Interactions of Diol Dehydrase and 3′,4′-Anhydroadenosylcobalamin: Suicide Inactivation by Electron Transfer†

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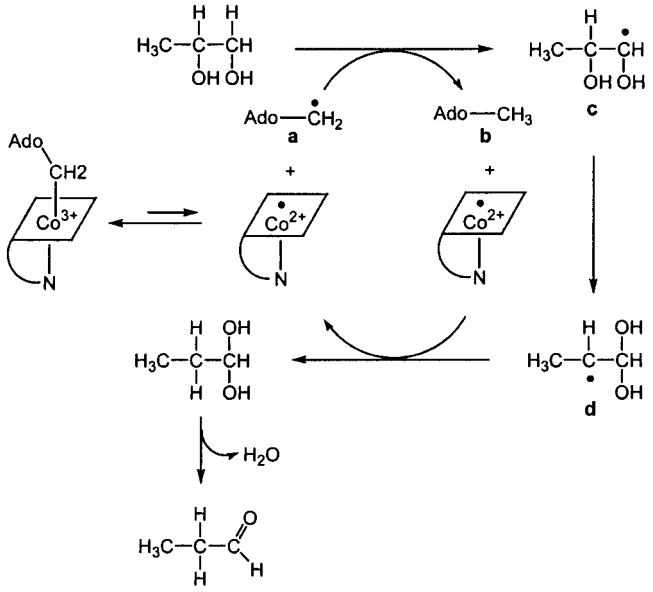
ABSTRACT: 3′,4′-Anhydroadenosylcobalamin (*an*AdoCbl) is an analogue of the adenosylcobalamin (AdoCbl) coenzyme (Magnusson, O.Th., and Frey, P. A. (2000) *J. Am. Chem. Soc. 122*, 8807−8813). This compound supports activity for diol dehydrase at 0.02% of that observed with AdoCbl. In a side reaction, however, *an*AdoCbl induces suicide inactivation by an electron-transfer mechanism. Homolytic cleavage of the Co−C bond of *an*AdoCbl at the active site of diol dehydrase was observed by spectrophotometric detection of cob(II)alamin. Anaerobic conversion of enzyme bound cob(II)alamin to cob(III)alamin, both in the absence and presence of substrate, indicates that the coenzyme derived 5′-deoxy-3′,4′-anhydroadenosine-5′-yl serves as the oxidizing agent. This hypothesis is supported by the stoichiometric formation of 3′,5′-dideoxyadenosine-4′,5′-ene as the nucleoside cleavage product, as determined by high-performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy. Experiments performed in deuterium oxide show that a single solvent exchangeable proton is incorporated into the product. These data are consistent with the intermediate formation of a transient allylic anion formed after one electron transfer from cob(II)alamin to the allylic 5′-deoxy-3′,4′-anhydroadenosyl radical. Selective protonation at C3′ was demonstrated by spectroscopic characterization of the purified product. This study provides an example of suicide inactivation of a radical enzyme brought about by a side reaction of an analogue of the radical intermediate.

DDH (diol dehydrase, EC:4.2.1.28) catalyzes the AdoCbl-dependent dehydration of small vicinal diols to the corresponding aldehydes (*1*). As in the cases of other AdoCbl-dependent enzymes, the reaction involves the participation of organic radical intermediates and a 1,2-migration of a functional substituent, in this case a hydroxyl group, with the concomitant transfer of a hydrogen atom. The minimal catalytic mechanism for DDH with 1,2-propanediol as a substrate is shown in Scheme 1 (*2*). Enzyme-induced cleavage of the Co−C bond of AdoCbl generates Cbl^{II} and a 5′-deoxyadenosyl radical **a**. The 5′-deoxyadenosyl radical abstracts a hydrogen atom from C1 of 1,2-propanediol to form the substrate radical **c**. The rearrangement of this radical to yield the product radical **d** occurs by an as yet unidentified

Scheme 1



mechanism. Several mechanistic proposals have been put forward (*3*−*5*). The reabstraction of a hydrogen atom from 5′-deoxyadenosine **b** yields the *gem*-diol product, which undergoes enzyme catalyzed dehydration, and the 5′-deoxyadenosyl radical, which recombines with Cbl^{II} to regenerate the coenzyme.

