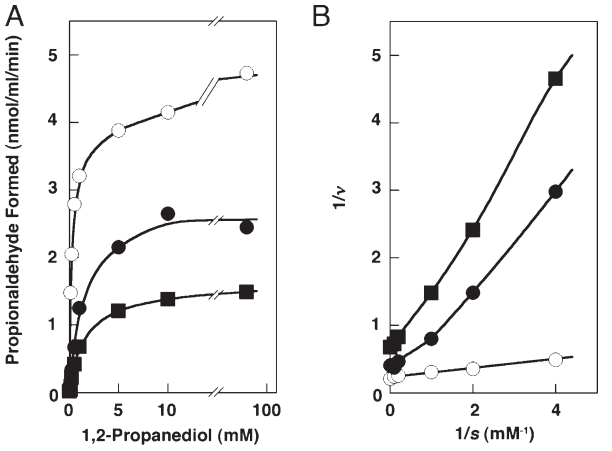

FIGURE 4: Type of inhibition of diol dehydratase by EDTA with respect to substrate. Recombinant apoenzyme (0.0064 unit) was preincubated at 37 °C for 5 min with the indicated concentrations of 1,2-propanediol in the absence (○) and presence of 0.008 (●) and 0.016 mM (■) EDTA in 0.9 mL of 0.03 M potassium phosphate buffer (pH 8.0) containing 0.05 M KCl. After preincubation at 37 °C for 5 min, AdoCbl was added to a concentration of 15 μM, and the remaining activity was measured: (A) $v-s$ curve and (B) double-reciprocal plots.

As shown in Figure 2A, strong inhibition was observed when purified apoenzyme from *K. oxytoca* was incubated at 37 °C for 5 min with EDTA in potassium phosphate buffer (pH 8.0) in the absence of substrate. The initial rate (k) of inhibition at 37 °C with 0.5 mM EDTA was ~0.13 min^{-1} (data not shown). The inhibition was prevented by the presence of 10 mM 1,2-propanediol, indicating that the substrate protects apoenzyme from the EDTA inhibition. Holoenzyme formed by the preincubation of apoenzyme with AdoCbl in the absence of substrate was also not susceptible to EDTA (Figure 2A). When recombinant apoenzyme was used instead of purified apoenzyme from *K. oxytoca*, essentially the same result was obtained (Figure S1A of the Supporting Information).

Of the other metal-binding reagents tested, EGTA also acted as a strong inhibitor of the enzyme (Figure S1B of the Supporting Information). However, the following compounds were not inhibitory at a concentration of 5 mM: 1,10-phenanthroline, α, α′-dipyridyl, citrate, succinate, tartrate, malate, and salicylate. It was reported that salicylate inhibits glycerol dehydratase, a similar AdoCbl-dependent dehydratase (*28*).

Some Characteristics of the EDTA Inhibition. The protective effect of 1,2-propanediol was examined in Tris-HCl buffer (pH 8.0) in the presence and absence of K^+. 1,2-Propanediol exhibited a protective effect on the enzyme from *K. oxytoca* only in the presence of K^+, although neither 1,2-propanediol nor K^+ alone was protective (Figure 2B). Similar results were obtained with recombinant apoenzyme as well (data not shown). Besides 1,2-propanediol, the following substrates or substrate analogues also exhibited the protective effect at a concentration of 50 mM against the inhibition with 0.2 mM EDTA in potassium phosphate buffer (pH 8.0): stylene glycol, glycerol, 2,3-butanediol, and 1,2-ethanediol (data not shown). All of them promote the reassociation of components F and S (*29*), which is facilitated in the presence of K^+ (*30*). It seems thus likely that the degree of EDTA inhibition is affected by the molecular assembly of subunits or components.

Figure 3 shows the time course of EDTA inhibition at 0 °C, indicating that the inhibition is time-dependent. When excess (2 mM) $CaCl_2$ (or $MgCl_2$) was added after incubation for 10 and 40 min, 87−91% of the enzyme activity was recovered. This indicates that the EDTA inhibition of apoenzyme is reversible at least in the initial phase. The inhibition was pH-dependent and less marked at pH <7 (Figure S2 of the Supporting Information). The pH of 50% inhibition was approximately 7.8, suggesting that EDTA does not exert the inhibitory effect at the lower pH where protons compete with Ca^{2+} for the ligand.

