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But-3-ene-1,2-diol: A Mechanism-Based Active Site Inhibitor for Coenzyme B_{12}-Dependent Glycerol Dehydratase

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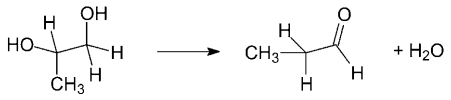
Dedicated to Professor Duilio Arigoni on the occasion of his 80th birthday

Coenzyme B_{12}-dependent glycerol dehydratase is a radical enzyme that catalyses the conversion of glycerol into 3-hydroxypropanal and propane-1,2-diol into propanal via enzyme-bound intermediate radicals. The substrate analogue but-3-ene-1,2-diol was studied in the expectation that it would lead to the 4,4-dihydroxylbut-2-en-1-yl radical, which is stabilised (allylic) and not reactive enough to retrieve a hydrogen atom from 5′-deoxyadenosine, thereby interrupting the catalytic cycle. Racemic and enantiomerically pure but-3-ene-1,2-diols and their [1,1-$^2H_2$], [2-^{2}H] and [4,4-$^2H_2$] isotopomers were synthesised and characterised by NMR spectroscopy. (S)-[4-^{14}C]but-3-ene-1,2-diol was also prepared. Kinetic measurements showed but-3-ene-1,2-diol to be a competitive inhibitor of glycerol dehydratase ($K_i = 0.21$ mM, $k_i = 5.0 \times 10^{-2}$ s^{-1}). With [4-^{14}C]but-3-ene-1,2-diol it was demonstrated that species derived from the diol become tightly bound to the enzyme's active site, but not covalently bound, because the radioactivity could be removed upon denaturation of the enzyme. EPR measurements with propane-1,2-diol as substrate generated sharp signals after 10 s that disappeared after about 1 min. In contrast, EPR resonances appeared and disappeared more slowly when but-3-ene-1,2-diol was incubated with the enzyme. Among the deuterated isotopomers, only [1,1-$^2H_2$]but-3-ene-1,2-diol showed a significantly different EPR spectrum from that of the unlabelled diol; this indicated that coupling between the unpaired electron and a deuterium at C-1 was stronger than with deuterium at C-2 or C-4. The experiments suggest the formation of the 1,2-dihydroxybut-3-en-1-yl radical, which decomposes to unidentified product(s).

Introduction

Diol and glycerol dehydratases are "radical enzymes"[1] that catalyse the conversion of simple 1,2-diols into an aldehyde (or ketone) and water (Scheme 1).[2] These enzymes were originally



Scheme 1. The diol/glycerol dehydratase reaction for propane-1,2-diol.

thought to be exclusively dependent on coenzyme B_{12} (adenosylcobalamin, AdoCbl), but the glycerol dehydratase from *Clostridium butyricum* was recently shown to be a glycyl radical enzyme activated by S-adenosylmethionine.[3] The glycerol dehydratases are of industrial interest because they offer a biotechnological route to propane-1,3-diol, a building block for polymers.[4]

The mechanism of action of coenzyme B_{12}-dependent diol/glycerol dehydratase has been elucidated by stereochemical studies,[5,6] experimental[7] and theoretical models,[8] and holoenzyme crystal structures.[9] The diol (for example, propane-1,2-diol), bound at the active site by a potassium ion and selected protein residues, is subject to attack at a C-1 hydrogen atom by the 5′-deoxyadenosyl radical released from the coenzyme by homolysis of the Co–C σ-bond. The resulting substrate radical undergoes a 1,2-oxygen shift[10] enabled by the "push-pull" effect of weakly acidic and basic groups of the enzyme.[8b] It has been proposed that partial deprotonating the nonmigrating OH at C-1 by a basic group (Gluα170 for diol dehydratase) facilitates migration of the C-2 OH of propane-1,2-diol, which is partially protonated by Hisα143.[8b] Recent studies by Toraya and co-workers,[9f] including QM/MM calculations,[8d] have shown that it is unprotonated Hisα143 that assists OH migration and also stabilises intermediate radicals. The so-formed product radical (propane-1,1-diol-1-yl) is converted into propane-1,1-diol by abstracting a hydrogen atom from the methyl group of 5′-deoxyadenosine. The final step is

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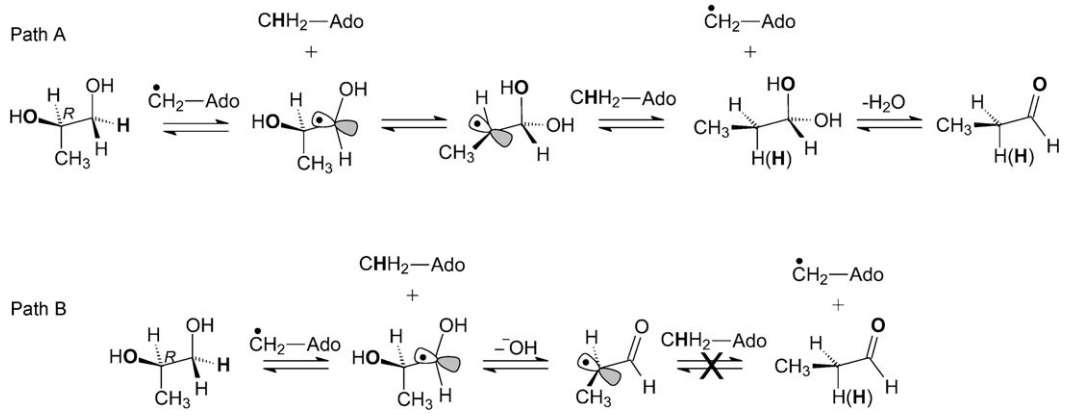


Scheme 2. Mechanisms for glycerol dehydratase acting on propane-1,2-diol (path A: via the propane-1,1-diol-1-yl radical; path B: via the propanal-2-yl radical).

the enzyme-mediated stereospecific dehydration of the 1,1-diol to propanal and water.[10] The overall sequence is summarised for (*R*)-propane-1,2-diol in Scheme 2, Path A.