Toraya and co-workers have extensively studied the structure−function relationship of the coenzyme. The β-D-ribofuranose moiety of the upper axial ligand is essential,

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[1] Abbreviations: AdoCbl, adenosylcobalamin; *an*AdoCbl, 3′,4′-anhydroadenosylcobalamin; anhydroadenosyl radical, 5′-deoxy-3′,4′-anhydroadenosine-5′-yl; Cbl^{II}, cob(II)alamin; Cbl^{III}, cob(III)alamin; DDH, diol dehydrase; DEAE, diethylamino ethyl; 5′-deoxyadenosyl radical, 5′-deoxyadenosine-5′-yl; EDTA, ethylenediamine tetraacetic acid; EPR, electron paramagnetic resonance; ESI-MS, electrospray ionization mass spectrometry; H_2O−Cbl, aquocob(III)alamin; HPLC, high-performance liquid chromatography; IPTG, isopropyl thio-β-D-galactoside; KP_i, potassium phosphate; LC−MS, liquid chromatography−mass spectrometry; NADH, β-nicotinamide dinucleotide; NMR, nuclear magnetic resonance; OH−Cbl, hydroxocob(III)alamin; PMSF, phenylmethyl sulfonyl fluoride.

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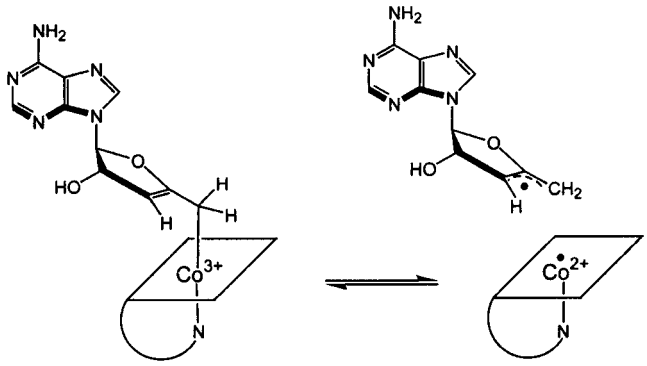
Scheme 2



possibly through its rigid structure, but minor modifications in the ribose can be tolerated, and both the 2′-deoxy and 3′-deoxy derivatives are functional coenzymes (*6*). The enzyme is also sensitive to the changes in the heterocyclic base, although slight alterations at the nitrogen atoms can be accommodated (*7*). The binding of 5′-deoxyadensoine and other related compounds to the apoenzyme has been demonstrated, and the enzyme was shown to be highly specific toward adenine nucleosides (*7*). Alterations in the corrin ring of the coenzyme, peripheral side chains, and nucleotide linker moiety have also been probed with varying effects, although the enzyme usually retains some or full activity in most of these cases (*8*, *9*).

Despite numerous structure−function studies, the mechanism of Co−C bond cleavage is poorly understood. The binding of substrate appears to trigger homolysis, and an appreciable amount of Cbl^{II} can be detected by either spectrophotometry or EPR spectroscopy. However, the enzyme undergoes slow irreversible cleavage of the coenzyme under aerobic conditions in the absence of substrate, leading to the formation of OH−Cbl, presumably due to O_2-mediated oxidation of Cbl^{II} (*10*). This observation suggests that Co−C bond cleavage can occur without substrate present and that the holoenzyme can be described in pre-equilibrium between intact and cleaved cofactor, which is shifted toward homolysis upon the binding of substrate.

We have previously described the synthesis and characterization of *an*AdoCbl, an analogue of AdoCbl designed to offer allylic stabilization of the 5′-deoxyadenosyl radical (Scheme 2) (*11*). The compound is unstable and decomposes rapidly at room temperature in the presence of oxygen or other radical scavengers (*11*). This property is attributed to a weak Co−C bond, and the bond dissociation energy is only ∼24 kcal mol^{-1} (*11*), which is ∼6 kcal mol^{-1} lower than has been measured for AdoCbl (*12*, *13*). The present study describes the interactions between DDH and *an*AdoCbl.

