Identification of the 1,2-Propanediol-1-yl Radical as an Intermediate in Adenosylcobalamin-Dependent Diol Dehydratase Reaction†

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ABSTRACT: The reaction catalyzed by adenosylcobalamin-dependent diol dehydratase proceeds by a radical mechanism. A radical pair consisting of the Co(II) of cob(II)alamin and an organic radical intermediate formed during catalysis gives EPR spectra. The high-field doublet and the low-field broad signals arise from the weak interaction of an organic radical with the low-spin Co(II) of cob(II)alamin. To characterize the organic radical intermediate in the diol dehydratase reaction, several deuterated and ^{13}C-labeled 1,2-propanediols were synthesized, and the EPR spectra observed in the catalysis were measured using them as substrate. The EPR spectra with the substrates deuterated on C1 showed significant line width narrowing of the doublet signal. A distinct change in the hyperfine coupling was seen with [1-^{13}C]-1,2-propanediol, but not with the [2-^{13}C]-counterpart. Thus, the organic radical intermediate observed by EPR spectroscopy was identified as the 1,2-propanediol-1-yl radical, a C1-centered substrate-derived radical.

Coenzyme B_{12} (AdoCbl[1])-dependent diol dehydratase (DL-1,2-propanediol hydrolyase, EC 4.2.1.28) of *Klebsiella oxytoca* ATCC 8724 catalyzes the conversion of 1,2-propanediol, 1,2-ethanediol, and glycerol to propionaldehyde, acetaldehyde, and β-hydroxypropionaldehyde, respectively (*1*, *2*). The formation of cob(II)alamin and an organic radical intermediate during catalysis was observed with AdoCbl-dependent enzymes (*3*, *4*). The high-field doublet and the low-field broad signals observed in EPR spectra with either diol dehydratase (*5*−*7*), glycerol dehydratase (*8*), or ethanolamine ammonia-lyase (*9*−*11*) were assigned to an organic radical intermediate and the low-spin Co(II) of cob(II)alamin, respectively. The EPR signals were interpreted to arise from a weak interaction of the organic radical with Co(II) (*12*−*14*). The EPR spectra of radical intermediates in the reactions catalyzed by methylmalonyl-CoA mutase (*15*−*18*), 2-methyleneglutarate mutase (*19*), and glutamate mutase (*20*, *21*) were also reported, but, in these cases, the signals were postulated to represent a strongly coupled cob(II)alamin−radical pair (*22*).

The organic radical intermediate and cob(II)alamin are formed in diol dehydratase and ethanolamine ammonia-lyase at a kinetically competent rate (*6*, *23*, *24*). The spin concentrations of the organic radical and Co(II) in the diol dehydratase reaction were reported to be 0.58 and 0.50 mol/mol of active site (*7*). Optical spectra indicated that approximately 77 and 90% of the active sites contain cob(II)alamin during the steady state of the reactions of diol dehydratase with 1,2-propanediol (*7*) and of ethanolamine ammonia-lyase with (*S*)-2-aminopropanol (*23*), respectively. It is widely accepted that all the AdoCbl-dependent rearrangements proceed by a common radical mechanism shown in Figure 1A and B (*3*, *4*). In the case of diol dehydratase (*24*), X is the hydroxyl group on C2, and H is a hydrogen atom on C1. The interaction between the apoenzyme and the coenzyme leads to the activation of the coenzyme Co−C bond for homolytic cleavage, forming cob(II)alamin and an adenosyl radical. In the absence of substrate, only a small fraction (<1%) of the coenzyme is present in the dissociated form. The addition of substrate to the holoenzyme shifts the equilibrium so that a major fraction of the coenzyme is converted to the dissociated form. The adenosyl radical formed abstracts a hydrogen atom from the substrate, producing a substrate-derived radical and 5′-deoxyadenosine. The substrate radical rearranges to the product-derived radical, which then abstracts a hydrogen atom back from 5′-deoxyadenosine. This leads to the formation of the final product and the adenosyl radical. The latter and cob(II)alamin recombine to regenerate the coenzyme. Recently, the X-ray structures of several AdoCbl-dependent enzymes have been reported (*25*−*29*). Figure 1C depicts the active site of diol dehydratase (*30*).