Figure 4A shows the effect of substrate concentration on EDTA inhibition. When enzyme activity was measured with varied concentrations of substrate in the presence of fixed concentrations of EDTA, the extent of inhibition was dependent on the substrate concentration; that is, the inhibition is more marked at a lower concentration of substrate. The double-reciprocal plot (Figure 4B) in the presence of EDTA did not provide straight lines, suggesting that the inhibition is not a simple competitive-type one with respect to substrate.

Because diol dehydratase consists of components F and S, both of which are essential for activity, dissociation of the enzyme into components might be a cause of the EDTA inhibition. This possibility was tested by nondenaturing PAGE analysis. However, even after purified recombinant apoenzyme was incubated at 37 °C for 10 min with an extraordinarily high concentration (50 mM) of K^+-EDTA in the absence of substrate, almost all ($>92\%$) of the enzyme migrated as a single band of $(\alpha\beta\gamma)_2$ in the absence of substrate (Figure S3A of the Supporting Information). Because the EDTA inhibition was observed in K^+-containing buffer, this eliminated the possibility that EDTA inhibited the apoenzyme by causing its dissociation into components or subunits.

In contrast, when treated with 50 mM Na^+-EDTA in the absence of substrate, approximately half of the apoenzyme dissociated (Figure S3B of the Supporting Information). Almost no dissociation took place when the sample was treated with only 125 mM KCl or NaCl. When the apoenzyme treated with 50 mM Na^+-EDTA in the absence of substrate (Figure S3C of the Supporting Information) was analyzed by two-dimensional electrophoresis, the band just below that of $(\alpha\beta\gamma)_2$ still contained a small amount of the β subunit, whereas the lowest band no

Table 1: Metal Analysis of Purified Diol Dehydratase from *K. oxytoca*

metal	diol dehydratase (mg/mL)	metal detected (mg/mL)	metal content[a] (mol/mol of enzyme)
Ca	2.57	0.925	1.9
	1.53	0.469	1.6
Mg	2.57	0.067	0.26
	1.53	0.034	0.19
Zn	0.80	0.032	0.12
Fe	2.57	0.063	0.09
Cu	0.80	0.046	0.16
Co	0.80	0.000	0.00
Mn	0.80	0.026	0.09
Cr	0.80	0.024	0.10
Mo	0.80	0.000	0.00

[a]Calculated on the basis of a molecular weight of 207268 (*6*).

longer contained the β subunit at all (data not shown). Because the component S obtained by DEAE-cellulose column chromatography also gave the major band in the same position, the lowest band might be that of $(\alpha\gamma)_2$ (component S). It was thus concluded that high concentrations of both Na^+ and EDTA are necessary for the marked dissociation of the apoenzyme into components in the absence of substrate. This dissociation might be followed by slow, irreversible inactivation or denaturation of the enzyme.

Metal Analysis of Diol Dehydratase. Although diol dehydratase shows selectivity in activation by monovalent cations, such as K^+ and NH_4^+ (*1*, *8*), no requirements of divalent metal ions for catalysis have been reported so far. To explain the inhibitory effects of EDTA and EGTA, atomic absorption analysis of the purified enzyme for metals was performed. Tables 1 and 2 show that both the enzyme purified from *K. oxytoca* and the recombinant enzyme contain about 2 mol of calcium per mole of enzyme. Since the enzyme exists as a dimer of heterotrimers, i.e., $(\alpha\beta\gamma)_2$ (*9*), it is likely that one calcium atom is bound to each $\alpha\beta\gamma$ heterotrimer unit. Magnesium was also detected, but in a stoichiometrically insignificant amount. Copper, iron, zinc, manganese, chromium, cobalt, and molybdenum were not detected at all or detected only in negligible quantities.