EXPERIMENTAL PROCEDURES

Materials. Sodium cholate, DEAE-Sephacel, AdoCbl, hydroxocobalamin, NADH, and yeast alcohol dehydrogenase were from Sigma. Deuterium oxide (99.9% D) and (*R*,*S*)-1,2-propanediol were from Aldrich. (*R*,*S*)-1,2-[$^2H_6$]propanediol (99.5% D) was obtained from CDN Isotopes. All other solvents, buffers, and chemicals were obtained either from Fisher or Aldrich and used as supplied. *an*AdoCbl and [ribose-$^{13}C_5$]*an*AdoCbl were synthesized as described elsewhere (*11*). The ^{13}C-labeled compound was prepared from a [ribose-$^{13}C_5$]*an*ATP precursor, the synthesis of which is described elsewhere (*14*).

DNA Expression and Protein Purification. The gene encoding DDH from *Salmonella typhimurium* was a generous gift from Dr. T. A. Bobik, University of Florida, Gainesville, FL. The cDNA for DDH in a pT7.7 vector was overexpressed in a BL21DE3 cell strain of *Escherichia coli*. Cell cultures were grown in shaker flasks in TB media containing 100 μg/mL of ampicillin until the absorbance at 600 nm reached 0.8−1.0, at which time the cells were induced by the addition of 0.5 mM IPTG. The cells were harvested 4 h later, frozen in liquid nitrogen, and stored at −70 °C. Approximately 50 g of wet cells were routinely obtained from 6 L cultures (12 × 500 mL).

All steps in the purification procedure were performed at 4 °C. Cell lysis was done by sonication, after suspending ∼50 g of the frozen cells in 180 mL of a 200 mM KP_i buffer (pH 8) containing 1% 1,2-propanediol, 1 mM EDTA, and 1 mM PMSF. The suspension was centrifuged at 160 000*g* for 40 min, after which <1% of the activity remained in the supernatant. The pellet was washed with the lysis buffer, and the suspension was sonicated and then centrifuged at 160 000*g* for 30 min. The pellet was resuspended in 120 mL of 10 mM KP_i (pH 8), 1 mM EDTA, 1% 1,2-propanediol, and 1% sodium cholate buffer; and the enzyme was extracted by gently stirring the suspension for 4 h. After ultracentrifugation, ∼90% of the enzyme activity was found in the supernatant fluid. The supernatant was diluted 4-fold with 10 mM KP_i (pH 8), 1% 1,2-propanediol, and 0.4% sodium cholate buffer and applied to a 2.5 × 30 cm DEAE-Sephacel anion exchange column that had been equilibrated in the same buffer. The enzyme was eluted from the column with a 2 L linear salt gradient (0−400 mM KCl), and fractions containing DDH were pooled based on absorbance at 278 nm and enzymatic activity. The purified protein was concentrated to ∼60 mg mL^{-1} by ultrafiltration using an Amicon concentrator with a 30 kDa molecular mass cutoff membrane. The protein was frozen in liquid nitrogen and stored at −70 °C. The purity of the enzyme was judged to be >90% on the basis of SDS−PAGE. The yields in terms of units of enzymatic activity ranged between 65% and 75%. Preparations of DDH exhibited specific activities of 35−40 IU mg^{-1} in the standard assay at 25 °C, where the protein concentration was measured using an extinction coefficient at 278 nm of 0.536 $(mg/mL)^{-1}$ (*15*).

In experiments with DDH and *an*AdoCbl, the enzyme was made anaerobic by repeated evacuation and flushing with O_2-free argon for at least 30 min, while the protein was kept on ice. Less than 10% activity loss was observed upon this treatment. Alternatively, the enzyme was dialyzed against 10 mM KP_i (pH 8), 1% 1,2-propanediol, and 0.5% sodium cholate buffer inside a Coy anaerobic chamber. The dialysis was performed in a double-wall beaker chilled with circulating icewater. This latter method was also used to prepare substrate-free enzyme, in which the buffer was devoid of 1,2-propanediol. Up to 20% loss in activity was measured after dialysis in substrate-free buffer. The anaerobic enzyme was frozen and stored in liquid nitrogen.

