J. Biochem. **144,** 437–446 (2008)
doi:10.1093/jb/mvn086

Mechanism-based Inactivation of Coenzyme B_{12}-dependent Diol Dehydratase by 3-Unsaturated 1,2-Diols and Thioglycerol

Tetsuo Toraya*, Naohisa Tamura, Takeshi Watanabe, Mamoru Yamanishi, Naoki Hieda and Koichi Mori

Department of Bioscience and Biotechnology, Graduate School of Natural Science and Technology, Okayama University, Tsushima-naka, Okayama 700-8530, Japan

Received June 5, 2008; accepted June 22, 2008; published online June 27, 2008

The reactions of diol dehydratase with 3-unsaturated 1,2-diols and thioglycerol were investigated. Holodiol dehydratase underwent rapid and irreversible inactivation by either 3-butene-1,2-diol, 3-butyne-1,2-diol or thioglycerol without catalytic turnovers. In the inactivation, the Co–C bond of adenosylcobalamin underwent irreversible cleavage forming unidentified radicals and cob(II)alamin that resisted oxidation even in the presence of oxygen. Two moles of 5′-deoxyadenosine per mol of enzyme was formed as an inactivation product from the coenzyme adenosyl group. Inactivated holoenzymes underwent reactivation by diol dehydratase-reactivating factor in the presence of ATP, Mg^{2+} and adenosylcobalamin. It was thus concluded that these substrate analogues served as mechanism-based inactivators or pseudosubstrates, and that the coenzyme was damaged in the inactivation, whereas apoenzyme was not damaged. In the inactivation by 3-unsaturated 1,2-diols, product radicals stabilized by neighbouring unsaturated bonds might be unable to back-abstract the hydrogen atom from 5′-deoxyadenosine and then converted to unidentified products. In the inactivation by thioglycerol, a product radical may be lost by the elimination of sulphydryl group producing acrolein and unidentified sulphur compound(s). H_2S or sulphide ion was not formed. The loss or stabilization of product radicals would result in the inactivation of holoenzyme, because the regeneration of the coenzyme becomes impossible.

Key words: adenosylcobalamin, coenzyme B_{12}, diol dehydratase, mechanism-based inactivation, radical enzyme.

Abbreviations: AdoCbl, adenosylcobalamin or coenzyme B_{12}; aqCbl, aquacobalamin; DDR, diol dehydratase-reactivating factor; EPR, electron paramagnetic resonance; MBTH, 3-methyl-2-benzothiazolinone hydrazone; THF, tetrahydrofuran.

In the intramolecular group-transfer reactions catalysed by adenosylcobalamin (AdoCbl) (coenzyme B_{12})-dependent enzymes, a hydrogen atom migrates from one carbon atom of the substrate to an adjacent carbon atom in exchange for group X that moves in the opposite direction (Fig. 1A) (*1–3*). In the case of reactions catalysed by diol dehydratase (*4*), X is the OH group on C2 of substrate (*5*) and H is a hydrogen atom on C1 (*6*, *7*), and a water molecule is subsequently removed from the resulting *gem*-diol. The essential early event in the catalysis is the homolytic cleavage of the coenzyme Co–C σ bond by which an adenosyl radical is formed [Fig. 1B (a)]. This radical serves as a catalytic radical and abstracts a hydrogen atom from the substrate producing a substrate-derived radical and 5′-deoxyadenosine [Fig. 1B (b)]. The substrate radical rearranges to the product radical by the group X migration from C2 to C1. The product radical then abstracts a hydrogen atom back from 5′-deoxyadenosine. This leads to the formation of the final product and the regeneration of the coenzyme.

The structural basis for this minimal mechanism has been provided from the X-ray structures of diol and glycerol dehydratases and other AdoCbl-dependent enzymes (*8–13*). It was suggested from a modelling study (*14*) and an actual structure (*15*) that the Co–C bond is activated and cleaved by steric strain (both angular strains and tensile force) that is induced by tight enzyme–coenzyme interactions at both the cobalamin moiety and the adenine ring of adenosyl group (Fig. 1C). This is consistent with previous speculations and suggestions (*16–18*). C5′, a radical centre of the adenosyl radical, is too far from C1 of substrate for direct hydrogen abstraction in the proximal conformation, but it accesses to substrate by ribosyl rotation (*14*) or pseudorotation (*15*) to the distal conformation. The radical centre now comes close to substrate (estimated distance between C5′ and the nearest hydrogen atom on C1 of substrate is 1.45–1.46 Å) (*14*, *19*) and would abstract it stereospecifically (*19*). The predicted stereospecificity of the hydrogen abstraction agrees with the experimentally observed one reported by Rétey *et al*. (*5*, *7*) and Abeles and co-workers (*6*). After the OH group migration from C2 to C1 (radical rearrangement), C2 of product radical, a new radical centre, comes close

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

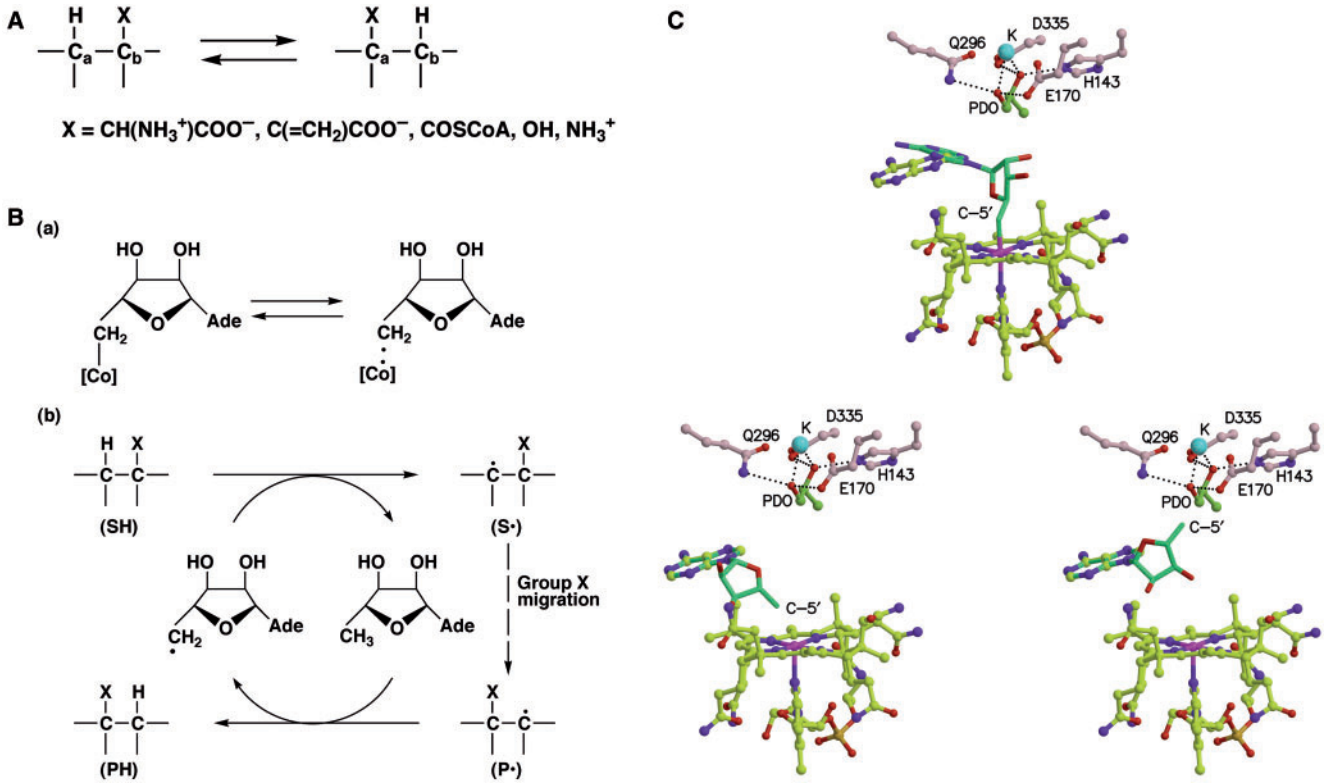

Fig. 1. **Rearrangements catalysed by AdoCbl-dependent enzymes (A), minimal mechanism of AdoCbl-dependent rearrangements (B) and steric strain and ribosyl rotation models based on the X-ray structures of diol dehydratase (C).** (B) (a) Homolysis of the Co–C bond of enzyme-bound AdoCbl. (b) Adenosyl-radical catalysed rearrangements. [Co], cobalamin; X, a generic migrating group. (C) (Upper) Superimposition of AdoCbl over that of enzyme-bound adeninylpentylcobalamin at the cobalamin moiety without cleavage of the Co–C bond. (Lower, left) The same superimposition at both the cobalamin moiety and the adenine ring with the Co–C bond cleaved and the Co–C distance kept at a minimum ('proximal' conformation). (Lower, right) Superimposed with the ribose moiety of adenosyl group rotated around the glycosidic linkage so that C5′ is closest to C1 of the substrate ('distal' conformation). The adenosyl group of AdoCbl is represented with stick models. Residue numbers in the α subunit.