By EPR measurements with ethanolamine ammonia-lyase using deuterated and ^{13}C-labeled substrates, the radical intermediates present during steady-state turnovers of 2-aminopropanol and ethanolamine were identified as the C1-centered substrate radical (*11*, *31*, *32*) and the C2-centered product radical (*33*), respectively. The organic radical observed by EPR in the glutamate mutase reaction was identified in a similar way as the 4-glutamyl radical that interacts with Co(II) from the distance of ca. 6.6 Å (*21*). The radical formed by the same enzyme from L-2-hydroxy

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[1] Abbreviations: AdoCbl, adenosylcobalamin; EPR, electron paramagnetic resonance; Tl^+, thallous ions.

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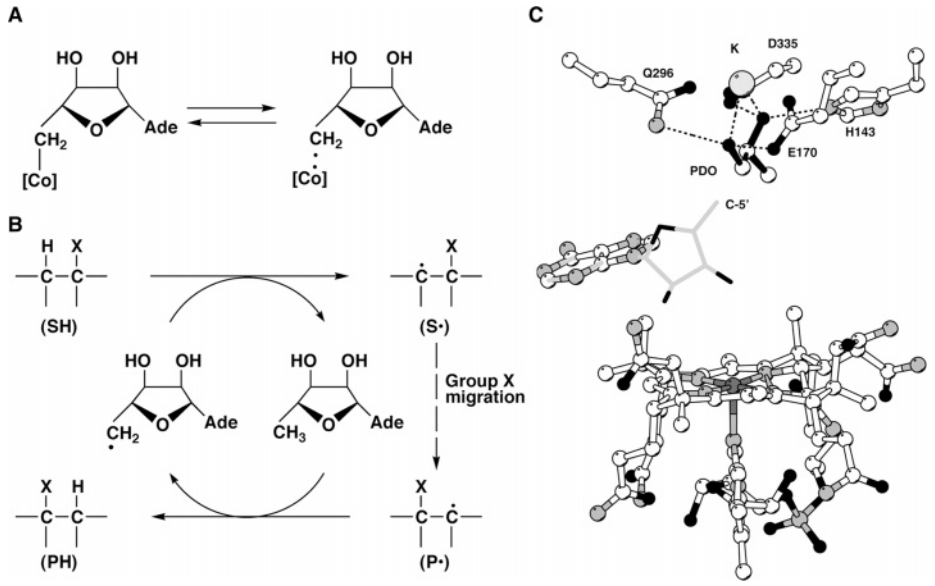

FIGURE 1: Minimal mechanism for AdoCbl-dependent enzymes and the active site of diol dehydratase. (A) Homolysis of the Co−C bond of the enzyme-bound AdoCbl. [Co], cobalamin. (B) Adenosyl-radical-mediated rearrangements. X, a generic migrating group. (C) The X-ray structure of diol dehydratase active site. The position of the ribose moiety in the "distal" conformation that was obtained by our ribosyl rotation model (*30*, *46*) is shown by a stick model.

glutarate, a very poor substrate, was identified as its C4-centered radical (*34*).

In this study, we synthesized several deuterated and ^{13}C-substituted substrates and measured the EPR spectra of holodiol dehydratase reacting with them. By comparison between the spectra with labeled and unlabeled substrates, we identified the organic radical intermediate in the diol dehydratase reaction as a substrate-derived C1-centered radical.

MATERIALS AND METHODS

Chemicals. Crystalline AdoCbl was a gift from Eisai Co. Ltd., Tokyo, Japan. $LiAlD_4$ and deuterium oxide were purchased from CEA, [D_2]sulfuric acid was from ISOTEC Inc., and [2-^{13}C]glycerol was from ICON. [1-^{13}C]- and [2-^{13}C]Pyruvic acids (sodium salts), [1,1,2,3,3,3-D_6]propylene oxide, [D_3]methylmagnesium iodide, and benzyloxyacetaldehyde were obtained from Aldrich. The isotope contents of these chemicals are >95%. All other chemicals were reagent-grade commercial products and were used without further purification.