Removal of Calcium from Apodiol Dehydratase. To investigate the role of calcium in catalysis, the removal of calcium from the recombinant apoenzyme was attempted. When the apoenzyme was incubated with 0.2 mM EDTA (pH 8.0) at 37 °C for 10 min and then ultrafiltered through a YM-10 membrane, the calcium content of the resulting enzyme decreased to almost zero (Table 2). In contrast, calcium was not removed at all by the EDTA treatment in the presence of substrate, followed by ultrafiltration.

Almost all (> 92 and 89%) of this calcium-deprived, EDTA-free apoenzyme migrated as a single band of $(\alpha\beta\gamma)_2$ in the presence and absence of substrate, respectively (data not shown).

Reconstitution of Calcium-Containing Apoenzyme from Calcium-Deprived Apoenzyme and Ca^{2+}. The calcium-deprived, EDTA-free apoenzyme thus obtained was reconstituted into the calcium-containing enzyme by the preincubation at 30 °C for 10 min with various concentrations of $CaCl_2$ in the absence of substrate. When assayed with 1 mM 1,2-propanediol, the enzyme activity was highly dependent on the Ca^{2+} concentration added (Figure 5). The calcium analysis indicated that the calcium contamination of the 2.5 M 1,2-propanediol stock solution was ~0.05 μM, although no calcium contamination of either AdoCbl, dithizone-treated 1 M potassium phosphate buffer (pH 8.0), or dithizone-treated 1 M KCl solution was detected. The calcium contamination of the assay mixture containing 1 mM 1,2-propanediol in the absence of added Ca^{2+} was estimated to be ~0.02 nM from the calcium contamination of the 1,2-propanediol stock solution. The enzyme concentration in the assay mixture was 0.27 nM (0.006 unit/mL). We therefore concluded that the calcium of this enzyme is essential for catalysis. A trace of enzyme activity observed in the absence of added Ca^{2+} might be due to a trace of calcium contamination in the assay mixture. From the activating effect of Ca^{2+}, the apparent K_m for Ca^{2+} was calculated to be 0.88 μM.

Table 2: Removal of Calcium from Recombinant Apoenzyme by EDTA Treatment, Followed by Ultrafiltration

treatment	conditions	calcium content[a] (mol/mol of enzyme)
no treatment	with 1,2-propanediol	2.2
	without 1,2-propanediol	2.4
EDTA treatment 1[b]	with 1,2-propanediol	2.5
	without 1,2-propanediol	0.3
EDTA treatment 2[c]	without 1,2-propanediol	0.0

[a]Calculated on the basis of a molecular weight of 207268 (*6*). [b]Incubated at 37 °C for 5 min with 0.1 mM EDTA, followed by ultrafiltration. [c]Incubated at 37 °C for 10 min with 0.2 mM EDTA, followed by ultrafiltration.

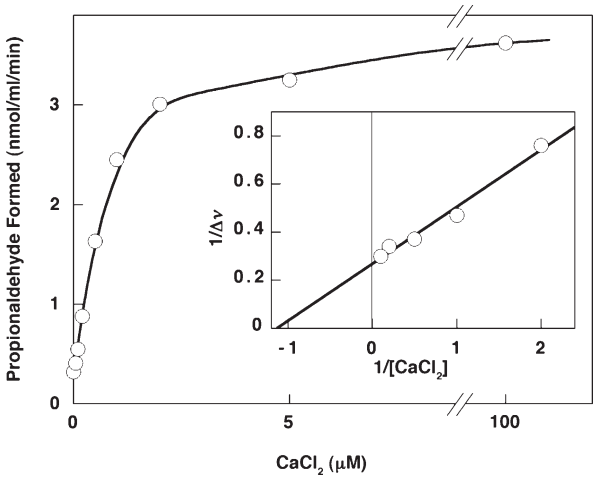

FIGURE 5: Activation of calcium-deprived apodiol dehydratase by preincubation with Ca^{2+}. Calcium-deprived apoenzyme (0.011 unit) obtained as described in the text was preincubated with the indicated concentrations of $CaCl_2$ in 1.5 mL of 0.04 M potassium phosphate buffer containing 0.03% Brij35. After preincubation at 30 °C for 10 min, 1 mM 1,2-propaendiol and 15 μM AdoCbl were added to 0.32 mL aliquots, and the remaining activity was measured. The inset is a double-reciprocal plot.