to 5′-deoxyadenosine (estimated distance between C2 and C5′ is 2.55–2.58 Å) and would back-abstract a hydrogen atom from it (*19*).

The radical intermediates present during steady-state turnovers of substrates were identified as the C1-centred substrate radical in the ethanolamine ammonia-lyase reaction (*20*, *21*), the 4-glutamyl radical in the glutamate mutase reaction (*22*), the C1-centred substrate radical in the diol dehydratase reaction (*23*) and the succinyl-CoA radical in the methylmalonyl-CoA mutase reaction (*24*).

However, the product radical in the diol dehydratase reaction has not yet been directly observed by electron paramagnetic resonance (EPR) spectroscopy, presumably because of its low steady-state concentration. Abeles and co-workers reported that diol dehydratase is inactivated by glycolaldehyde (*25*) and chloroacetaldehyde (*26*). Frey and co-workers have identified the radical derived from these inactivators as the *cis*-ethanesemidione radical (*27*, *28*). The formation of this stabilized radical supports the formation of a C2-centred radical from a product analogue.

In the present article, we attempted to obtain evidence for the formation of C2-centred product radical from substrate analogues in the diol dehydratase reaction. We used 3-butene-1,2-diol and 3-butyne-1,2-diol as substrate analogues, because these 3-unsaturated 1,2-diols can be expected to delocalize an unpaired electron if the C2-centred product radicals are formed from them. In addition, thioglycerol was used because the SH group neighbouring to the radical centre might be eliminated from the C2-centred product radical. Bachovchin *et al.* (*29*) have reported that thioglycerol brings about irreversible inactivation, although the detailed analysis of the reaction was not done.

EXPERIMENTAL PROCEDURES

Materials—Crystalline AdoCbl was a gift from Eisai, Co. Ltd. (Tokyo, Japan). (*R*,*S*)-3-Butene-1,2-diol and thioglycerol were purchased from Acros Organics, Geel, Belgium and Wako Pure Chemical Industries, Ltd., Osaka, Japan, respectively. All other chemicals were analytical grade reagents and used without further purification.

Diol dehydratase and diol dehydratase-reactivating factor (DDR) were purified from *Escherichia coli* JM109 cells harbouring expression plasmid pUSI2E(DD) (*30*) and *E. coli* B834 cells harbouring pUSI2E(6/5b) (*31*), respectively, as described before (*32*, *33*).

Synthesis of 3-Butyne-1,2-diol—To the solution of 10 ml of glycerol in 100 ml of trichloromethane was added

12.9 ml of acetic anhydride. One drop of concentrated H_2SO_4 was added, and the mixture was allowed to react at room temperature for 3 h with gentle stirring. The reaction was terminated by adding saturated aqueous solution of $NaHCO_3$. The product, glycerol 1-acetate, was extracted with diethyl ether and purified by silica gel column chromatography in 67% yield. To the solution of 22.9 g of $NaIO_4$ in 80 ml of tetrahydrofuran (THF)-H_2O (12 : 5, v/v) was added 11.9 g of glycerol 1-acetate. After stirring overnight at room temperature, reaction was quenched by adding saturated aqueous solution of $NaHCO_3$. The desired product, glycol aldehyde acetate, was extracted by diethyl ether and purified by silica gel column chromatography in 84% yield. To the solution of 1.6 g of glycol aldehyde acetate in 45 ml of THF was added 40.5 ml of 0.5 M ethynylmagnesium bromide in THF. After 3 h, at room temperature, the reaction was terminated by adding saturated aqueous solution of NH_4Cl. The reaction mixture was filtered through a celite column and concentrated. The product, 3-butyne-1,2-diol, was obtained by distillation under reduced pressure in 30% yield. ^1H NMR ($CDCl_3$): δ 2.50 (d, 1H, J = 2.2 Hz), 3.73 (m, 2H), 4.46 (ddd, 1H, J = 2.2, 3.85, 6.32 Hz) p.p.m.

Enzyme Assay—Diol dehydratase activity was routinely assayed in the dark by the 3-methyl-2-benzothiazolinone hydrazone (MBTH) method (*34*). The standard reaction mixture contained an appropriate amount of apoenzyme, 15 μM AdoCbl, 0.1 M 1,2-propanediol, 50 mM KCl and 35 mM potassium phosphate buffer (pH 8.0), in a total volume of 1.0 ml. After incubation at 37°C for 10 min, reaction was terminated by adding 1 ml of 0.1 M potassium citrate buffer (pH 3.6). 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 amount of aldehyde formed was determined by measuring the absorbance at 305 nm. One unit is defined as the amount of enzyme activity that catalyses the formation of 1 μmol of propionaldehyde per minute at 37°C under the standard assay conditions.

Protein Assay—Protein concentrations of purified diol dehydratase and DDR were determined by measuring the absorbance at 280 nm. The molar absorption coefficients at 280 nm, calculated by the method of Gill and von Hippel (*35*) from their deduced amino acid composition and subunit structure, were 120,500 and 58,140/M/cm, respectively (*32*).

Kinetic Analysis with Substrate Analogues—Substrate-free apoenzyme was obtained by dialysis at 4°C for 48 h against 200 volumes of 10–50 mM potassium phosphate buffer (pH 8.0) containing 20 mM sucrose monocaprate with two buffer changes. The concentrations of 1,2-propanediol remaining in dialysates were negligible (<0.1 μM) when diluted >100-fold. Residual activity after incubation with a substrate analogue for appropriate time periods was determined by adding 0.1 M 1,2-propanediol to the mixture or by dilution into the reaction mixture containing 0.1 M 1,2-propanediol, followed by incubation for additional 10 min. The concentration of aldehyde formed was measured by the MBTH method, as described above. K_i values were determined from residual activity upon incubation of holoenzymes at 37°C for 1 min with varied concentrations of substrate analogues. The k_{inact} values were calculated from a change in the slope of a tangent to the time course curves of residual activity upon incubation of holoenzyme with each substrate analogue at 37°C for various time periods.

Fate of the Adenosyl Group of AdoCbl—To identify adenosyl group-derived product(s) formed from AdoCbl in the inactivation of holoenzyme by substrate analogues, substrate-free apoenzyme (100 U, 4.6 nmol) was incubated at 37°C for 30 min in the dark with 15 μM AdoCbl in the presence of 10 mM 1,2-butanediol or 1 mM other analogues (3-butene-1,2-diol, 3-butyne-1,2-diol or thioglycerol). Ethanol was then added to a final concentration of 80% to denature the enzyme protein. After heating at 90°C for 10 min, the mixture was centrifuged, and the supernatant was evaporated to dryness and then dissolved in 0.5 ml of 15% methanol containing 1% acetic acid. The nucleoside product from the adenosyl group was identified as 5′-deoxyadenosine by HPLC using a Cosmosil C_{18} column (0.46 × 15 cm) (Nacalai Tesque, Kyoto, Japan) with 15% methanol containing 1% acetic acid as a mobile phase. The amount of 5′-deoxyadenosine formed was determined from its peak height when the calibrated column was developed at a flow rate of 0.4 ml/min. The retention time of 5′-deoxyadenosine was ~10 min under the conditions employed.