Enzyme Assays. Enzymatic activity of DDH was measured using a coupled assay with (*R*,*S*)-1,2-propanediol as the substrate in which the propionaldehyde was reduced by

NADH upon the action of alcohol dehydrogenase (*8*). Standard assays were performed in 50 mM KP_i (pH 8) buffer containing 1% 1,2-propanediol, 1% sodium cholate, 250 μM NADH, 20 μM AdoCbl, and 10 IU of alcohol dehydrogenase. The reactions were initiated by the appropriate amount of DDH, and the reaction progress was monitored by the decrease in absorbance at 340 nm at 25 °C. The initial rates were calculated from the linear portion of the progression curves using an extinction coefficient of 6.22 mM^{-1} cm^{-1} at 340 nm for NADH. The reactions were monitored with either a Hewlett-Packard Model 8102 diode array spectrophotometer equipped with a circulating temperature bath or a Shimadzu dual-beam spectrophotometer with a Peltier temperature controller. In assays with *an*AdoCbl as a coenzyme, buffer and assay reagents were prepared with anaerobic water that had been deoxygenated by purging with O_2-free argon. Kinetic isotope effects were measured with (*R*,*S*)-1,2-[$^2H_6$]propanediol as substrate.

UV−*Vis Spectrophotometry*. Anaerobic samples of DDH and *an*AdoCbl were prepared inside the anaerobic chamber. Samples in the presence of substrate contained 80 μM DDH, 50 μM *an*AdoCbl, and 125 mM 1,2-propanediol in a 50 mM KP_i and 0.5% sodium cholate buffer (pH 8). Samples without 1,2-propanediol were prepared with dialyzed DDH in the same manner except that a 20 min preincubation in the presence of 0.5 μM AdoCbl was performed before the addition of *an*AdoCbl to convert any residual substrate carried over with the enzyme solution to product. Samples were also made from reagents prepared in D_2O. The enzyme was exchanged into anaerobic D_2O buffer by repeated concentration and dilution using Centricon 30 spin concentrators that were fitted into sealed glass tubes to prevent exposure to air. The samples were estimated to have a 1:4 ratio of H_2O/D_2O. All samples were rapidly transferred to 100 μL microcuvettes fitted with a sealed Teflon stopper, and absorption spectra were recorded at different timepoints using a diode array spectrophotometer at 25 °C. Rates for Cbl^{II} formation or dissipation were fitted to either single or double exponential equations using Kaleidagraph (Synergy Software).

Product Analysis by HPLC and Mass Spectrometry. At the conclusion of the reactions described in the previous section, the products were analyzed by reversed-phase HPLC. First, the protein was precipitated by perchloric acid. The samples were centrifuged, and the clear supernatant was removed and neutralized with KOH. The precipitated $KClO_4$ was removed by centrifugation, and the samples were subjected to HPLC analysis. A C_{18} Bondapak (Waters) column (3.9 × 150 mm) was used with a Beckman Model 125 instrument. A H_2O/MeOH elution system containing 0.03% CH_3COOH was employed. A 30 min linear gradient (0−100% MeOH) was used with a flow rate of 0.8 mL/min and with detection at 260 nm.

Samples were also subjected to LC−MS analysis in order to characterize the reaction products. An Agilent 110D series HPLC−ESI-MS system equipped with an autosampler, online diode-array UV detector, and a single quadrupole mass detector was employed, using the same column, eluent, and flow conditions as described previously. Data were collected in the positive ion mode.

Analysis by NMR Spectroscopy. Samples of the nucleoside product for NMR analysis were prepared by mixing 80 μM DDH and 70 μM *an*AdoCbl in a total volume of 12 mL in the absence of dioxygen. The anaerobic reaction progress was monitored by spectrophotometry, as described previously. After the partial removal of protein by TCA precipitation and neutralization with KOH, the remaining protein was separated from smaller molecules by ultrafiltration using Centriprep 10 (Amicon). The filtrate was concentrated by rotary evaporation, and the nucleoside product was purified by reversed-phase HPLC. A semipreperative C_{18} column (Phenomenex; 250 × 10 mm) with a linear gradient of 10−80% MeOH containing 5 mM sodium acetate (pH 4.8) in 25 min was employed. The desired nucleoside product emerged in 21 min under these conditions, and the pooled fractions were lyophilized and resuspended in DMSO-d_6. The following ^1H NMR spectrum was obtained with a 400 MHz Bruker instrument: (δ) 8.26, 8.15 (s, adenine H-2 and H-8), 7.34 (s, NH_2), 6.15 (d, 1′-H, $J_{1',2'} = 3.5$ Hz), 4.86 (dt, 2′-H, $J_{2',3'a} = 7$ Hz, $J_{2',3'} = 3.5$ Hz, $J_{2',1'} = 3.5$ Hz), 4.22 (d, 5′-H_a, $J_{gem} = 1.5$ Hz), 3.97 (d, 5′-H_b, $J_{gem} = 1.5$ Hz).