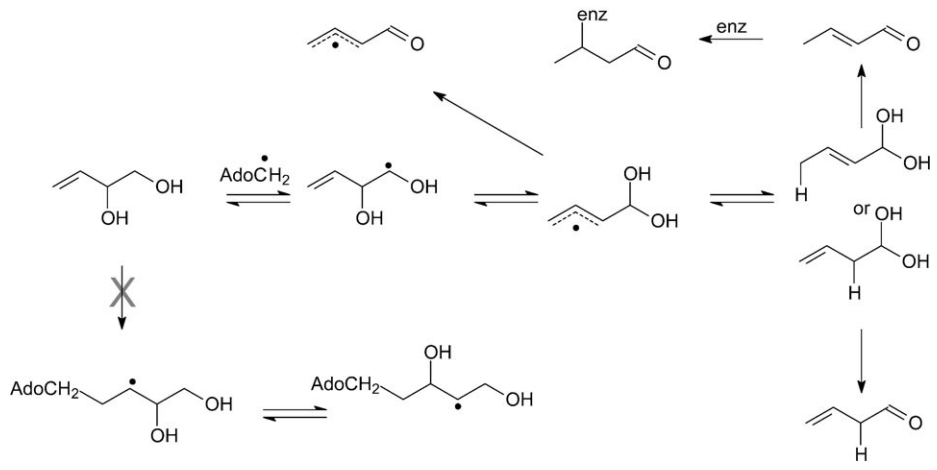
A variant of the mechanism described has the substrate radical eliminate hydroxide (or water) with the generation of a formyl-substituted alkyl radical (propanal-2-yl, Scheme 2, Path B), which recaptures the hydroxide (or water) with formation of the product-related radical.[1] It has been proposed[11] that diol and glycerol dehydratase follow the more "elaborate" path of intramolecular 1,2-migration, validated for the dehydratase by ^{18}O labelling of the C-2 OH group,[10] because it is absolutely necessary to generate a product-related radical that is a poorly stabilised methylene radical rather than a formyl-stabilised radical.[11] Only the former can achieve the relatively high energy process of abstracting a hydrogen atom from 5′-deoxyadenosine. Calculations have recently quantified these effects.[12]

But-3-ene-1,2-diol is a potentially valuable molecule for probing the mechanism of glycerol dehydratase. If this molecule were perceived as a substrate by glycerol dehydratase and followed the first two steps of the mechanism shown in Scheme 2 (path A), this would lead from the 1,2-dihydroxybut-3-en-1-yl radical to the resonance-stabilised[12b] 4,4-dihydroxybut-2-en-1-yl radical (Scheme 3). The latter is expected to be unable to remove a hydrogen atom from 5′-deoxyadenosine and may either survive as an observable species or dehydrate to the even more stable and potentially observable 4-oxobut-2-en-1-yl radical. All possible radicals could in principle react with the protein. In this paper we present a study of the behaviour of but-3-ene-1,2-diol and specifically labelled analogues (^2H, ^{14}C) with recombinant glycerol dehydratase from *Citrobacter freundii*.[13] These studies show the initial formation of the 1,2-dihydroxybut-3-en-1-yl radical followed by time-dependent inactivation of the protein.

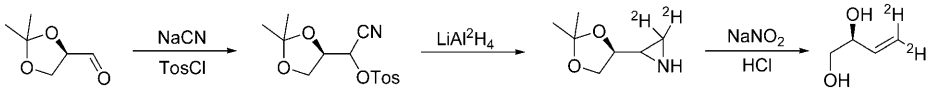
Results and Discussion

Synthesis of (*S*)-but-3-ene-1,2-diol and isotopically labelled but-3-ene-1,2-diols

Several syntheses have been reported for the enantiomeric but-3-ene-1,2-diols.[14,15] For the preparation of (*S*)-but-3-ene-1,2-diol, we used a starting material from the chiral pool in order to ensure a high level of enantiopurity. The diol was prepared from (+)-2,3-di-*O*-isopropylidene-D-glyceraldehyde, readily available from D-mannitol, using a similar procedure to that described.[16] The Wittig step on the isopropylidene-glyceraldehyde was performed in the published manner.[17] The hydrolysis of the intermediate acetal [(*S*)-di-*O*-isopropylidene-but-3-ene-1,2-diol] was done using Dowex 50W-X8 resin (H^+ form) in methanol.[18] (*S*)-[4-^{14}C]But-3-ene-1,2-diol was synthesised by performing the Wittig step with the ylid derived from [^{14}C]methyl-triphenylphosphonium iodide, which was pre-



Scheme 3. Possible intermediates and products from the action of glycerol dehydratase on but-3-ene-1,2-diol.