Determination of Acrolein and Crotonaldehyde—To identify the products upon inactivation by thioglycerol and 3-butene-1,2-diol, the reaction mixtures were analysed by HPLC. Substrate-free apoenzyme (100 U, 4.6 nmol) was incubated at 37°C for 20 min in the dark with 25 μM AdoCbl in the presence of 1 mM thioglycerol or 3-butene-1,2-diol. Methanol was then added to the reaction mixture to a final concentration of 10%. After centrifugation, 50 μl of the supernatant solution was injected to Aquapore RP-300 C8 (7 μm) column (0.21 × 22 cm) (Applied Biosystems, CA, USA) and developed at a flow rate of 0.2 ml/min using 10% methanol as a mobile phase (UV Detector, 210 nm). The retention times of acrolein and crotonaldehyde were ~7.5 and 11.7 min, respectively, under these conditions.

Other Analytical Procedures—The amount of H_2S or sulphide ion formed in the reaction with thioglycerol was determined by the method of Beinert (*36*). Optical spectra were obtained on a JASCO V-560 recording spectrophotometer under aerobic conditions. The cobalamin concentration was determined spectrophotometrically after conversion to dicyanocobalamin ($\varepsilon_{367} = 30.4 \times 10^3$/M/cm) (*37*).

For EPR measurements, apoenzyme solution was mixed at 0°C with AdoCbl solution in a quartz EPR tube (o.d. 5 mm) stoppered with a rubber septum. After replacement of the air in the tube with argon (purity, 99.999%) by repeated evacuation and flushing with argon three times, holoenzymes were formed and reacted with substrate or its analogues, as described in the legend to Fig. 3. The sample was rapidly frozen in an isopentane bath which had been preliminarily cooled to its melting point (approximately −160°C) and kept in liquid nitrogen until EPR measurements. The sample was then transferred to the EPR cavity and cooled with a cold nitrogen gas flow controlled by an Eurotherm B-VT 2000

temperature controller. EPR spectra were taken as described previously (*23*, *38*) at −130°C on a Bruker ESP-380E spectrometer modified with a Gunn diode X-band microwave unit. EPR microwave frequency, 9.481–9.499 GHz; modulation amplitude, 1 mT; modulation frequency, 100 kHz; microwave power, 10 mW.

Holoenzymes inactivated by substrate and substrate analogues were formed and subjected to reactivation by DDR (*32*, *39*), as described in the legend to Fig. 4.

RESULTS

Behaviours of Substrate Analogues toward Diol Dehydratase—As summarized in Table 1, 3-butene-1,2-diol, 3-butyne-1,2-diol and thioglycerol did not show detectable substrate activity, although 1,2-butanediol showed ~2% activity of 1,2-propanediol. The enzyme activity was lost upon the incubation of holoenzyme with these substrate analogues in a time-dependent manner. The inactivation rate followed the pseudo-first-order reaction kinetics (data not shown). K_i values obtained from residual activity upon incubation of holoenzyme with varying concentrations of substrate analogues as well as k_{inact} values obtained from the time course changes of residual activity upon incubation of holoenzyme with them are also shown in Table 1. K_i values for 3-unsaturated 1,2-diols were lower than that for 1,2-butanediol and almost the same as the K_m value for 1,2-propanediol (*29*, *40*, *41*). K_i for thioglycerol was much lower than K_m for glycerol (*29*, *40*, *41*). 1,2-Butanediol also induced inactivation during catalysis, but at a much slower rate than the others. The amounts of aldehyde formed per mol of enzyme upon inactivation with 3-butene-1,2-diol, 3-butyne-1,2-diol and thioglycerol were less than one per active site. These results indicated that 3-unsaturated 1,2-diols and thioglycerol are efficient mechanism-based inactivators or pseudosubstrate for diol dehydratase. The formation of an equimolar amount of acrolein (~1.2 mol/mol of enzyme) upon incubation with thioglycerol was observed by HPLC analysis. The amount of H_2S or sulphide ion formed in the reaction with thioglycerol was negligible (<0.07 mol/mol of enzyme), suggesting that the inactivation is not accompanied by the elimination of SH group as SH^-, although the sulphur compound(s) produced were not identified. It was demonstrated that crotonaldehyde was not formed at all upon inactivation by 3-butene-1,2-diol, but the identification of the organic products from 3-unsaturated 1,2-diols was not successful yet.

Spectral Change of AdoCbl upon Mechanism-based Inactivation by Substrate Analogues—Upon aerobic incubation of AdoCbl with apoenzyme in the presence of 1,2-propanediol, the absorbance at 375 nm decreased and a new peak at 478 nm appeared (Fig. 2B). This peak is characteristic of cob(II)alamin (so-called B_{12r}) that is bound to diol dehydratase (*42*), indicating that the Co–C bond of AdoCbl was homolytically cleaved during catalysis. The steady-state concentration of cob(II)alamin was ~80%, which is roughly consistent with the previously reported value (*43*). In the reaction with 1,2-butanediol, AdoCbl did not undergo significant spectral change (Fig. 2C). The formation of cob(II)alamin was suggested from very slight increase of the absorbance at 478 nm, but its steady-state concentration was almost negligible, as expected from its low substrate activity (*29*, *44*). The coenzyme was gradually converted to enzyme-bound hydroxocobalamin upon inactivation. In contrast, in the reaction with either 3-butene-1,2-diol or 3-butyne-1,2-diol, the spectrum of coenzyme rapidly changed to enzyme-bound cob(II)alamin (Fig. 2D and E). The spectrum suggested the complete conversion of AdoCbl to cob(II)alamin within 4 min of incubation. Since the inactivation was also completed by this time, it is evident that the coenzyme undergoes irreversible cleavage of its Co–C bond upon mechanism-based inactivation by these substrate analogues. The cob(II)alamin species were stable even under aerobic conditions, but it was oxidized to aquacobalamin (aqCbl) upon denaturation of enzyme. The spectrum no longer changed upon photoillumination. Since the Co–C bond of AdoCbl is rapidly cleaved by photoillumination, this indicates that the conversion of AdoCbl to cob(II)alamin was quantitative. Thioglycerol also induced similar spectral changes—that is, the enzyme-bound AdoCbl was completely converted to cob(II)alamin by this substrate analogue as well (Fig. 2F). The spectrum obtained after denaturation was that of thiolate cobalamin, but not that of aqCbl. Thiolate cobalamin must be formed by the reaction of aqCbl with thioglycerol outside of active sites. It can thus be summarized that cob(II)alamin is one of the products derived from AdoCbl upon mechanism-based inactivation by these substrate analogues.

Table 1. **Kinetic parameters of diol dehydratase for substrate and substrate analogues.**[a]

Substrate or its analogue	k_{cat} (s^{-1})	K_m (mM)	K_i (mM)	k_{inact} (min^{-1})	k_{cat}/k_{inact} or aldehyde formed/active site (mol/mol)[b]
1,2-Propanediol	366[c]	0.08[d]		0.014[e]	1.57×10^6
1,2-Butanediol	7.8[c]	2.5[f]	1.97[d]	0.066	8.7×10^3
3-Butene-1,2-diol			0.03	1.08	<1
3-Butyne-1,2-diol			0.02	1.02	<1
Thioglycerol			0.1	1.14	<1

[a]Determined at 37°C. Experimental details are described in the text. [b]The k_{cat}/k_{inact} values show the average numbers of catalytic turnovers before inactivation and thus correspond to the amounts of aldehyde formed per active site (mol/mol). [c]From (*41*). [d]From (*44*). [e]From (*43*). [f]From (*40*).