RESULTS

Purification of DDH. DDH from *S. typhimurium* was overproduced in *E. coli* and purified by a modification of a method for DDH from *Klebsiella oxytoca* (*16*). The enzyme from *S. typhimurium* shares 98% sequence identity with the *K. oxytoca* enzyme (*17*). The procedure takes advantage of the peripheral membrane association of the enzyme, which provides a convenient method of separating DDH from soluble cytosolic proteins. Extraction of membranes with sodium cholate solubilizes the protein, and the detergent is required to prevent aggregation in subsequent purification steps and all handling of the enzyme. Addition of 1,2-propanediol in purification buffers is also required to prevent dissociation of the hexameric $\alpha_2\beta_2\gamma_2$ structure, a treatment that is also required for DDH from other organisms. The purified protein exhibits a specific activity of 35−40 IU mg^{-1} at 25 °C, which is similar to that reported for the overproduced enzyme from *K. oxytoca* when differences in the assay conditions are taken into consideration (90 IU mg^{-1} at 37 °C) (*16*).

Activation of DDH by anAdoCbl. A progression curve for the reaction of DDH with *an*AdoCbl as coenzyme using the coupled assay with alcohol dehydrogenase is shown in Figure 1. As evidenced by the decrease in absorbance at 340 nm due to NADH oxidation, the enzyme is turning over the substrate, demonstrating that *an*AdoCbl is a functional coenzyme for DDH. Control experiments without the addition of either DDH or *an*AdoCbl showed a decrease in absorbance at 340 nm of less than 0.01 OD after 30 min (data not shown). The initial rate was determined from the linear portion of the curve (6−8 min), which yielded a

[2] This study made use of the National Magnetic Resonance Facility at Madison, which is supported by NIH Grant RR02301 from the Biomedical Research Technology Program, National Center for Research Resources. Equipment in the facility was purchased with funds from the University of Wisconsin, the NSF Biological Instrumentation Program (DMB 8415048), NSF Academic Research Instrumentation Program (BIR-9214394), NIH Biomedical Research Technology Program (RR02301), NIH Shared Instrumentation Program (RR02781 and RR02301), and the U.S. Department of Agriculture.

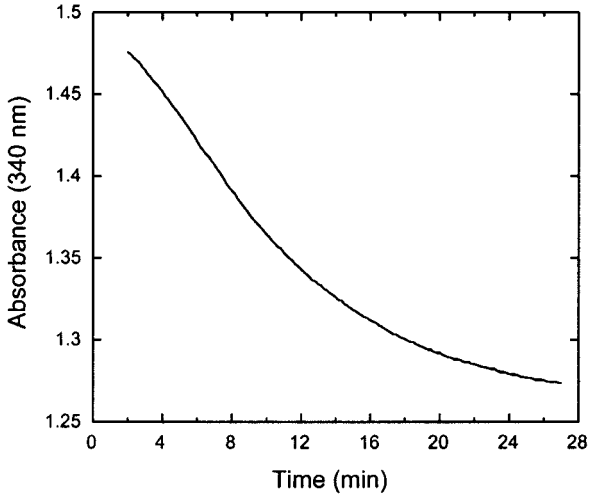


FIGURE 1: Activation of DDH with *an*AdoCbl. 1,2-Propanediol is the substrate at 25 °C. The coupled assay is based on reduction of the product, propionaldehyde, to 1-propanol catalyzed by alcohol dehydrogenase. The concomitant oxidation of NADH is monitored by the decrease in absorbance at 340 nm. The composition of the standard assay solution is given in the Experimental Procedures. No rate was observed in the absence of either DDH or *an*AdoCbl.