Stability of Calcium-Deprived Apoenzyme. Calcium-deprived apoenzyme was unstable when incubated even at 37 °C in the absence of both Ca^{2+} and substrate (Figure 6). $CaCl_2$ significantly protected the apoenzyme from inactivation. Substrate fully stabilized the apoenzyme even in the absence of $CaCl_2$, and the addition of both $CaCl_2$ and substrate showed not only a stabilizing effect but also some activating effect (~30%) upon preincubation.

DISCUSSION

As reported in this paper, we found that diol dehydratase is almost completely inhibited by the preincubation with EDTA or

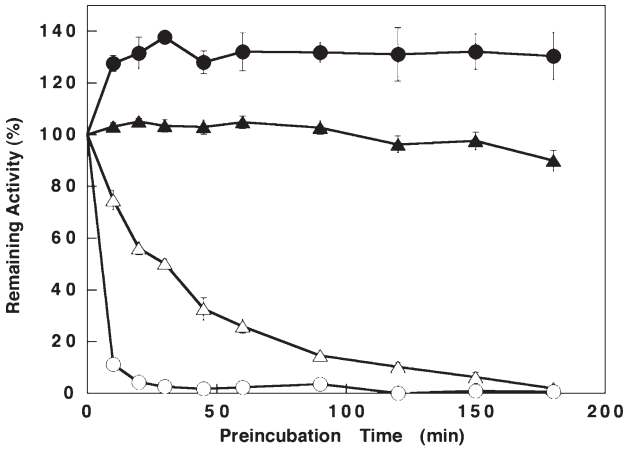

FIGURE 6: Effect of Ca^{2+} and substrate on the stability of calcium-deprived diol dehydratase. Calcium-deprived apoenzyme (corresponding to 0.0012 unit) obtained as described in the text was preincubated at 37 °C for the indicated time periods in 0.16 mL of 0.04 M potassium phosphate buffer (pH 8.0) in the presence and absence of 10 μM $CaCl_2$ and/or 0.1 M 1,2-propanediol. The substrate 1,2-propanediol and AdoCbl were added to concentrations of 0.5 M and 15 μM, respectively, and the remaining activity was measured. Additions: none (○), $CaCl_2$ (△), 1,2-propanediol (▲), and $CaCl_2$ with 1,2-propanediol (●). The activity of apoenzyme kept at 0 °C under respective conditions were taken to be 100%. Average ± standard deviation (n = 3).

EGTA in the absence of substrate. This became a new clue for addressing the problem. The metal analysis revealed that approximately 2 mol of calcium is tightly bound to diol dehydratase, while the contents of other metals were almost negligible.

In the X-ray structure analysis of diol dehydratase in complex with CN-Cbl, a large electron density was observed near the two OH groups of the substrate (*10*, *11*). It is generally not easy to discriminate between K^+ and Ca^{2+} from the electron density and its shape even at a resolution of 1.7 Å. The coordination distances between this metal and ligands, including O1 and O2 of the substrate OH groups, are 2.2, 2.3, 2.3, 2.4, 2.4 (O1), 2.4 (O2), and 2.5 Å (Figure 1B) [Protein Data Bank (PDB) entry 1EGM], which are slightly shorter than those expected for six-coordinated K^+ and rather suitable for Ca^{2+}. Despite these considerations, we assigned the electron density to seven-coordinate K^+ (*10*), partly because this was the only kind of metal ion that bound to the enzyme and partly because K^+ was the only metal ion that has been established to be essential for catalysis (*1*, *8*). Because the data of coordination distances for seven-coordinate K^+ were not available at that time, we assumed that coordination distances in the seven-coordinated "entatic" state might be different from those for six-coordinate K^+. Later, we succeeded in the X-ray analysis of the diol dehydratase−AdePeCbl complex (*11*). We found that another kind of metal ion is bound near the adenine ring of the adenosyl group in addition to the substrate-coordinated one (Figure 1A). This metal ion is six-coordinate, and the coordination distances between this and ligands are 2.7, 2.8, 2.8 (H_2O), 2.9, 3.0, and 3.0 (H_2O) Å (Figure 1C) (PDB entry 1EEX), which are typical for six-coordinate K^+. Therefore, this metal ion was also identified as K^+ (*11*). Recent QM/MM calculations, however, indicated that the coordination distances in the X-ray structures are significantly shorter than the calculated K^+−ligand distances and better fit to the calculated Ca^{2+}−ligand distances (*21*). The coordination distances for seven-coordinate K^+ in arsenate reductase have recently been reported (PDB entry 1JF8) (*31*), but they are not significantly shorter than those for six-coordinate K^+.