EPR Spectra Observed upon Incubation of Holoenzyme with Substrate Analogues—Essentially, no EPR signals were observed in the absence of AdoCbl (Fig. 3A). When holodiol dehydratase was incubated with 1,2-propanediol at 4°C for 1 min under anaerobic conditions, the typical EPR spectrum of reacting holoenzyme was obtained (Fig. 3B). The high-field doublet signal was identified as 1,2-propanediol-1-yl radical (*23*), and the low-field broad signal was assigned to low-spin Co(II) of cob(II)alamin. This spectrum bears striking resemblances to those observed in what is known as AB systems in proton NMR, that is, a system consisting of exchange-coupled spins having relatively similar 'chemical shifts' or *g*-values. Such a spectrum arises from weak coupling in the Co(II)-organic radical pair (*45–47*). Schepler *et al.* (*45*) explained that such an EPR spectrum arises

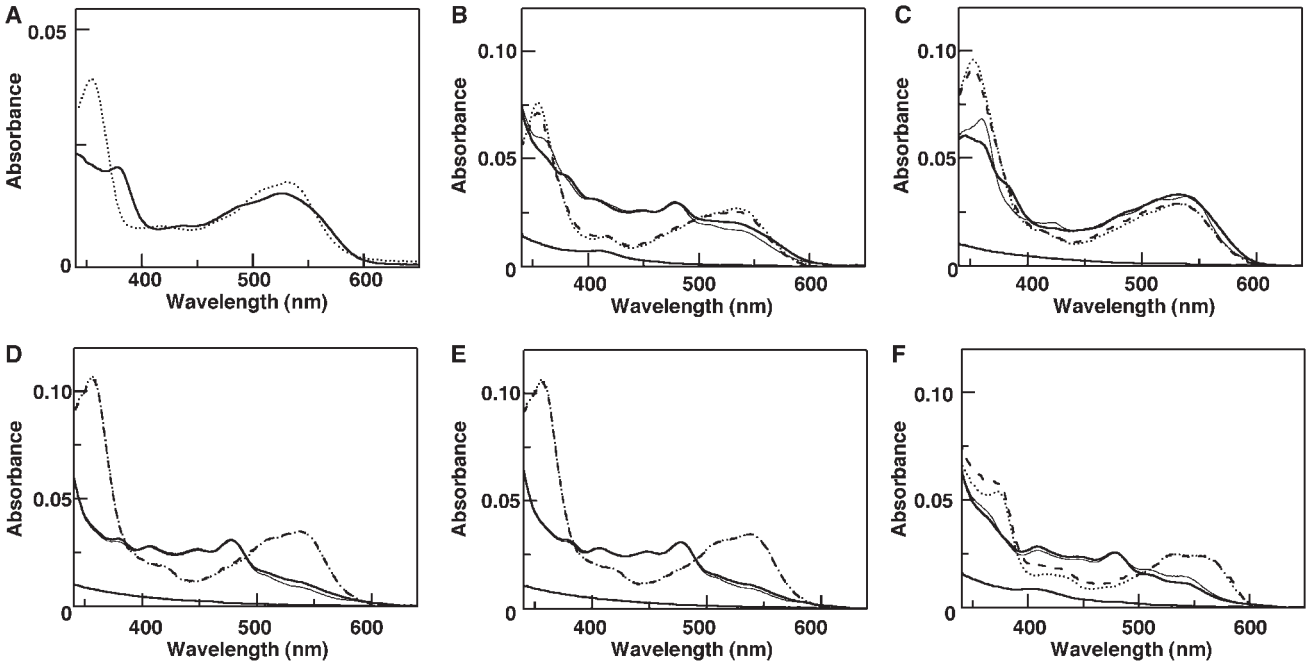

Fig. 2. **Spectral changes of AdoCbl upon aerobic incubation with apodiol dehydratase in the presence of substrate and substrate analogues.** (A) Free AdoCbl (3.5 μM) in 35 mM potassium phosphate buffer (pH 8.0) containing 1M 1,2-propanediol (solid line). The spectrum after photolysis was also taken (dotted line). (B–F) Apoenzyme (100 U, 4.6 nmol) was incubated at 30°C with 3.5 μM AdoCbl in 40 mM potassium phosphate buffer (pH 8.0) containing 0.8% Brij35 and 1.0 M 1,2-propanediol (B), 10 mM 1,2-butanediol (C), or 1 mM 3-butene-1,2-diol (D), 3-butyne-1,2-diol (E) or thioglycerol (F), in a volume of 1.0 ml. Spectra were taken at 4 min (thick solid lines) and 16 min (thin solid lines) of incubation after the addition of AdoCbl. Enzymes was then denatured by adding 6 M guanidine·HCl and 0.06 M citric acid. After incubation at 37°C for 10 min, the mixture was neutralized to pH 8.0 by adding 200 μl of 1 M potassium phosphate buffer (pH 8.0) and 70 μl of 5 N KOH. After spectral measurement (broken lines), the mixture was photoilluminated in an ice-water bath for 5 min with a 250-W tungsten light bulb from a distance of 20 cm, and the spectrum was taken (dotted lines). Apoenzyme control (bottom lines). Spectra are corrected for dilution.

predominantly from an isotropic exchange interaction between an organic radical and low spin Co(II) of cob(II)alamin. Buettner and Coffman (*46*) and Boas *et al.* (*47*) interpreted the EPR spectra on the basis of a coupling model which includes both isotropic exchange and dipolar interactions and established a lower limit of 10 Å for the organic radical-Co(II) separation. The splitting of the doublet signals was 13.8 mT. The signals lasted at least 3 min of incubation at 25°C. With 1,2-butanediol, a small doublet signal with a splitting of 6.0 mT was observed by 1 min incubation at 4°C, and it lasted for at least 3 min at 25°C (Fig. 3C). In clear contrast to them, 3-butene-1,2-diol gave a prominent singlet signal with a *g*-value of 2, together with the peaks due to cob(II)alamin that is coupled with an organic radical (Fig. 3D). The intensity of the singlet signal was almost double of the doublet signal observed with 1,2-propanediol. The spin density of the latter signal was reported to be ~50–60% of the active site (*43*). This indicates that the EPR-observable organic radical species formed in the reaction with 3-butene-1,2-diol is a kind of stabilized radical, which is not capable of back-abstracting a hydrogen atom from 5′-deoxyadenosine but still coupled with the cob(II)alamin species. The singlet signal decreased in the intensity with time of incubation and completely disappeared after 33 min of incubation at 25°C. The low-field signal observed after 33 min of incubation at 25°C was typical of cob(II)alamin without an organic radical coupling partner (*38*), and it increased with time of incubation. The EPR spectra observed with 3-butyne-1,2-diol was similar, but the singlet-like signal with a *g*-value of ~2 was much smaller (Fig. 3E). After incubation at 25°C for 3 min, the singlet-like signal was almost completely lost, and a typical signal of cob(II)alamin appeared in the low-field region. With thioglycerol, however, a typical signal of cob(II)alamin without an organic radical coupling partner was clearly observed even after 1 min of incubation at 4°C (Fig. 3F). A singlet-like signal around $g = 2$ was almost not observed with this substrate analogue. These changes observed in EPR spectra would be related with the rapid inactivation of holoenzyme by 3-butene-1,2-diol, 3-butyne-1,2-diol and thioglycerol.