Scheme 4. Synthesis of [4,4-$^2H_2$]but-3-ene-1,2-diol.

pared from [^{14}C]iodomethane in a similar manner to that described for the unlabelled compound.[19] (*S*)-[4,4-$^2H_2$]But-3-en-1,2-diol was synthesised (Scheme 4) from the cyanohydrin of (+)-2,3-di-*O*-isopropylidene-D-glyceraldehyde, which was prepared by a variant of the method of Ichimura.[20] (*R*,*S*) [2-^{2}H]-but-3-ene-1,2-diol was prepared from unlabelled (*R*,*S*)-but-3-ene-1,2-diol. After protection of the primary OH group as a *tert*-butyldiphenylsilyl ether, the product was oxidised at C-2 to the corresponding ketone, which was reduced by LiAl$^2H_4$. Removal of the protecting group by treatment with tetrabutylammonium fluoride gave (*R*,*S*)-[2-^{2}H]but-3-ene-1,2-diol. (*R*,*S*)-[1,1-$^2H_2$]but-3-ene-1,2-diol was prepared from acrolein, the cyanohydrin of which was protected by acetylation. After converting the cyano group to the corresponding methyl ester, the latter was reduced with LiAl$^2H_4$ to afford (*R*,*S*)-[1,1-$^2H_2$]but-3-ene-1,2-diol.

The structures of the ^{2}H-labelled but-3-ene-1,2-diols were validated by ^{1}H and ^{13}C NMR spectroscopy. The spectra were similar to those of unlabelled but-3-ene-1,2-diol except for the lack of resonances from proton(s) replaced by deuterons and the modified appearance of some resonances due to the effect of neighbouring ^{2}H.

Enzyme purification

Glycerol dehydratase was obtained using *Escherichia coli*, expression system BL21(DE3)/pET-28a + gdh.[13,21] The enzyme was purified with the help of an N-terminal His$_6$-tag, which was subsequently removed by thrombin. The purification was completed by gel filtration chromatography, which raised the specific activity with the substrate *rac*-propane-1,2-diol to 16.2 U mg^{-1}. The enzyme with the His$_6$-tag on was also active, but had a lower V_{max} value (11.1 U mg^{-1}). The K_m value was not significantly affected by the His-tag (191 μM versus 194 μM after thrombin treatment and gel filtration chromatography).

Inhibition kinetics with *rac*-but-3-ene-1,2-diol

Glycerol dehydratase was kinetically characterised using *rac*-propane-1,2-diol. For determination of the inhibition constant K_i and rate constant k_i for inhibition, glycerol dehydratase was incubated with various concentrations of *rac*-but-3-ene-1,2-diol for 4 min at 37 °C, thereby establishing the binding equilibrium. The inhibition process was started by addition of AdoCbl. After various time intervals the inhibition reaction was interrupted by addition of an excess of *rac*-propane-1,2-diol and the remaining activity was determined under standard assay conditions. Different inhibition times and inhibitor concentrations were used. The inhibition reaction followed first-order kinetics as can be appreciated from Figure 1 A. Plotting the inhibition rate constants (k_{iobs}) against the inhibitor concentration showed saturation behaviour (Figure 1 B): the double reciprocal plot of k_{iobs} against inhibitor concentration gave a straight line. Assuming reversible binding followed by irreversible inactivation, an inhibition constant (K_i) of 0.21 mM with a rate constant (k_i) of 5.0×10^{-2} s^{-1} was observed.

Noncovalent binding of the inhibitor as shown by radioactive labelling

Glycerol dehydratase was incubated for 2.5 h with a 17-fold excess of (2*S*)-[4-^{14}C]but-3-ene-1,2-diol. After removal of the excess of inhibitor by membrane filtration, the resulting solution of protein was radioactive and indicated that one molecule of but-3-ene-1,2-diol bound per active site. The protein

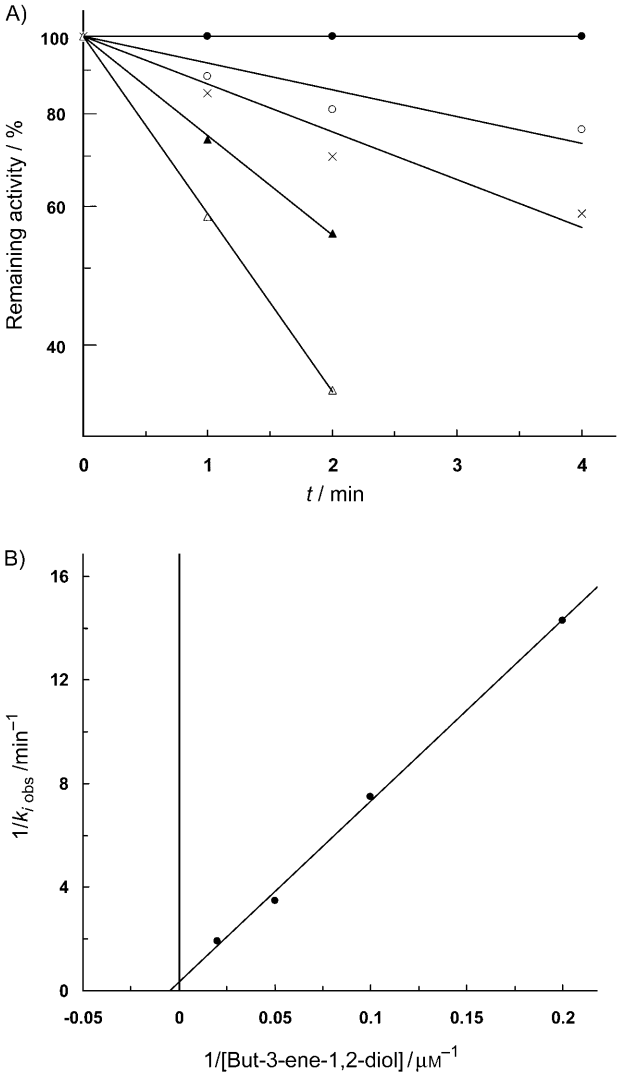


Figure 1. A) Inhibition of *Citrobacter freundii* holo glycerol dehydratase by various concentrations of but-3-ene-1,2-diol as a function of preincubation time. No (●), 5 μM (○), 10 μM (×), 20 μM (▲), and 50 μM (△) but-3-ene-1,2-diol. B) Double reciprocal plot of the observed rate of inactivation as a function of the inhibitor concentration.

was denatured, centrifuged and fractionated by gel filtration giving two protein bands containing about one third of the original radioactivity. Upon SDS-electrophoresis the eluted protein bands were devoid of radioactivity.