specific activity at 25 °C of $(5 \pm 1) \times 10^{-3}$ IU mg^{-1}. This activity corresponds to ~0.017% of that measured with AdoCbl as coenzyme (29 IU mg^{-1}) using the same enzyme preparation. Note the progressive decrease in activity that starts at about 10 min. This observation is not due to product inhibition or to the depletion of either 1,2-propanediol or NADH because a similar timecourse with AdoCbl as coenzyme is linear within the same absorbance range (data not shown). This inactivation process is due to a slow oxidation of Cbl^{II} to Cbl^{III} during the catalytic cycle of the enzyme, as will be demonstrated in the following sections. Diol dehydrase could not be reactivated by addition of AdoCbl, which shows that the cobalamin species remains tightly bound to the enzyme as is the case for a number of other coenzyme analogues that lead to the inactivation of DDH (*18*).

Because the preparation of *an*AdoCbl is not perfectly pure (*11*), a point of concern is the possibility that the low activity may be due to AdoCbl as a trace contaminant, although the kinetic behavior described previously would argue against that. This issue was clarified by incubating a sample of *an*AdoCbl in the dark at room temperature for 3 h after exposure to air. Authentic AdoCbl and other known active analogues are stable under these conditions, whereas *an*AdoCbl undergoes thermal degradation because of irreversible cleavage of the Co−C bond (*11*). No enzymatic activity could be detected after this treatment, proving that the observed activity could not have been due to AdoCbl but must have been due to the action of *an*AdoCbl as a coenzyme.

Kinetic Isotope Effects. Reaction rates were measured with (*R*,*S*)-1,2-[$^2H_6$]propanediol as the substrate. An isotope effect, $^DV = 13.6 \pm 0.5$, was determined with AdoCbl as the coenzyme. The reaction with *an*AdoCbl as the coenzyme was too slow to obtain rates with deuterated substrate, owing to the competing inactivation reaction.

Cleavage of the Co−C Bond and Oxidation of Cbl^{II}. Experiments with DDH and *an*AdoCbl in the absence of

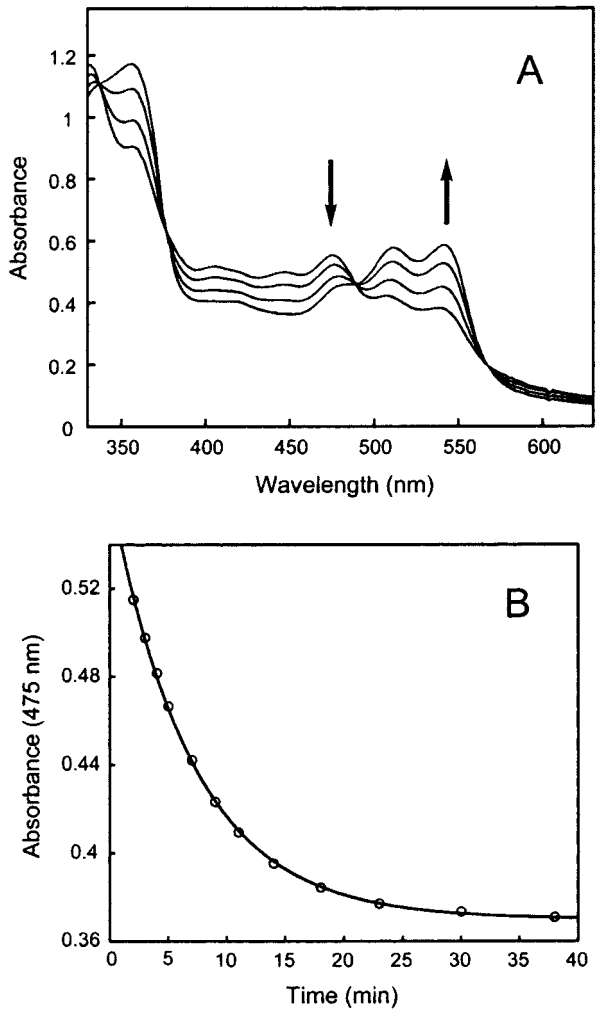


FIGURE 2: Reaction of DDH and *an*AdoCbl in the absence of 1,2-propanediol. (A) Representative UV−vis spectra measured at 2, 4, 7, and 11 min after mixing in an anaerobic chamber, respectively. The decrease in absorbance at 475 nm (arrow) is accompanied by increase in absorbance at 542 nm (arrow). (B) Single exponential fit to the decrease in absorbance at 475 nm, which gave $k_{obs} = (0.143 \pm 0.002)$ min^{-1}.