Fate of Adenosyl Group of AdoCbl—Upon incubation of holoenzyme with substrate analogues, the coenzyme underwent the irreversible cleavage of its Co–C bond. To investigate the fate of the upper axial ligand of AdoCbl in the inactivation, adenosyl group-derived product(s) from AdoCbl was identified. After the mechanism-based inactivation by substrate analogues, the inactivated holoenzymes were denatured, and product(s) formed from the coenzyme in the inactivation were extracted and analysed by HPLC on a reversed phase column. The nucleoside product from the adenosyl group was identified as

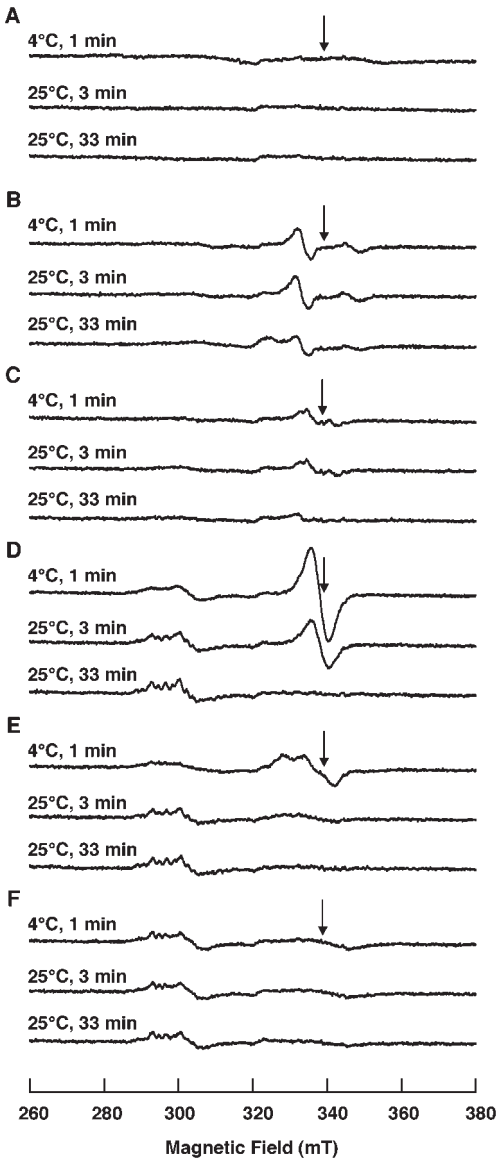

Fig. 3. **EPR spectra observed upon incubation of holodiol dehydratase with substrate and substrate analogues.** The arrows correspond to $g = 2.0$. Holoenzyme was formed under an argon atmosphere by incubating substrate-free apoenzyme (150 U, 6.9 nmol) at 25°C for 3 min with (B–F) or without (A) 50 nmol of AdoCbl in 0.65 ml of 46 mM potassium phosphate buffer (pH 8.0) containing 18 mM sucrose monocaprate. The enzyme reaction was started by adding 71 mM 1,2-propanediol (A and B), 10 mM 1,2-butanediol (C) or 1 mM 3-butene-1,2-diol (D), 3-butyne-1,2-diol (E) or thioglycerol (F). After 1 min at 4°C, the reaction mixture was rapidly frozen in an isopentane bath that had been preliminarily cooled to approximately −160°C and then in a liquid nitrogen bath. EPR spectra were taken at −130°C. After the first measurement of EPR spectrum, the mixture was incubated at 25°C for 3 min and frozen again, as described above for the second measurement. The mixture was incubated at 25°C for additional 30 min for the third measurement; *mT*, millitesla.

5′-deoxyadenosine (data not shown). The formation of adenine, adenosine, 4′,5′-anhydroadenosine, 5′,8-cyclic adenosine or adenosine 5′-aldehyde was not observed at all. It is thus evident that the hydrogen abstraction from substrate analogues by coenzyme adenosyl radical, i.e. the initial event of the catalysis, took place. Therefore, we concluded that the inactivation is mechanism-based, suicidal one. As shown in Table 2, the amounts of 5′-deoxyadenosine formed in the inactivation by 3-butene-1,2-diol, 3-butyne-1,2-diol and thioglycerol corresponded to ~2 mol/mol of enzyme. These data, together with the consideration that diol dehydratase exists as a dimer of $\alpha\beta\gamma$ heterotrimer (*9*), suggested that both heterotrimeric units functioned in the formation of 5′-deoxyadenosine. The amount of 5′-deoxyadenosine formed in the reaction with 1,2-butanediol was about a half of others. This may reflect that the rate of inactivation by this substrate is slower than those with other analogues, and some AdoCbl survived as suggested by optical spectra.

Table 2. **Formation of 5′-deoxyadenosine in the inactivation of diol dehydratase by substrate analogues.**[a]

Substrate analogue	5′-Deoxyadenosine formed (nmol)	5′-Deoxyadenosine/diol dehydratase (mol/mol)
1,2-Butanediol	5.8	1.3
3-Butene-1,2-diol	9.7	2.1
3-Butyne-1,2-diol	10.3	2.2
Thioglycerol	10.5	2.3

[a]Experimental details are described in the text. The amount of diol dehydratase used in each experiment was 4.6 nmol.

Reactivation of Inactivated Holoenzymes by DDR—To examine whether apoenzyme undergoes damage upon inactivation, it was examined whether the holoenzymes inactivated by substrate analogues can be reactivated by DDR. DDR is known to reactivate inactivated holoenzymes or activate inactive cobalamin–apoenzyme complexes by releasing a damaged cofactor or adenine-lacking cobalamins from them (*32*, *39*). This results in (re)activation through the exchange of these cobalamins for intact AdoCbl. As shown in Fig. 4, all the holoenzymes inactivated in the reactions with glycerol (Fig. 4A), one of the physiological substrates for the enzyme, and substrate analogues (Fig. 4B–E) underwent rapid reactivation by DDR in the presence of AdoCbl, ATP and Mg^{2+}. In the presence of AdoCbl, ATP and Mg^{2+}, but in the absence of DDR, such reactivation was not seen at all. As compared with the apoenzyme control, 60, 50, 76, 72 and 78% of the original activity were recovered from the holoenzymes inactivated by glycerol, 1,2-butanediol, 3-butene-1,2-diol, 3-butyne-1,2-diol and thioglycerol, respectively. These results indicated that, in the mechanism-based inactivation by these substrate analogues as well as by glycerol, the apoenzyme itself was not damaged in the inactivation procedure. In other words, only the coenzyme underwent inactivation through the irreversible cleavage of the Co–C bond in the reaction with substrate analogues, and the damaged cofactor remained tightly bound to the active site and was not displaceable by exogenously added intact coenzyme, which resulted in the inactivation of enzyme.

DISCUSSION

All the data presented in this article indicated that diol dehydratase was suicidally inactivated by 3-unsaturated