These results indicate that a species derived from but-3-ene-1,2-diol and AdoCbl becomes tightly but not covalently bound to the active site of glycerol dehydratase. This species could be derived from the allyl radical 4,4-dihydroxybut-2-en-1-yl (Scheme 3), the formation of which might explain the inhibitory effect of but-3-ene-1,2-diol on the enzyme. The resonance-stabilised allyl radical is unable to reabstract a H-atom from deoxyadenosine. Previous studies have shown the formation of resonance-stabilised radicals from coenzyme[22] and substrate[23] analogues that prevent turnover and inhibit the enzyme. The possibility that the 5′-deoxyadenosyl radical adds to the double bond of but-3-ene-1,2-diol (see Scheme 3) is considered unlikely because this would not explain the modified EPR resonance observed with [1,1-$^2H_2$]but-3-ene-1,2-diol (see below).

EPR spectroscopy

Incubation of propane-1,2-diol with glycerol dehydratase for 10 s followed by rapid freeze-quench gave an EPR spectrum consisting of a doublet composed of two sharp derivative-shaped lines at $g = 2.038 \pm 0.001$ and $g = 1.925 \pm 0.003$, and an additional broad signal with $g = 2.20$ (see top trace in Figure 2 A). These signals disappeared with longer incubation times (Figure 2 A, middle and lower traces). The spectrum shown is very similar to that reported by Toraya et al. from incubating diol dehydratase from *Klebsiella oxytoca* with propane-1,2-diol.[24] The initial spectrum can be interpreted as arising from the interaction of an organic radical ($g = 2.00$) derived from the substrate with a low-spin Co^{II} ($g_\perp = 2.23$, weak hyperfine coupling, and $g_\parallel = 2.00$, exhibiting hyperfine coupling comprising eight triplets, $A_\parallel^{Co} = 10.5$ mT and $A_\parallel^{N} = 2.0$ mT) with isotropic exchange and dipolar coupling. This assumes a distance of about 10 Å between the organic radical and Co^{II}.[25]

Experiments were carried out with but-3-ene-1,2-diol under similar conditions to those described for propane-1,2-diol (Figure 2 B). A strong signal appeared at $g = 2.002$, which increased (45 s) but then decreased upon longer incubation (2.5 and 7.5 min) with formation of signals typical for enzyme-bound cob(II)alamin (that is, well resolved cobalt and nitrogen hyperfine compared to free cob(II)alamin). In other experiments the cob(II)alamin was seen to persist up to 30 min (data not shown). The enzyme-bound cob(II)alamin had the following EPR parameters: $g_\perp = 2.34$–2.18, with resolved hyperfine couplings and $g_\parallel = 2.00$, split into eight triplets by $A_\parallel^{Co} = 10.2$ mT and $A_\parallel^{N} = 2.07$ mT. All previously studied AdoCbl-dependent enzymes produced such enzyme-bound cob(II)alamin signals upon inactivation or photolysis (particularly under aerobic conditions).[24]

The usual organic radical-Co^{II} EPR spectrum exhibits doublet-like lines or a structured derivative-shaped EPR spectrum around $g = 2.02$ due to isotropic exchange and dipolar coupling.[24,25] The 5.0 mT line width (peak to trough) of the species giving the $g = 2.002$ EPR signal from but-3-ene-1,2-diol bound to glycerol dehydratase/AdoCbl indicates a coupling process, even though the *g*-value is identical to that of the free-electron. Decay of this species seems to be associated with an increase of enzyme-bound cob(II)alamin signals. Therefore the microwave power saturation of the $g = 2.002$ signal was compared with the signal at $g = 2.038$ observed with propane-1,2-diol (Figure 3.). The $g = 2.002$ signal could be from a radical close to diamagnetic Co^{III} or from a radical at a longer distance (that is, > 15 Å) from Co^{II}. For these cases saturation should occur at liquid nitrogen temperature (that is, power $P_{1/2} \ll 1$ mW). However, if the radical is close to enzyme-bound cob(II)alamin then the power saturation will be less pronounced, as for the propane-1,2-diol $g = 2.038$ signal (that is, $P_{1/2} \gg 1$ mW). Saturation of the but-3-ene-1,2-diol-induced