Synthesis of Labeled 1,2-Propanediols. Isotopically labeled substrates were synthesized from the following starting materials using general techniques of organic syntheses, purified by column chromatography on silica gel and/or distillation, and characterized by ^1H- and ^{13}C NMR with 1D and 2D techniques. [1,1-D_2]-, [2-D]-, And [1,1,2-D_3]-1,2-propanediols were synthesized by the reduction of ethyl lactate, hydroxyacetone, and ethyl pyruvate, respectively, with $LiAlD_4$. [1,1,2,3,3,3-D_6]-1,2-Propanediol was synthesized by the hydrolysis of [1,1,2,3,3,3-D_6]propylene oxide in deuterium oxide containing [D_2]sulfuric acid. For the synthesis of [3,3,3-D_3]-1,2-propanediol, [D_3]methylmagnesium iodide was added dropwise to the ethereal solution of benzyloxyacetaldehyde. The protecting group of the resulting 1-benzyloxy-2-propanol was removed by catalytic hydrogenation with Pd/C. Remaining amounts of ^1H in the synthetic deuterated 1,2-propanediols were estimated by ^1H NMR. It was confirmed that all the deuterated substrates contained >90% deuterium in the position(s) of substitution.

[1-^{13}C]- And [2-^{13}C]-1,2-propanediols were synthesized by the reduction of corresponding pyruvic acids with $LiAlH_4$. The free pyruvic acids were obtained by passing their sodium salts through a DOWEX G50 (H^+ form) column and concentrated in vacuo.

Diol Dehydratase. The apoenzyme of recombinant diol dehydratase of *Klebsiella oxytoca* ATCC8724 was purified from overexpressing *Escherichia coli* JM109 cells harboring expression plasmid pUSI2E(DD) (*35*), as described previously (*36*). The enzyme fractions with specific activity larger than 80 units/mg were used in this study. Substrate-free and K^+-free apoenzymes were obtained by dialysis at 4 °C against 100 volumes of 50 mM potassium phosphate buffer (pH 8.0) and 50 mM Tris phosphate buffer (pH 8.0), respectively, containing 20 mM sucrose monocaprate with two buffer changes. Essentially no activity (<1%) was detected in the K^+-free apoenzyme without addition of K^+. The activity of diol dehydratase was determined by the 3-methyl-2-benzothiazolinone hydrazone method (*37*). 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.

EPR Measurements. EPR experiments were carried out essentially as described before (*38*−*40*). Substrate-free apoenzyme solution [unless otherwise indicated, 100 units in 0.6 mL of 50 mM potassium phosphate buffer (pH 8.0) containing 20 mM sucrose monocaprate] was mixed at 0 °C with aqueous AdoCbl solution (50 nmol in 0.05 mL) in a quartz EPR tube (o.d. 5 mm) stoppered with a rubber septum. After replacement of the air in the tube with nitrogen by repeating evacuation/nitrogen introduction three times, the mixture was incubated at 25 °C for 3 or 5 min for the formation of holoenzyme. After being cooled in an ice−water bath, aqueous 1,2-propanediol solution (50−100 mmol in 0.05 mL) was added to the mixture by a syringe through the septum. After mixing, the mixture was allowed to stand