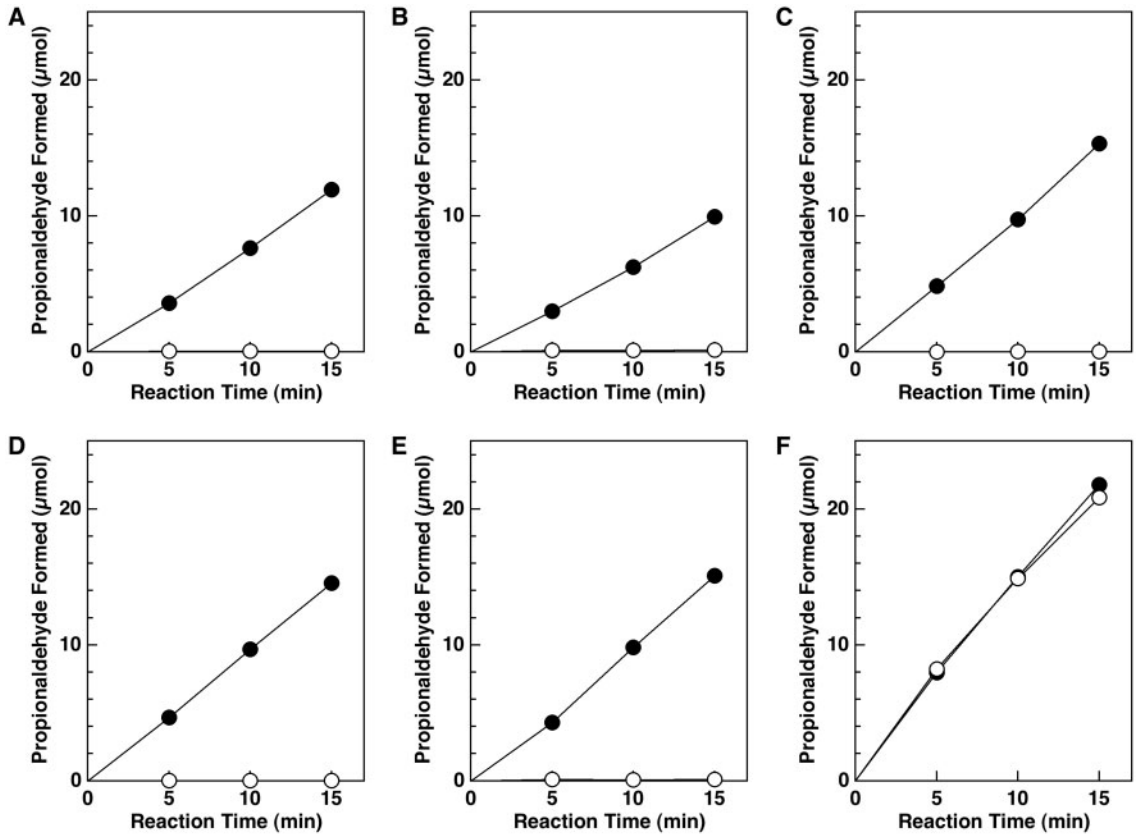

Fig. 4. **Reactivation of inactivated holodiol dehydratases by DDR.** (A–E) Holoenzymes inactivated by glycerol (A), 1,2-butanediol (B), 3-butene-1,2-diol (C), 3-butyne-1,2-diol (D) or thioglycerol (E). (F) Apoenzyme control. The holoenzymes inactivated by substrate or substrate analogues were formed by incubating apoenzyme (100 U, 4.6 nmol) with 40 nmol of AdoCbl at 37°C for 20 min in 0.3 ml of 6 mM potassium phosphate buffer (pH 8.0) containing 0.1% Brij35 and 1.2 M glycerol, 10 mM 1,2-butanediol or 1 mM 3-butene-1,2-diol, 3-butyne-1,2-diol or thioglycerol. Substrate analogues were replaced with 1,2-propanediol through repeated concentration by ultrafiltration and dilution. The inactivated holoenzymes corresponing to 1.5 U were incubated at 37°C for the indicated time periods with (closed circles) and without (open circles) 47 μg of DDR in the presence of 1.2 M 1,2-propanediol, 21 μM AdoCbl, 12 mM ATP and 12 mM $MgCl_2$, in a total volume of 50 μl. Reaction was terminated by the addition of 0.05 M potassium citrate buffer (pH 3.6). After the reaction mixtures were diluted and centrifuged, the amount of propionaldehyde formed was determined by the MBTH method after appropriate dilution.

1,2-diols, such as 3-butene-1,2-diol and 3-butyne-1,2-diol, and thioglycerol during catalysis. The coenzyme underwent damage by the irreversible cleavage of the Co–C bond, and the damaged cofactor remains tightly bound to the active site of the enzyme. The apoenzyme was not damaged in this inactivation process, because all the inactivated holoenzymes underwent reactivation by DDR in the presence of ATP, Mg^{2+} and AdoCbl. DDR reactivates inactivated holodiol dehydratases by the exchange of damaged cofactors for intact coenzyme (*32*, *39*). When C2-centred product radicals are formed from these 3-unsaturated 1,2-diols, they are expected to delocalize an unpaired electron, resulting in the stabilization of product radicals. If such stabilized product radicals are formed, they would be unable to abstract a hydrogen atom back from the CH_3 group of 5′-deoxyadenosine. Thus, it can be expected that these substrate analogues serve as mechanism-based inactivators for diol dehydratase, although the identification of organic products upon inactivation by 3-unsaturated 1,2-diols was not yet successful. Crotonaldehyde formation from 3-butene-1,2-diol was not observed. The proposed mechanism of inactivation by these substrate analogues are illustrated in Fig. 5. Thioglycerol also caused the mechanism-based inactivation of the enzyme. Equimolar amount of acrolein was detected in the reaction with thioglycerol, but the formation of H_2S or sulphide ion was not observed, suggesting that the inactivation is not accompanied by the elimination of the SH group as SH^-. Thus, in this case, the inactivation may be caused by an undesired side reaction of the product radical—that is, elimination of the SH group forming acrolein and unidentified sulphur compound(s) such as H_2S_2. The stabilization or side reaction of product radicals leads to the inactivation of enzyme, because the regeneration of the coenzyme becomes impossible. The EPR spectra of the inactivated holoenzymes indicated that the signals due to organic radicals are singlet or singlet-like. The signal of cob(II)alamin is somewhat different from that of cob(II)alamin without organic radical coupling partners (*38*), indicating that some magnetic coupling with organic radicals exists. These results may suggest that the product radicals derived from these substrate analogues might be still bound at or near the active site of the

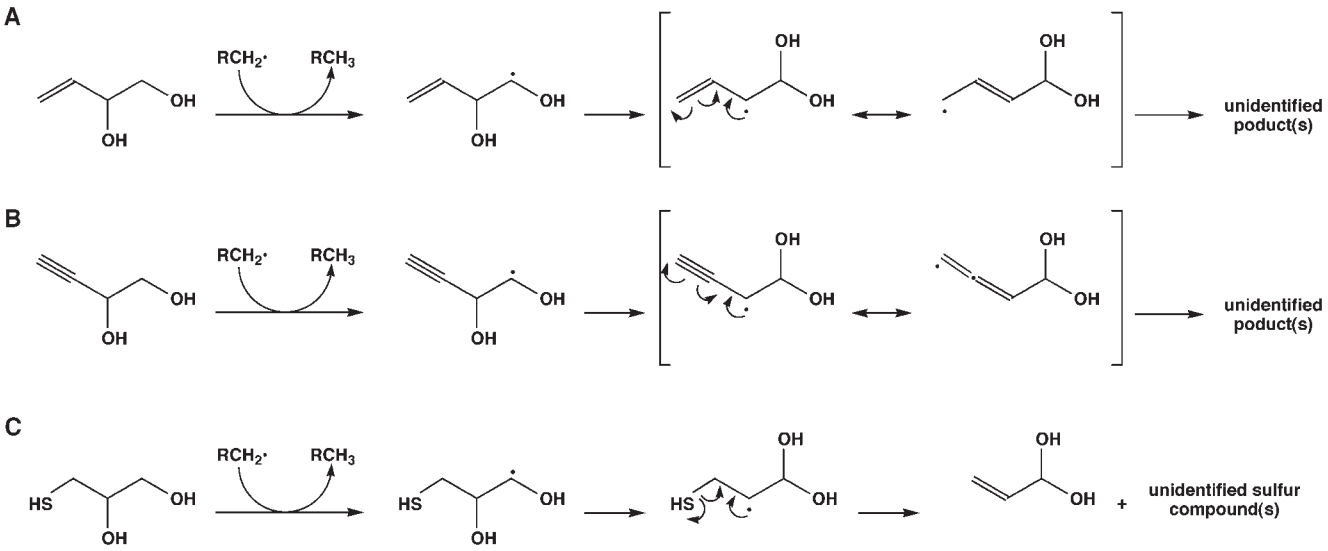

Fig. 5. **Possible mechanisms of suicidal inactivation of diol dehydratase by 3-butene-1,2-diol (A), 3-butyne-1,2-diol (B) and thioglycerol (C).** RCH_2, adenosyl radical; RCH_3, 5′-deoxyadenosine.

enzyme. This is reasonable because they would retain their affinity for the active site until the dehydration of 1,1-diol radicals.