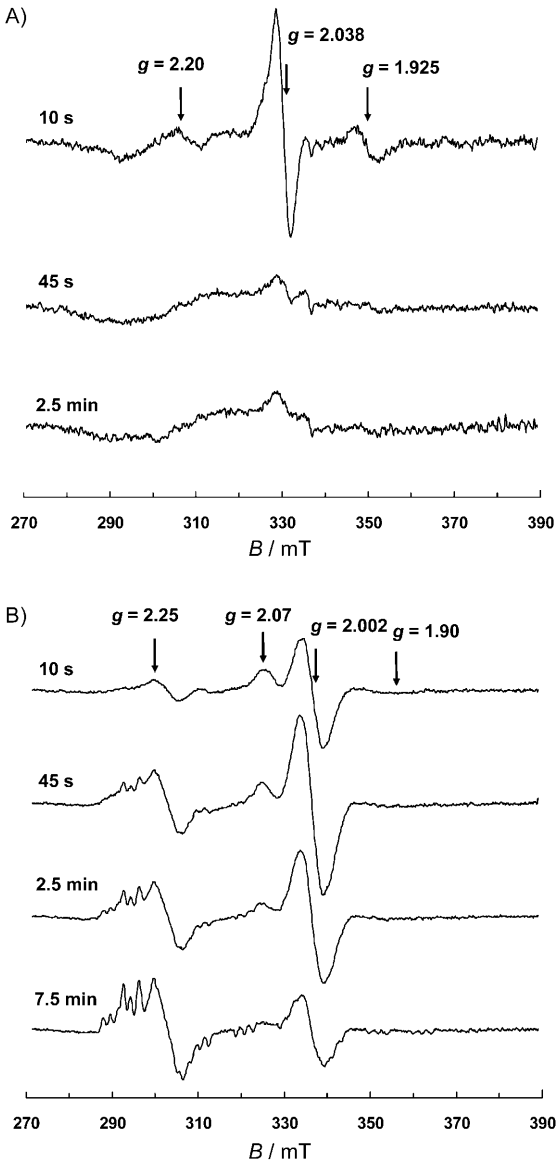


Figure 2. EPR spectra recorded at 77 K of *Citrobacter freundii* holo glycerol dehydratase which was incubated with A) propane-1,2-diol and B) but-3-ene-1,2-diol at 6 °C for the indicated times.

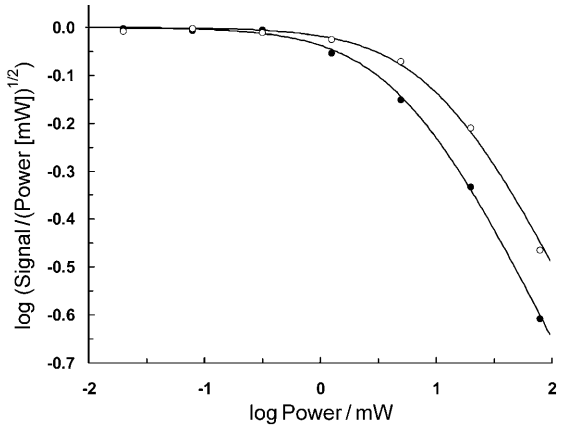


Figure 3. Saturation at 77 K of the EPR signals generated by incubation of *Citrobacter freundii* holo glycerol dehydratase at 6 °C with propane-1,2-diol for 10 s [●, fit with $P_{1/2}=5.3$ mW ($b=1$)] and but-3-ene-1,2-diol for 45 s [○, fit with $P_{1/2}=11.5$ mW ($b=1$)]. See Figure 2A (top trace) and Figure 2B (second trace) for spectra.

signal at $g=2.002$ (Figure 3) shows similar characteristics to those of the well-characterised doublet of a coupled system.[24,25] It is therefore concluded that the $g=2.002$ signal is from a species having Co^{II} at a distance of approximately 10 Å.

To help identify the species derived from but-3-ene-1,2-diol, measurements were performed using [1,1-$^2H_2$], [2-^{2}H] and [4,4-$^2H_2$] isotopomers and the data compared with that for the unlabelled diol. Only [1,1-$^2H_2$]but-3-ene-1,2-diol showed a significantly different EPR spectrum (Figure 4), suggesting the formation of the 1,2-dihydroxybut-3-en-1-yl radical. In the study of Toraya et al.,[24] deuterium labelling at C-1 of propane-1,2-diol also caused a significant narrowing of the signal observed with unlabelled propane-1,2-diol, whereas the effect of labelling of C-2 was barely detectable. Their findings are remarkably similar to our results: narrowing upon deuterium labelling of C-1 and no significant change upon labelling of C-2. The increased resolution observed with [1,1-$^2H_2$]but-3-ene-1,2-diol compared to [1,1-$^2H_2$]propane-1,2-diol allows the complexity of the EPR signal to be resolved. Though we were not able to simulate the spectrum, the shape of the signal is reminiscent of a Pake doublet,[23,26] with resolved structure reflecting the isotropic exchange and dipole-dipole coupling parameters rather than (or in addition to) proton hyperfine coupling. Contributions from species other than the 1,2-dihydroxybut-3-en-1-yl radical can additionally explain the complexity and asymmetry of the signal.

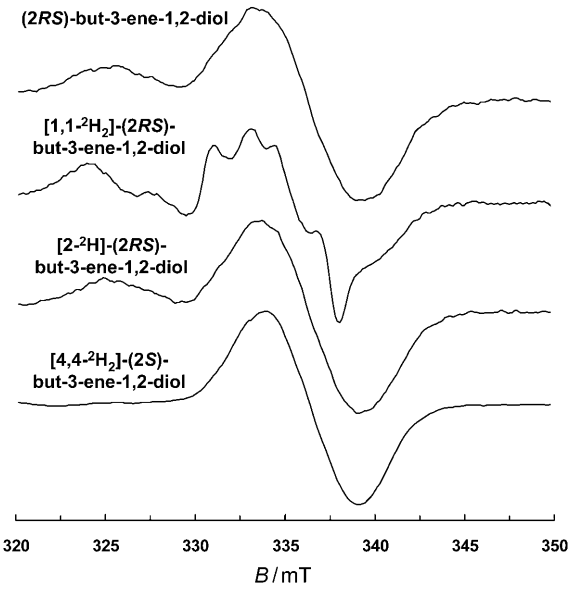


Figure 4. EPR spectra recorded at 77 K of *Citrobacter freundii* holo glycerol dehydratase which was incubated at 6 °C for 45 s with the indicated isotopomers of but-3-ene-1,2-diol.