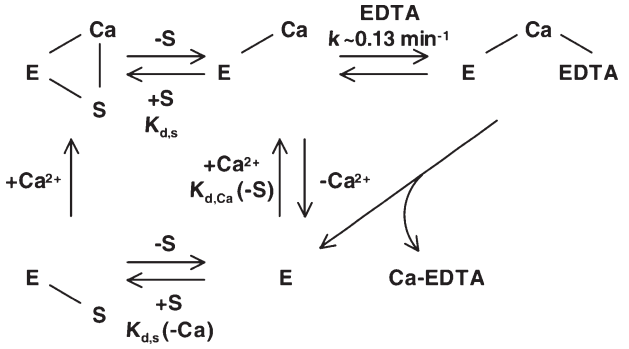

FIGURE 7: Schematic representation of EDTA inhibition of apodiol dehydratase. Abbreviations: E, apoenzyme; S, substrate; $K_{d,s}$, dissociation constant of the calcium-containing apoenzyme for the substrate; $K_{d,s}(-Ca)$, dissociation constant of the calcium-deprived apoenzyme for the substrate; $K_{d,Ca}(-S)$, dissociation constant of the substrate-free apoenzyme for Ca^{2+}. k was estimated from the initial rate of inhibition at 37 °C with 0.5 mM EDTA.

The finding that diol dehydratase is a calcium metalloenzyme raised the possibility that the substrate-coordinated metal ion might be not K^+ but calcium. The K_m of the enzyme for Ca^{2+} determined by reconstitution experiments in the absence of substrate was 0.88 μM. Because the affinity of the enzyme for Ca^{2+} is so high and calcium-free apoenzyme is unstable, calcium-deprived apoenzyme was difficult to obtain by dialysis. Reconstitutable calcium-deprived apoenzyme was obtained here for the first time by the EDTA treatment, followed by ultrafiltration. Calcium-deprived enzyme lost the activity almost completely, but its activity was recovered by the addition of Ca^{2+}. This would be reasonable if the enzyme-bound calcium is directly involved in substrate binding. Collectively from all the data available at present, we reassign the substrate-coordinated metal ion to calcium and propose that, in addition to K^+, this calcium is essential for catalysis. Figure 1A shows the active site structure of the diol dehydratase−AdePeCbl complex (*11*), in which calcium (M) and K^+ are coordinated by the substrate and located near the adenine ring of the adenosyl group, respectively.

It is likely from the experimental results that EDTA causes inhibition by its coordination to the substrate-coordinated metal ion (Figure 7). This reaction occurs in the absence of substrate but is blocked by the substrate. The resulting complex with EDTA would have a sufficiently long lifetime to be significant for enzyme activity. The inhibition seems to be reversible at least in an early stage, because it was mostly reversed by the addition of either Ca^{2+} or Mg^{2+}. The calcium-deprived apoenzyme was less stable than the calcium-containing one but was stabilized partially by Ca^{2+} and fully by substrate (Figure 6). The X-ray structure of the enzyme revealed that the substrate is bound in the negatively charged inner part of the active site cavity in component S ($\alpha_2\gamma_2$ complex). Besides coordination to the metal ion, it forms hydrogen bonds with four amino acid residues (*10*) from four β-strands composing the $(\beta/\alpha)_8$ (TIM) barrel of the α subunit. The substrate binding would thus participate in stabilizing the barrel structure. Therefore, it is reasonable that the removal of substrate from the active site brings about a conformational change of component S, which would result in weakening of the interaction with component F (β subunit) and thus promote its gradual dissociation. This might lead to the slow, irreversible inactivation or denaturation of the enzyme.