There are different types of mechanism-based inactivation of AdoCbl-dependent enzymes. One is that stabilized radicals are formed and unable to abstract a hydrogen atom back from 5′-deoxyadenosine. This type of suicide inactivation was first reported by Frey and co-workers using chloroacetaladehyde and glycolaldehyde as inactivators for diol dehydratase (*28*, *48*) and ethanolamine ammonia-lyase (*27*). The inactivation of ethanolamine ammonia-lyase by hydroxyethylhydrazine (*49*), of glutamate mutase by 2-thioglutarate (*50*), and of diol dehydratase by 3-unsaturated 1,2-diols reported in this article also belongs to this type. The loss of radical intermediates by undesired side reactions would also lead to the same result. The inactivation by thioglycerol seems to be this type of inactivation. Another type of inactivation involves the electron transfer from cob(II)alamin to a radical derived from substrate analogues or coenzyme analogues. Typical examples are the inactivation of lysine 5,6-aminomutase by substrate (*51*) and of diol dehydratase by 3′,4′-anhydroadenosylcobalamin (*52*) reported by Frey and co-workers. The inactivation of methylmalonyl-CoA mutase by allylmalonyl-CoA (*53*) and its R207Q mutants by butyryl-CoA (*54*) also belongs to this type. The other unique type of suicide inactivation was reported by Marsh and co-workers (*55*). They proposed that an adduct is formed between substrate analogue 2-methyleneglutarate and the adenosyl radical in the glutamate mutase reaction, followed by electron transfer from cob(II)alamin. In all cases, the regeneration of the coenzyme Co–C bond becomes impossible by side reactions. It has not always been investigated whether apoenzymes are damaged or not, but in the cases of mechanism-based inactivation of diol dehydratase by coenzyme analogues (*56–58*) or by glycerol (T. Toraya and T. Tobimatsu, unpublished results), active apoenzyme is recovered by resolution of inactivated holoenzymes by acid ammonium sulphate treatment.

AdoCbl-dependent rearrangements involve radical processes, and the coenzyme-derived adenosyl radical has to undergo conformational changes during catalysis, such as ribosyl rotation (*14*) or pseudo-rotation (*15*) between proximal (close-to-Co) and distal (close-to-substrate) conformations. It would thus be reasonable that AdoCbl-dependent enzymes are susceptible to mechanism-based suicidal inactivation in general. Mechanism-based inactivators are not always artificial. Some natural or physiological substrates, such as glycerol for diol dehydratase (*29*, *40*) and ethanolamine for ethanolamine ammonia-lyase (*59*), serve as both substrate and inactivators. When side reactions leading to the inactivation of enzymes take place *in vivo*, some enzymes undergo rapid reactivation by their own reactivating proteins. These proteins are designated reactivating factors: diol dehydratase-reactivating factor (DDR) (*32*, *39*), glycerol dehydratase-reactivating factor (GDR) (*60*, *61*) and ethanolamine ammonia lyase-reactivating factor (EALR) (*59*). It remains to be investigated whether these reactivating factors can reactivate every type of inactivated holoenzymes.

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

REFERENCES

1. Toraya, T. (2003) Radical catalysis in coenzyme B_{12}-dependent isomerization (eliminating) reactions. *Chem. Rev.* **103**, 2095–2127