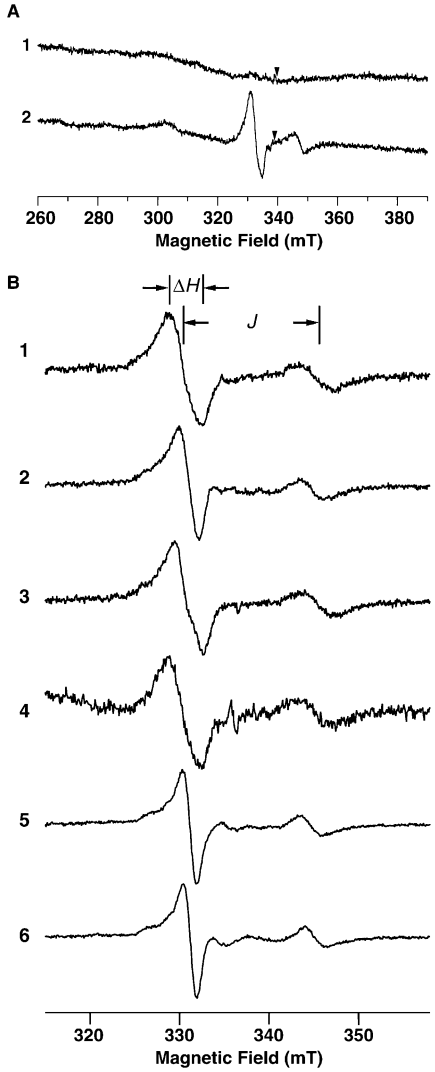

FIGURE 2: Doublet signals of holodiol dehydratase reacting with deuterated substrates. (A) EPR spectra observed in the presence and absence of substrate: (1) without 1,2-propanediol and (2) with 1,2-propanediol; microwave power, 10 mW; modulation amplitude, 1 mT. (B) Doublet signals: (1) unlabeled 1,2-propanediol, (2) [1,1-D_2]-1,2-propanediol, (3) [2-D]-1,2-propanediol, (4) [3,3,3-D_3]-1,2-propanediol, (5) [1,1,2-D_3]-1,2-propanediol, and (6) [1,1,2,3,3,3-D_6]-1,2-propanediol; microwave power, 5 mW; modulation amplitude, 0.5 mT.

at 4 °C for an additional 0.5−1 min to initiate the reaction. The mixture was then frozen in an isopentane bath that had been preliminarily cooled to its melting point (ca. −160 °C). The freeze-quenched samples were stored in a liquid nitrogen bath until EPR measurements.

Spectra were obtained on a Bruker EPS-380E EPR spectrometer equipped with a nitrogen gas flow−temperature controller. Conditions for EPR measurements are as follows: temperature, −128 °C; microwave frequency, 9.4 GHz (X-band); microwave power, 1−10 mW; modulation amplitude, 0.1−1 mT; modulation frequency, 100 kHz. Each spectrum was the average of four scans.

Optimization of EPR Conditions. Figure 2A shows the EPR spectra of K^+-holodiol dehydratase. In the absence of substrate, no EPR signals were observed. Upon the addition of unlabeled 1,2-propanediol, high-field asymmetric doublet and low-field broad signals due to an organic radical and low-spin Co(II), respectively (*5*−*7*), appeared. These signals are characteristic of a weakly coupled cob(II)alamin−organic radical system (*12*−*14*), that is, arising from an AB-type of coupling between them. The intensity of the doublet signal was almost saturated at an irradiation power of 10 mW. The intensity of the doublet signal was increased with modulation amplitude. As the results of optimization, irradiation power of 5 or 10 mW and modulation amplitude of 0.5 or 1 mT were used for the EPR measurements with labeled substrates. Under these conditions, the broad signal due to cob(II)alamin was weak, and a signal-to-noise (*S*/*N*) ratio was satisfactory.

Table 1: Magnitude of Spin−Spin Exchange Interaction (J) and the Line Width (ΔH) of the Doublet Signal[a]

substrate	J (mT)	ΔH (mT)
unlabeled 1,2-propanediol	14.7	3.9
[1,1-D_2]-1,2-propanediol	13.9	2.3
[2-D]-1,2-propanediol	14.6	3.2
[3,3,3-D_3]-1,2-propanediol	14.9	3.7
[1,1,2-D_3]-1,2-propanediol	13.7	1.7
[1,1,2,3,3,3-D_6]-1,2-propanediol	14.0	1.6

[a] The J and ΔH values were obtained as indicated in Figure 2B.