Conclusions

We have described herein a study of the interaction of the substrate analogue but-3-ene-1,2-diol with coenzyme B_{12}-dependent glycerol dehydratase. The observed inactivation of the enzyme was ascribed to the formation of the 1,2-dihydroxybut-3-en-1-yl radical and possibly additional species. Generation of the 1,2-dihydroxybut-3-en-1-yl radical was indicated by EPR spectroscopy with the aid of specifically deuterated but-3-ene-1,2-diols. However, this radical rapidly decayed, possibly with intermediate formation of a stabilised radical (4,4-dihydroxylbut-2-en-1-yl radical or 1-formylallyl—see Scheme 3) and cob(II)alamin, the latter being observed by EPR. Based on the absence of effects on the EPR spectrum in the $g=2$ region upon deuterium labelling of C-4, the 1-formylallyl radical (Scheme 3) is not a major species. If a stabilised radical is formed, it is unable to retrieve a hydrogen atom from 5′-deoxyadenosine and this interrupts the catalytic cycle. Species derived from but-3-ene-1,2-diol and AdoCbl are apparently tightly but not covalently bound to the enzyme, as shown with the aid of radiolabelling.

Experimental Section

Materials: All chemicals, including coenzyme B_{12}, were supplied by Aldrich Chemical Co., Lancaster Syntheses, or Fluka Chemie AG. Solvents were AnalaR or laboratory grade, and were dried when necessary according to standard procedures. Medium pressure chromatography was carried out on Fison's Matrix silica gel 60 (35–70 microns) with the indicated eluent. Thin layer chromatography (TLC) was performed on E. Merck silica gel (Kieselgel 60 F254) aluminium-backed plates. ($^2H_3$)Methyl iodide, lithium aluminium deuteride (LiAl^2H_4), and thrombin were from Sigma–Aldrich; NADH and yeast alcohol dehydrogenase (ADH) were from Roche Diagnostics (Mannheim, Germany). Glycerol dehydratase was isolated as described previously[13,21] from overexpressing *Escherichia coli* cells containing the genomic DNA for glycerol dehydratase from *Citrobacter freundii*. Glycerol dehydratase was expressed also with an N-terminal His_6-tag, which facilitated purification. The His-tag could be removed by treatment with thrombin to give glycerol dehydratase of very high purity.

Synthesis of but-3-ene-1,2-diols: (R,S)-But-3-ene-1,2-diol: This was obtained in 28% yield by treatment of (*Z*)-but-2-ene-1,4-diol with mercury(II) sulfate.[27]

(*S*)-But-3-ene-1,2-diol: (+)-2,3-di-*O*-isopropylidene-D-glyceraldehyde was prepared in 50–60% yield by cleavage of the diol moiety of 1,2;5,6-di-*O*-isopropylidene-D-mannitol with $Pb(OAc)_4$.[16] The Wittig reagent from methyl-triphenylphosphonium iodide was prepared using dimsyl sodium in DMSO.[17] An equal volume of pentane was added to the resulting solution, with vigorous stirring. A solution of (+)-2,3-di-*O*-isopropylidene-D-glyceraldehyde in pentane was added dropwise with stirring to the intense yellow suspension. The end of the reaction was indicated by the disappearance of the colour. Most of the product was in the pentane phase and was isolated after further extractions with pentane. The by-product, triphenylphosphine oxide, remained in the DMSO phase. Removal of the protecting group from the isopropylidene-(*S*)-but-3-ene-1,2-diol occurred quantitatively with DOWEX 50W-X8 (H^+ form) in methanol[18] to afford the title compound as a colourless oil (24% overall from the Wittig step). The ^1H and ^{13}C NMR spectra of this compound were identical to that of (*rac*)-but-3-ene-1,2-diol. ^1H NMR (300 MHz, $CDCl_3$): $\delta = 3.43$ (dd, 1H), 3.59 (d, 1H), 3.9 (br, OH), 4.2 (br, 1H), 5.15 (d, 1H), 5.29 (d, 1H), 5.78 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 65.8$, 72.8, 115.9, 136.6.

(*S*)-[4-^{14}C]But-3-ene-1,2-diol: This was synthesised in the manner described for the unlabelled compound using [^{14}C]methyl-triphenylphosphonium iodide (404 mg, 1 mmol, 250 μCi, specific activity: 0.25 mCi mmol^{-1}), which was prepared from triphenylphosphine and [^{14}C]methyl iodide using the procedure described for unlabelled material.[19] The labelled diol was analysed by thin layer chromatography and radioautography, which both showed a spot corresponding to but-3-ene-1,2-diol. For the inhibition experiments 13.3 μCi of the radiolabelled diol was used.

(*S*)-[4,4-^{2}H$_2$]But-3-ene-1,2-diol: This was synthesised from the cyanohydrin of (+)-2,3-di-*O*-isopropylidene-D-glyceraldehyde, which was prepared by a variant of the method of Ichimura.[20] Sodium cyanide in H_2O (2 mL) was added dropwise at 0 °C to *p*-toluenesulfonyl chloride in acetonitrile and 50 mM K_2HPO_4 (pH 7.4). The reaction afforded two diastereoisomers in a 2:1 ratio, which were reduced with $LiAl^2H_4$. The intermediate primary amine spontaneously eliminated tosylate giving an aziridine (Scheme 4) as a mixture of diastereoisomers. Finally, the aziridine was transformed to (2*S*)-[4,4-^{2}H$_2$]but-3-ene-1,2-diol (20% overall yield) by nitrosation with $NaNO_2$/conc. acetic acid, under which conditions the protecting group was simultaneously removed. The ^1H and ^{13}C NMR spectra of this compound were similar to those of authentic but-3-ene-1,2-diol, except for the lack of the H-4 resonances and simplification of the H-3 resonance, and the appearance of C-4 as a multiplet.