substrate show that the Co−C bond undergoes homolysis, as evidenced by the absorption spectra of the holoenzyme (Figure 2A), in particular the band at 475 nm that is a signature for Cbl^{II} formation (*19*). Prolonged incubation, however, leads to dissipation of this band and the formation of a new chromophore that has the spectral characteristics of Cbl^{III}. The characteristic γ band at 358 nm and the overall appearance of the spectra suggest that this new chromophore represents enzyme bound OH−Cbl and rules out an alkylcobalamin species (*19*). The formation of Cbl^{III} occurs without the buildup of another intermediate, as is evident by the clean isosbestic points at 337 nm, 377, 490, and 567 nm, respectively. The process can be fitted to a single exponential equation with the first-order rate constant $k_{obs} = 0.143 \pm 0.002$ min^{-1} at 25 °C, as measured by the decrease in absorbance at 475 nm (Figure 2B). The formation of Cbl^{II} was complete before the first scan was recorded. Because the sample was prepared within an anaerobic chamber and had to be transferred to the spectrophotometer for data acquisition, timepoints within 2 min could not be obtained in this study. The rate of formation of Cbl^{II} could, therefore, not be obtained.

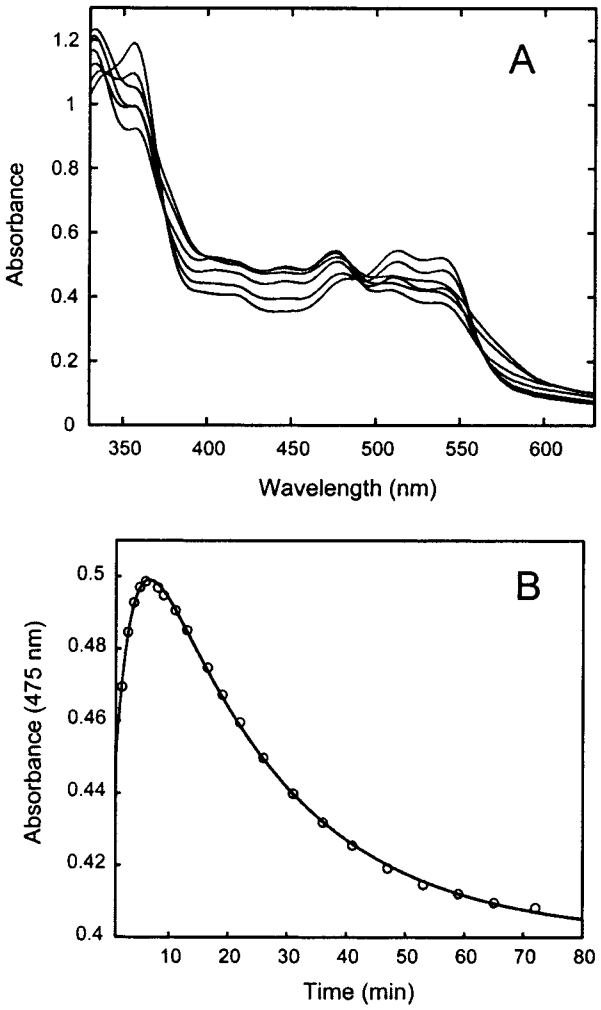


FIGURE 3: Reaction of DDH and *an*AdoCbl in the presence of 1,2-propanediol. (A) Representative UV−vis spectra obtained after 2, 3, 8, 19, 36, and 72 min, respectively. The lack of isosbestic points demonstrates the inclusion of more than two chromophores. (B) A double exponential fit to the change in absorbance at 475 nm. First-order rate constants, $k_{obs} = (0.33 \pm 0.02)$ min^{-1} and $k_{obs} = (0.043 \pm 0.001)$ min^{-1} were obtained.