As a result of the new assignment described above, K^+ bound near the adenine ring of the coenzyme adenosyl group (upper

axial ligand) must be essential for activity. Why is this K^+ or another monovalent cation with an ionic radius similar to that of K^+ absolutely required for diol dehydratase catalysis (*1*, *8*)? We have previously reported that such a monovalent cation plays an important role in the tight binding of cobalamin to the apoenzyme (*8*). Recently, Frey and co-workers have reported that K^+ is essential for the homolytic cleavage of the coenzyme Co−C bond (*9*). It should be noted that this K^+ is six-coordinate by four amino acid residues and two H_2O molecules, one of which is hydrogen-bonded to N1 of the coenzyme adenine moiety (Figure 1C). It is therefore evident that K^+ is one of the constituents of the so-called "adenine-binding pocket" that plays an essential role in the coenzyme Co−C bond cleavage and thus in catalysis through tight binding and proper positioning of the adenine ring (*11*). In addition, the coordination of the four amino acid residues (Gα261, Sα264, Eα265, and Eα280) and two H_2O molecules (hydrogen bonded to Gα263, Eα265, Mα276, and Vα225) to K^+ would be important for keeping the local orientation of strands β7 and β8 and helices α14 and α15, which may be important for the cobalamin binding as well. The sensitivity of cofactor activity to ionic radii might also be explained on the basis of this structure. The difference between K^+ and Na^+ in inducing the apoenzyme dissociation in the presence of a high concentration of EDTA suggests that the binding of a monovalent cation with an appropriate ionic radius to this site may contribute to the association of subunits or components to some extent.

Glycerol dehydratase, an AdoCbl-dependent isofunctional enzyme, was also reported to be inhibited by EDTA (*28*). This enzyme is very similar to diol dehydratase in various properties (*32*), although they are immunologically distinct enzymes (*33*). In contrast to diol dehydratase, glycerol dehydratase is inhibited with EDTA even in the presence of substrate (*28*). This paper suggests that the EDTA inhibition of glycerol dehydratase is due to dissociation of subunits. However, there might be a possibility that it is also related to metal binding, because the three-dimensional structure and the action mechanism of glycerol dehydratase are similar to those of diol dehydratase (*13*, *34*).

ACKNOWLEDGMENT

We thank Mr. Tokuji Tanaka (Kyoto University) for metal analyses of purified diol dehydratase from *K. oxytoca* and Prof. Jian Feng Ma and Ms. Sanae Rikiishi (Okayama University) for calcium analysis of recombinant diol dehydratase. We thank Dr. Naoki Shibata (University of Hyogo, Hyogo, Japan) for helpful discussion and Ms. Yukiko Kurimoto for her assistance in manuscript preparation.