(*R*,*S*)-[1,1-^{2}H$_2$]But-3-ene-1,2-diol: Acetic anhydride (9.5 mL, 100 mmol) was added at −10 °C with vigorous stirring to freshly distilled acrolein (6.7 mL, 5.6 g, 100 mmol) in toluene (20 mL), NaCN (7.35 g, 150 mmol) in H_2O (40 mL) was added dropwise while keeping the temperature at −10 °C to the resulting mixture. After stirring for 2 h the phases were separated and the aqueous phase was extracted with toluene (3×5 mL). The combined organic layers were washed with 1 M acetic acid (2×5 mL), saturated Na_2CO_3 solution (2×5 mL), and H_2O (2×5 mL). After drying ($MgSO_4$) the solvent was removed yielding 2-acetoxy-1-cyanobut-3-ene (9.5 g, 76%). Saturated HCl in methanol (15 mL) and conc. HCl (4 mL) were added to a refluxing solution of the nitrile in methanol (15 mL). After boiling at reflux for 3.5 h the mixture was cooled to 0 °C. The precipitated NH_4Cl was filtered off and the solvent was removed. The residue was taken up in diethyl ether (30 mL) and the solution was washed with saturated Na_2CO_3 solution (2×5 mL) and H_2O (2×5 mL). The aqueous phase was extracted with diethyl ether (4×5 mL). The combined organic phases were dried ($MgSO_4$) and the solvent was removed. The crude product was fractionated in vacuo to give methyl 2-hydroxybut-3-enoate (2.3 g, 31%) as a clear liquid, bp 61 °C at 20 mbar.

$LiAl^2H_4$ (250 mg, 6.0 mmol) was added in small portions at 0 °C to a solution of the ester (730 mg, 6.38 mmol) in diethyl ether (15 mL). After the completion of the addition, the mixture was stirred at 25 °C for 25 h. The reaction was terminated by addition of wet diethyl ether (5 mL) followed by saturated potassium tartrate solution (7 mL). The precipitated orange solid was redissolved by adding diethyl ether (50 mL) to the mixture. The aqueous phase was extracted with diethyl ether (4×50 mL). The combined organic phase was dried ($MgSO_4$) and the solvent was removed. The crude product was purified by medium-pressure chromatography on silica (10 g) using CH_2Cl_2/CH_3OH (98:2, *v*/*v*) as eluent, yielding pure (*R*,*S*)-[1,1-^{2}H$_2$]but-3-ene-1,2-diol (384 mg, 71%). ^1H NMR (500 MHz, $CDCl_3$): $\delta = 3.71$ (br, OH), 4.21 (s, 1H), 5.19 (d, 1H), 5.32 (d, 1H), 5.81 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 72.9$, 116.5, 136.5.

(*R*,*S*)-[2-^{2}H$_1$]But-3-ene-1,2-diol: Tert-butyldiphenylsilyl chloride (2.08 mL, 2.19 g, 7.98 mmol) was added dropwise at 20 °C to (*R*,*S*)-but-3-ene-1,2-diol (586 mg, 6.65 mmol) in dry pyridine (3 mL). After stirring for 1 h at 20 °C, CH_2Cl_2 (20 mL) was added and the mixture was washed with ice-cold 5% HCl (10 mL). The organic layer was washed with water (10 mL) and dried ($MgSO_4$). The solvent was removed giving (*R*,*S*)-but-3-ene-1,2-diol 1-*O*-tert-butyldiphenylsilyl ether (2.08 g, 93%). Oxalyl chloride (390 μL, 4.41 mmol) was added dropwise at −78 °C to DMSO (655 μL, 9.22 mmol) in dichloromethane (30 mL) and the mixture was stirred for 1 h. The silyl ether (1.31 g, 4.01 mmol) in CH_2Cl_2 (7 mL) was added and the mixture was stirred for 1 h at −78 °C. Finally triethylamine (2.8 mL, 20.1 mmol) was added dropwise. The mixture was kept at −78 °C for a further 30 min and then allowed to warm to RT. The resulting mixture was washed with water (2×10 mL) and dried ($MgSO_4$). Removal of the solvent afforded (*R*,*S*)-1-tert-butyldiphenylsilyloxy-but-3-en-2-one (1.23 g). The ketone (1.23 g, 3.79 mmol) in diethyl ether (5 mL) was added to a solution of $LiAl^2H_4$ (190 mg, 4.54 mmol) in diethyl ether (20 mL) at −78 °C. After stirring for 15 min the reaction was worked up as before. The crude product was purified by medium pressure chromatography on silica (10 g) using hexane/ethyl acetate (95:5, *v*/*v*) as eluent. Tetrabutylammonium fluoride (433 mg, 1.37 mmol) in THF (5 mL) at 20 °C was added to (*R*,*S*)-[2-^{2}H$_1$]but-3-ene-1,2-diol 1-tert-butyldiphenylsilyl ether (225 mg, 0.68 mmol) in THF (10 mL). After stirring for 3.5 h water was added (100 μL). The solvent was removed under reduced pressure and the crude product was purified by medium pressure chromatography on silica (10 g) using hexane:ethyl acetate (50:50, *v*/*v*) as eluent yielding (*R*,*S*)-[2-^{2}H$_1$]but-3-ene-1,2-diol (36 mg, 59%) as a pale yellow oil. ^1H NMR (500 MHz, $CDCl_3$): $\delta = 3.43$ (d, 1H), 3.61 (d, 1H), 3.77 (br, OH), 5.16 (dd, 1H), 5.30 (dd, 1H), 5.79 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 66.1$, 72.8, 116.5, 136.6.