RESULTS

EPR Spectra with Deuterated Substrates. Figure 2B shows the doublet signals obtained with various deuterated substrates. With either substrate, the doublet signal was observed. The line widths of the doublet signal with [1,1-D_2]-, [1,1,2-D_3]-, and [1,1,2,3,3,3-D_6]-1,2-propanediols were significantly narrower than those with unlabeled, [2-D]-, and [3,3,3-D_3]-1,2-propanediols, although the splitting of the doublet was almost not affected by the isotopic labeling.

The separation between the high-field doublet peaks gives the magnitude of spin−spin exchange interaction (J) between the organic radical and cob(II)alamin, and the separation between the magnetic fields giving the maximum and the minimum (ΔH) is proportional to the line width of the absorption. Table 1 summarizes the J and ΔH values observed with unlabeled and deuterated substrates. Although the J values were almost the same as that (14.4 mT) reported before (*39*), the ΔH values varied significantly. It is evident that the replacement of the hydrogens on C1 by deuterium resulted in distinct line width narrowing. It was thus suggested that the unpaired electron of the organic radical is centered on C1, from which the adenosyl radical abstracts a hydrogen atom. The substitution of the hydrogen on C2 for deuterium brought about significant line width narrowing, but the substitution of the methyl hydrogens for deuterium did not at all (Figure 2B and Table 1). These experimental results are consistent with the conclusion that the observed organic radical is the C1-centered substrate radical.

EPR Spectra with ^{13}C-Substituted Substrates. To obtain more conclusive results, [1-^{13}C]- and [2-^{13}C]-1,2-propanediols were synthesized, and the EPR spectra were measured with holodiol dehydratase using them as substrate. Figure 3 shows that the EPR spectrum obtained with [1-^{13}C]-1,2-propanediol is quite different in the doublet signal region from that obtained with unlabeled substrate, that is, each peak of the doublet signal was split into two. In contrast, the doublet signal obtained with [2-^{13}C]-1,2-propanediol was essentially the same as that obtained with unlabeled substrate. Furthermore, when [2-^{13}C]glycerol was used as substrate, the EPR

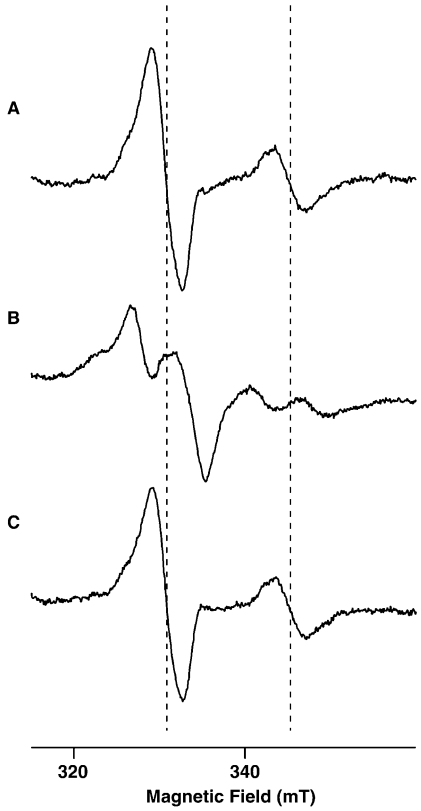

FIGURE 3: Doublet signals of holodiol dehydratase reacting with ^{13}C-labeled substrates (microwave power, 10 mW; modulation amplitude, 1 mT): (1) unlabeled 1,2-propanediol, (2) [1-^{13}C]-1,2-propanediol, and (3) [2-^{13}C]-1,2-propanediol. Vertical lines indicate the peak positions of the doublet signals (330.8 and 345.3 mT).

spectrum obtained was almost the same as that with unlabeled glycerol (data not shown). These results offer evidence indicating that the EPR-observed organic radical species is a C1-centered substrate-derived radical. This conclusion is in agreement with that from the EPR spectra with deuterated substrates. The hyperfine coupling constant for ^{13}C (A_{13C}), which represents the magnitude of interaction of unpaired electron with the ^{13}C nucleus ($I = 1/2$) of C1, was measured to be ~6 mT by a ruler.