2. Banerjee, R. (2003) Radical carbon skeleton rearrangements: catalysis by coenzyme B_{12}-dependent mutases. *Chem. Rev.* **103**, 2083–2094
3. Brown, K.L. (2005) Chemistry and enzymology of vitamin B_{12}. *Chem. Rev.* **105**, 2075–2149
4. 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
5. Rétey, J., Umani-Ronchi, A., Seibl, J., and Arigoni, D. (1966) On the mechanism of the propanediol dehydrase reaction. *Experientia* **22**, 502–503
6. 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
7. Rétey, J., Umani-Ronchi, A., and Arigoni, D. (1966) On the stereochemistry of the propanediol dehydrase reaction. *Experientia* **22**, 72–73
8. Mancia, F., Keep, N.H., Nakagawa, A., Leadlay, P.F., McSweeney, S., Rasmussen, B., Bösecke, P., Diat, O., and Evans, P.R. (1996) How coenzyme B_{12} radicals are generated: the crystal structure of methylmalonyl-coenzyme A mutase at 2 Å resolution. *Structure* **4**, 339–350
9. Shibata, N., Masuda, J., Tobimatsu, T., Toraya, T., Suto, K., Morimoto, Y., and Yasuoka, N. (1999) A new mode of B_{12} binding and the direct participation of a potassium ion in enzyme catalysis: X-ray structure of diol dehydratase. *Structure* **7**, 997–1008
10. Reitzer, R., Gruber, K., Jogl, G., Wagner, U.G., Bothe, H., Buckel, W., and Kratky, C. (1999) Glutamate mutase from *Clostridium cochlearium*: the structure of a coenzyme B_{12}-dependent enzyme provides new mechanistic insights. *Structure* **7**, 891–902
11. Sintchak, M.D., Arjara, G., Kellogg, B.A., Stubbe, J., and Drennan, C.L. (2002) The crystal structure of class II ribonucleotide reductase reveals how an allosterically regulated monomer mimics a dimer. *Nat. Struct. Biol.* **9**, 293–300
12. 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
13. Berkovitch, F., Behshad, E., Tang, K.-H., Enns, E.A., Frey, P.A., and Drennan, C.L. (2004) A locking mechanism preventing radical damage in the absence of substrate, as revealed by the x-ray structure of lysine 5,6-aminomutase. *Proc. Natl. Acad. Sci. USA* **101**, 15870–15875
14. 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
15. Gruber, K., Reitzer, R., and Kratky, C. (2001) Radical shuttling in a protein: ribose pseudorotation controls alkyl-radical transfer in the coenzyme B_{12} dependent enzyme glutamate mutase. *Angew. Chem. Int. Ed. Engl.* **40**, 3377–3380
16. Pratt, J.M. (1982) Coordination chemistry of the B_{12} dependent isomerase reactions in *B_{12}* (Dolphin, D., ed.) Vol. 2, pp. 325–392, John Wiley & Sons, New York
17. Toraya, T., Watanabe, N., Ichikawa, M., Matsumoto, T., Ushio, K., and Fukui, S. (1987) Activation and cleavage of the carbon-cobalt bond of adeninylethylcobalamin by diol dehydrase. *J. Biol. Chem.* **262**, 8544–8550
18. Kräutler, B., Keller, W., and Kratky, C. (1989) Coenzyme B_{12} chemistry: The crystal and molecular structure of cob(II)alamin. *J. Am. Chem. Soc.* **111**, 8936–8938
19. 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
20. Warncke, K., Schmidt, J.C., and Ke, S.-C. (1999) Identification of a rearranged-substrate, product radical intermediate and the contribution of a product radical trap in vitamin B_{12} coenzyme-dependent ethanolamine deaminase catalysis. *J. Am. Chem. Soc.* **121**, 10522–10528. Correction (2008) **138**, 6055.
21. LoBrutto, R., Bandarian, V., Magnusson, O.T., Chen, X., Schramm, V.L., and Reed, G.H. (2001) 5'-Deoxyadenosine contacts the substrate radical intermediate in the active site of ethanolamine ammonia-lyase: ^{2}H and ^{13}C electron nuclear double resonance studies. *Biochemistry* **40**, 9–14
22. Bothe, H., Darley, D.J., Albracht, S.P.J., Gerfen, G.J., Golding, B.T., and Buckel, W. (1998) Identification of the 4-glutamyl radical as an intermediate in the carbon skeleton rearrangement catalyzed by coenzyme B_{12}-dependent glutamate mutase from *Clostridium cochlearium*. *Biochemistry* **37**, 4105–4113
23. Yamanishi, M., Ide, H., Murakami, Y., and Toraya, T. (2005) Identification of the 1,2-propanediol-1-yl radical as an intermediate in adenosylcobalamin-dependent diol dehydratase reaction. *Biochemistry* **44**, 2113–2118
24. Mansoorabadi, S.O., Padmakumar, R., Fazliddinova, N., Vlasie, M., Banerjee, R., and Reed, G.H. (2005) Characterization of a succinyl-CoA radical-cob(II)alamin spin triplet intermediate in the reaction catalyzed by adenosylcobalamin-dependent methylmalonyl-CoA mutase. *Biochemistry* **44**, 3153–3158
25. Wagner, O.W., Lee, H.A. Jr., Frey, P.A., and Abeles, R.H. (1966) Studies on the mechanism of action of cobamide coenzymes. Chemical properties of the enzyme-coenzyme complex. *J. Biol. Chem.* **241**, 1751–1762
26. Finlay, T.H., Valinsky, J., Sato, K., and Abeles, R.H. (1972) Studies on the mechanism of action of coenzyme B_{12}. The formation of 5'-deoxyadenosine and $B_{12(r)}$ in the reaction of dioldehydrase with chloroacetaldehyde. *J. Biol. Chem.* **247**, 4197–4207
27. Abend, A., Bandarian, V., Reed, G.H., and Frey, P.A. (2000) Identification of *cis*-ethanesemidione as the organic radical derived from glycolaldehyde in the suicide inactivation of dioldehydrase and of ethanolamine ammonia-lyase. *Biochemistry* **39**, 6250–6257
28. 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
29. Bachovchin, W.W., Eagar, R.G. Jr., Moore, K.W., and Richards, J.H. (1977) Mechanism of action of adenosylcobalamin: glycerol and other substrate analogues as substrates and inactivators for propanediol dehydratase – kinetics, stereospecificity, and mechanism. *Biochemistry* **16**, 1082–1092
30. 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
31. Mori, K., Tobimatsu, T., Hara, T., and Toraya, T. (1997) Characterization, sequencing, and expression of the genes encoding a reactivating factor for glycerol-inactivated adenosylcobalamin-dependent diol dehydratase. *J. Biol. Chem.* **272**, 32034–32041
32. Toraya, T. and Mori, K. (1999) A reactivating factor for coenzyme B_{12}-dependent diol dehydratase. *J. Biol. Chem.* **274**, 3372–3377
33. 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. Biphys.* **347**, 132–140
34. 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
35. Gill, S.C. and von Hippel, P.H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**, 319–326
36. Beinert, H. (1983) Semi-micro methods for analysis of labile sulfide and of labile sulfide plus sulfane sulfur in unusually stable iron-sulfur proteins. *Anal. Biochem.* **131**, 373–378
37. Barker, H.A., Smyth, R.D., Weissbach, H., Munch-Petersen, A., Toohey, J.I., Ladd, J.N., Volcani, B.E., and Wilson, R.M. (1960) Assay, purification, and properties of the adenylcobamide coenzyme. *J. Biol. Chem.* **235**, 181–190
38. Yamanishi, M., Yamada, S., Muguruma, H., Murakani, Y., Tobimatsu, T., Ishida, A., Yamauchi, J., and Toraya, T. (1998) Evidence for axial coordination of 5,6-dimethylbenzimidazole to the cobalt atom of adenosylcobalamin bound to diol dehydratase. *Biochemistry* **37**, 4799–4803
39. Mori, K. and Toraya, T. (1999) Mechanism of reactivation of coenzyme B_{12}-dependent diol dehydratase by a molecular chaperone-like reactivating factor. *Biochemistry* **38**, 13170–13178
40. 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
41. Toraya, T. (1999) Diol dehydratase and glycerol dehydratase in *Chemistry and Biochemistry of B_{12}* (Banerjee, R., ed.) pp. 783–809, John Wiley & Sons, New York
42. Toraya, T., Watanabe, N., Ushio, K., Matsumoto, T., and Fukui, S. (1983) Ligand exchange reactions of diol dehydrase-bound cobalamins and the effect of the nucleoside binding. *J. Biol. Chem.* **258**, 9296–9301
43. Toraya, T., Krodel, E., Mildvan, A.S., and Abeles, R.H. (1979) Role of peripheral side chains of vitamin B_{12} coenzymes in the reaction catalyzed by dioldehydrase. *Biochemistry* **18**, 417–426
44. Toraya, T. and Fukui, S. (1972) Coenzyme B_{12}-dependent propanediol dehydratase systems. Ternary complex between apoenzyme, coenzyme, and substrate analog. *Biochim. Biophys. Acta* **284**, 536–548
45. Schepler, K.L., Dunham, W.R., Sands, R.H., Fee, J.A., and Abeles, R.H. (1975) A physical explanation of the EPR spectrum observed during catalysis by enzymes utilizing coenzyme B_{12}. *Biochim. Biophys. Acta* **397**, 510–518
46. Buettner, G.R. and Coffman, R.E. (1977) EPR determination of the Co(II)-free radical magnetic geometry of the "doublet" species arising in a coenzyme B_{12}-enzyme reaction. *Biochim. Biophys. Acta* **480**, 495–505
47. Boas, J.F., Hicks, P.R., Pilbrow, J.R., and Smith, T.D. (1978) Interpretation of electron spin resonance spectra due to some B_{12}-dependent enzyme reactions. *J. Chem. Soc. Faraday II* **74**, 417–431
48. Sandala, G.M., Smith, D.M., Coote, M.L., Golding, B.T., and Radom, L. (2006) Insights into the hydrogen-abstraction reactions of diol dehydratase: relevance to the catalytic mechanism and suicide inactivation. *J. Am. Chem. Soc.* **128**, 3433–3444
49. Bandarian, V., Poyner, R.R., and Reed, G.H. (1999) Hydrogen atom exchange between 5'-deoxyadenosine and hydroxyethylhydrazine during the single turnover inactivation of ethanolamine ammonia-lyase. *Biochemistry* **38**, 12403–12407
50. Yoon, M., Patwardhan, A., Qiao, C., Mansoorabadi, S.O., Menefee, A.L., Reed, G.H., and Marsh, E.N.G. (2006) Reaction of adenosylcobalamin-dependent glutamate mutase with 2-thioglutarate. *Biochemistry* **45**, 11650–11657
51. Tang,, K.-H, Chang, C.H., and Frey, P.A. (2001) Electron transfer in the substrate-dependent suicide inactivation of lysine 5,6-aminomutase. *Biochemistry* **40**, 5190–5199
52. Magnusson, O.T. and Frey, P.A. (2002) Interactions of diol dehydrase and 3',4'-anhydroadenosylcobalamin: suicide inactivation by electron transfer. *Biochemistry* **41**, 1695–1702
53. Padovani, D. and Banerjee, R. (2006) Alternative pathways for radical dissipation in an active site mutant of B_{12}-dependent methylmalonyl-CoA mutase. *Biochemistry* **45**, 2951–2959
54. Vlasie, M.D. and Banerjee, R. (2004) When a spectator turns killer: suicidal electron transfer from cobalamin in methylmalonyl-CoA mutase. *Biochemistry* **43**, 8410–8417
55. Huhta, M.S., Ciceri, D., Golding, B.T., and Marsh, E.N.G. (2002) A novel reaction between adenosylcobalamin and 2-methyleneglutarate catalyzed by glutamate mutase. *Biochemistry* **41**, 3200–3206
56. Toraya, T. and Abeles, R.H. (1980) Inactivation of dioldehydrase in the presence of a coenzyme-B_{12} analog. *Arch. Biochem. Biophys.* **203**, 174–180
57. Toraya, T. and Ishida, A. (1991) Roles of the D-ribose and 5,6-dimethylbenzimidazole moieties of the nucleotide loop of adenosylcobalamin in manifestation of coenzymic function in the diol dehydrase reaction. *J. Biol. Chem.* **266**, 5430–5437
58. Ishida, A. and Toraya, T. (1993) Adenosylcobinamide methyl phosphate as a pseudocoenzyme for diol dehydrase. *Biochemistry* **32**, 1535–1540
59. Mori, K., Bando, R., Hieda, N., and Toraya, T. (2004) Identification of a reactivating factor for adenosylcobalamin-dependent ethanolamine ammonia lyase. *J. Bacteriol.* **186**, 6845–6854
60. Kajiura, H., Mori, K., Tobimatsu, T., and Toraya, T. (2001) Characterization and mechanism of action of a reactivating factor for adenosylcobalamin-dependent glycerol dehydratase. *J. Biol. Chem.* **276**, 36514–36519
61. Seifert, C., Bowien, S., Gottschalk, G., and Daniel, R. (2001) Identification and expression of the genes and purification and characterization of the gene products involved in reactivation of coenzyme B_{12}-dependent glycerol dehydratase of *Citrobacter freundii*. *Eur. J. Biochem.* **268**, 2369–2378