Enzyme assays: Reactions were performed at 37 °C in darkness in 20 mM K_2HPO_4 buffer (pH 8.0) containing 0.1% (*RS*)-propane-1,2-diol, NADH (10 μL of a solution containing 22 mg mL^{-1}), yeast alcohol dehydrogenase (10 μL of a solution containing 3 mg mL^{-1}) and 10 μL of glycerol dehydratase solution. The reactions were started by the addition of AdoCbl (0.1 mL of a 5 mM solution) to give total volume of 0.5 mL. The decrease of the absorption at 340 nm was measured. A mean rate for the enzymatic reaction, measured over

the interval $t = 1$–2 min of the assay, was used for the calculation of the kinetic constants.

Kinetic measurements with substrates and inhibitors: For kinetic measurements at exact substrate concentrations the enzyme was freed from residual substrate (required for stabilisation) by dialysis against 20 mM K_2HPO_4 (pH 8). The kinetic constants, K_m and V_{max}, were calculated from Lineweaver–Burk plots. For measurements of the inhibition kinetics the enzyme was preincubated with (*RS*)-but-3-ene-1,2-diol for 4 min at 37 °C. The reaction was started by the addition of AdoCbl. After 1, 2, 4, and 8 min the remaining activity was measured by addition of an excess of (*RS*)-propane-1,2-diol. The experiments were carried out in triplicate at different inhibitor concentrations (5, 10, 20, and 50 μM). The inhibition reaction followed first-order kinetics (see Figure 1 A). Plotting the individual $k_{i\,obs}$ values against the inhibitor concentration showed saturation behaviour (Figure 1 B). On the assumption that a reversible binding is followed by an irreversible inactivation, Equations (1) and (2) were applied for calculation of the inhibition constant K_i.

$$[E_{inhib}] = [E_0]\, e^{-k_{i\,obs}\, t} \quad (1)$$

$$k_{i\,obs} = \frac{k_i}{1 + (K_i/[I])} \quad (2)$$

[E] = enzyme concentration before [E_0] and after inhibition [$E_{inhib.}$] as measured by the catalytic activity; [I] = inhibitor concentration in μM; K_i = inhibition constant; k_i = rate constant for inhibition.

The plot of the reciprocals of the observed inhibition rate constants ($k_{i\,obs}$) against the reciprocals of the inhibitor concentrations gave a straight line (Figure 1 B). The inhibition constant K_i was 0.21 mM (from the intersection on the *x* axis at $-1/K_i$) and the rate constant for inhibition k_i was 3.0 min^{-1} (5.0×10^{-2} s^{-1}; intersection on the *y* axis at $1/k_i$).

Inhibition with (*S*)-[4-^{14}C]but-3-ene-1,2-diol: A solution of (*S*)-[4-^{14}C]but-3-ene-1,2-diol (1 mL, 5.3 μmol, 1.3 μCi) followed by a solution of AdoCbl (40 μL, 15 mM, 0.6 μmol) were added in the dark to substrate-free glycerol dehydratase (30 mg, 0.15 μmol, 0.3 μmol active centres) in K_2HPO_4 (0.6 mL, 20 mM, pH 8.0). After incubation for 2.5 h at 37 °C the solution was concentrated to approximately one tenth with a Centricon device (< 5 kDa exclusion, Millipore Ultrafree-4 Centrifugal Filter Unit). After four dilution/concentration cycles with K_2HPO_4 (20 mM), only traces of radioactivity was found in the protein-free wash buffer. The concentrated protein solution showed 0.075 μCi corresponding to 0.25 μCi μmol^{-1}, which was consistent with the expected value for 0.3 μmol inhibited active centres. The concentrated protein solution was treated with SDS and 2-mercaptoethanol followed by heating at 100 °C for 5 min. The denatured protein suspension was centrifuged at 16000 *g*. Two thirds of the radioactivity were in the upper layer, which was gel filtered. The main portion of the radioactivity was found in three peaks. In two of them, still containing about one third of the original radioactivity, the protein was precipitated by ammonium sulfate; the third contained no protein and was most likely released inhibitor. The radioactive protein fractions were investigated by SDS electrophoresis. Examination of the SDS PAGE in a phospho-imager revealed that the protein bands were devoid of radioactivity.

Time-dependence of the generation of EPR signals with propane-1,2-diol and but-3-ene-1,2-diol: Aerobic reaction mixtures were of total volume 250 μL containing 0.1 M propane-1,2-diol (or but-3-ene-1,2-diol, unlabelled or labelled) and glycerol dehydratase (5 mg mL^{-1}) in 20 mM KH_2PO_4 buffer, pH 8. Using glycerol dehydratase (1.25 mg, 54 U), it was calculated that under standard conditions at RT the propane-1,2-diol (25 μmol) would be depleted after 28 s. To avoid the depletion of the propane-1,2-diol and generation of detrimental amounts of propanal the mixing was performed by rapid addition of substrate solution at 4 °C to a solution of enzyme containing a fivefold molar excess of AdoCbl, which had been preincubated in the EPR tube at 6 °C. The fastest time of mixing and then quenching by immersion in ethanol, precooled in liquid nitrogen, was maximally 15 s. Other incubation times (45 s, 2.5 min, and 7.5 min) were more easily and reliably obtained. The experiments were carried out in darkness to avoid formation of hydroxocobalamin by photolysis.

EPR spectroscopy: The instrument and further EPR conditions were identical to those previously described.[28] Temperature, 77 K; microwave power, 1.2 mW; modulation amplitude, 1.0 mT; microwave frequency, 9.431 GHz.

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