DISCUSSION

All the data reported in this paper indicated that the organic radical intermediate observed by EPR spectroscopy is a C1-centered substrate-derived radical. The deuterium kinetic isotope effect (KIE) (k_H/k_D) on the overall diol dehydratase reaction is 10 (*41*), indicating that breaking of the C−H bond is rate-determining in the reaction. This suggests that the activation energy for the OH group migration from C2 to C1 is smaller than that for the hydrogen abstraction or hydrogen recombination (back abstraction) and that the substrate radical and the product radical may be in equilibrium. Since the organic radical species observed by EPR spectroscopy was identified as the substrate radical in this paper, it was suggested that the energy of the substrate radical is lower than that of the product radical. If it is assumed that the EPR spectrum of the substrate radical is distinguishable from that of the product radical, the fraction of the latter would be <10% that of the former. Thus, it seems likely that a difference in the energy between substrate-derived and product-derived radicals is >1.4 kcal/mol at 37 °C.

The hyperfine coupling constant for the α-proton ($A_{H\alpha}$) is expressed as follows:

$$A_{H\alpha} = Q_\alpha \rho_c$$

where ρ_c and Q_α are a spin density on radical carbon and a proportion constant, respectively. Q_α is 2.30 mT for methyl radical. Since the spin density on C1 (ρ_c) obtained by density-functional-theory calculations (*42*, *43*) is 0.8, the predicted value of $A_{H\alpha}$ would be 1.8 mT. Figure 2B shows that the hyperfine coupling constant for the β-proton ($A_{H\beta}$) is small. This seems reasonable, because the spin density on C2 of the substrate radical was predicted to be almost zero (0.0) by theoretical calculations.

The geometry of the radical intermediate in the ethanolamine ammonia-lyase reaction was analyzed in detail by pulsed electron nuclear double resonance (ENDOR) spectroscopy (*31*). From the dipole−dipole coupling, the distance between C1 of the radical and C5′ of the labeled cofactor component was estimated to be 3.4 ± 0.2 Å. It was thus suggested that C5′ of the adenosyl radical moves ~7 Å from its position as part of AdoCbl to a position where it is in contact with C1 of the substrate, which lies ~10−12 Å (*13*, *32*, *44*) from the Co(II) of cob(II)alamin. It was also demonstrated by X-band two-pulse electron spin−echo envelope modulation (ESEEM) spectroscopy that the unpaired electron on C1 of the substrate radical and C5′ are separated by 3.2 Å, suggesting that C5′ of the adenosyl radical directly mediates radical migration between cobalt in cobalamin and the substrate/product site over a distance of 5−7 Å in the active site of this enzyme (*45*). These estimations of movement of C5′ during catalysis are based on the assumption that C1, C5′, and Co(II) are collinear. The geometry of reactant centers determined by using orientation-selection-ESEEM spectroscopy demonstrated that this is true; namely, C5′ is located close to the Co(II)−C1 axis (7.8 Å from Co(II) and 3.3 Å from C1) (*44*). In diol dehydratase, C1 of the substrate, Co of cobalamin, and C5(Pe) of the adeninylpentyl group are collinear, as judged from the X-ray structure of the diol dehydratase·adeninylpentylcobalamin complex (*30*). The C1−C5′−Co bond angle in the "distal" conformation (Figure 1C) was about 120° according to our ribosyl rotation model.

We have proposed the mechanism of action of diol dehydratase on the assumption that K^+ in the active site remains coordinated to the substrate and all radical intermediates throughout the course of the reaction (*24*, *26*, *30*, *46*). However, there is an argument about this point. Diol dehydratase requires K^+ or other monovalent cations having similar ionic radii (*1*, *47*). Tl^+ activates the enzyme as efficiently as K^+ (*1*). Frey and co-workers have reported that both EPR and pulsed EPR experiments with Tl^+-holoenzyme fail to detect the interaction of the magnetic nuclei of Tl^+ with the unpaired electron in the inhibitor-derived *cis*-ethanesemidione radical (*48*). It is not clear whether this conclusion is applicable to the case of catalytic reaction.

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