SUPPORTING INFORMATION AVAILABLE

Inhibition of purified recombinant apodiol dehydratase by preincubation with EDTA and EGTA (Figure S1), pH dependence of EDTA inhibition (Figure S2), and PAGE analysis of EDTA-treated apodiol dehydratase (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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) Substrate specificity of coenzyme B_{12}-dependent diol dehydrase: Glycerol as both a good substrate and a potent inactivator. *Biochem. Biophys. Res. Commun. 69*, 475–480.
3. 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.
4. McGee, D. E., and Richards, J. H. (1981) Purification and subunit characterization of propanediol dehydratase, a membrane-associated enzyme. *Biochemistry 20*, 4293–4298.
5. 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.
6. 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.
7. 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.
8. 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*, 3475–4384.
9. 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.
10. Shibata, N., Masuda, J., Tobimatsu, T., Toraya, T., Suto, K., Morimoto, Y., and Yasuoka, N. (1999) A mew mode of B_{12} binding and the direct participatin of a potassium ion in enzyme catalysis: X-ray structure of diol dehydratase. *Structure 7*, 997–1008.
11. 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.
12. Kinoshita, K., Kawata, M., Ogura, K., Yamasaki, A., Watanabe, T., Komoto, N., Hieda, N., Yamanishi, M., Tobimatsu, T., and Toraya, T. (2008) Histidine-α143 assists 1,2-hydroxyl group migration and protects radical intermediates in coenzyme B_{12}-dependent diol dehydratase. *Biochemistry 47*, 3162–3173.
13. Toraya, T. (2003) Radical catalysis in coenzyme B_{12}-dependent isomerization (eliminating) reactions. *Chem. Rev. 103*, 2095–2127.
14. Rétey, J., Umani-Ronchi, A., Seibl, J., and Arigoni, D. (1966) On the mechanism of the propanediol dehydrase reaction. *Experientia 22*, 502–503.
15. Rétey, J., Umani-Ronchi, A., and Arigoni, D. (1966) On the stereochemistry of the propanediol dehydrase reaction. *Experientia 22*, 72–73.
16. 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. 241*, 3028–3035.
17. 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.
18. 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.
19. Schwartz, P., LoBrutto, R., Reed, G. H., and Frey, P. A. (2003) Suicide inactivation of dioldehydrase by 2-chloroacetaldehyde: Formation of the '*cis*-ethanesemidione' radical, and the role of a monovalent cation. *Helv. Chim. Acta 86*, 3764–3775.
20. 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.
21. Kamachi, T., Takahata, M., Toraya, T., and Yoshizawa, K. (2009) What is the identity of the metal ions in the active sites of coenzyme B_{12}-dependent diol dehydratase? A computational mutation analysis. *J. Phys. Chem. B 113*, 8435–8438.
22. 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.
23. Gill, S. C., and von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem. 182*, 319–326.
24. Toraya, T., and Mori, K. (1999) A reactivating factor for coenzyme B_{12}-dependent diol dehydratase. *J. Biol. Chem. 274*, 3372–3377.

25. Vallee, B. L., Stein, E. A., Sumerwell, W. N., and Fischer, E. H. (1959) Metal content of α-amylases of various origins. *J. Biol. Chem. 234*, 2901–2905.
26. Davis, B. J. (1964) Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci. 121*, 404–427.
27. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680–685.
28. Schneider, Z., Larsen, E. G., Jacobson, G., Johnson, B. C., and Pawelkiewicz, J. (1970) Purification and properties of glycerol dehydratase. *J. Biol. Chem. 245*, 3388–3396.
29. Toraya, T., and Fukui, S. (1982) Diol dehydrase. In B_{12} (Dolphin, D., Ed.) pp 233−262, John Wiley & Sons, New York.
30. Toraya, T., Ohashi, K., and Fukui, S. (1975) Immobilized diol dehydrase and its use in studies of cobalamin binding and subunit interaction. *Biochemistry 14*, 4255–4260.
31. Lah, N., Lah, J., Zegers, I., Wyns, L., and Messens, J. (2003) Specific potassium binding stabilizes pI258 arsenate reductase from *Staphylococcus aureus*. *J. Biol. Chem. 278*, 24673–24679.
32. Stroinski, A., Pawelkiewicz, J., and Johnson, B. C. (1974) Allosteric interactions in glycerol dehydratase. Purification of enzyme and effects of positive and negative cooperativity for glycerol. *Arch. Biochem. Biophys. 162*, 321–330.
33. Toraya, T., and Fukui, S. (1977) Immunochemical evidence for the difference between coenzyme-B_{12}-dependent diol dehydratase and glycerol dehydratase. *Eur. J. Biochem. 76*, 285–289.
34. 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 propane-1,2-diol. *Eur. J. Biochem. 269*, 4484–4494.