When DDH is incubated with *an*AdoCbl in the presence of the substrate 1,2-propanediol, formation of Cbl^{II} is also apparent followed by oxidation to Cbl^{III}. This process, however, displays different kinetic behavior than that seen in the absence of substrate, and it appears to be biphasic (Figure 3A). The data can be fitted to two sequential first-order processes (Figure 3B), where buildup of Cbl^{II} with a rate constant of $k_{obs} = 0.33 \pm 0.02$ min^{-1} is followed by the slower formation of Cbl^{III} with a rate constant of $k_{obs} = 0.043 \pm 0.001$ min^{-1} at 25°.

Identification of Nucleoside Products. The UV−vis spectra discussed previously show that one-electron oxidation of Cbl^{II} occurs in samples of DDH and *an*AdoCbl, both in the presence and absence of substrate. The fate of the other homolysis partner during this process, the anhydroadenosyl radical, was investigated by analysis and characterization of the reaction mixtures by HPLC, LC−ESI-MS, and NMR spectroscopy.

After the removal of the protein by acid precipitation, the spectrophotometric analysis of products separated by HPLC revealed two prominent peaks absorbing at 260 nm, with elution times of 13 and 20 min, respectively (data not shown). The chromophore that emerged at 13 min was shown to be Cbl^{III} (H_2O−Cbl) by coelution with authentic material and by its absorption spectrum obtained by use of an HPLC instrument with a diode-array detector. The compound that emerged at 20 min displayed a single, broad absorption band centered at 260 nm, indicative of an adenosine-like compound. By using the extinction coefficients ($\epsilon_{260\ nm}$) for H_2O−Cbl (20 500 M^{-1} cm^{-1}) and adenosine (14 700 M^{-1} cm^{-1}), a stoichiometry of 1:1 between these two compounds was established. The data, therefore, show that the formation of these two compounds is related and that the material emerging at 20 min is derived from the upper axial ligand of *an*AdoCbl. Control experiments with free *an*AdoCbl, oxidized in aerobic solution, yielded H_2O−Cbl and a few other peaks that absorbed at 260 nm. The peak at 20 min was absent; instead, the most intense peak emerged after 16 min and had the spectral characteristics of an adenosine-like compound.

Mass spectral analysis by LC−ESI-MS confirmed that the nucleoside product is an adenosine derivative. A prominent ion at $m/z = 234.1$ (Figure 4A) corresponds to the mass of either 3′,5′-dideoxyadenosine-3′,4′-ene (**a**) or 3′,5′-dideoxyadenosine-4′,5′-ene (**b**) in Scheme 3, respectively. Performance of the reaction in ∼80% D_2O (Figure 4B) shifts the mass by one unit ($m/z = 235.1$), which shows that a single solvent exchangeable proton/deuteron is incorporated into the molecule. The peak at $m/z = 234.1$ that is also seen in the spectrum of Figure 4B represents molecules quenched by residual H_2O present in the sample. Other minor peaks in both spectra were not identified specifically. The spectrophotometric and mass spectrometric results suggest that the anhydroadenosyl radical formed upon homolysis of *an*AdoCbl oxidizes Cbl^{II} by one electron, leading to the transient formation of an allylic anion that is subsequently quenched by solvent or a solvent exchangeable site on the protein.

The ^{1}H NMR spectrum proves the selective protonation at the 3′ position, yielding 3′,5′-dideoxyadenosine-4′,5′-ene (**b** in Scheme 3). Although the transitions for the 3′ hydrogens did not appear in the spectrum, owing to overlap with an intense peak for HOD at ∼3.4 ppm, the coupling information obtained from 2′-H (see Experimental Procedures) provides information concerning the identity of the 3′ hydrogens and supports the structural assignment.

The compound with the retention time of 16 min in the control experiment with free *an*AdoCbl (no DDH) gave $[M + H]^+ = 266.1$, which corresponds to the mass of the hydrated form of 3′,4′-anhydroadenosine-5′-carboxyaldehyde (**c** in Scheme 3). The formation of a 5′-aldehyde is analogous to what occurs upon aerobic photolysis or thermolysis of AdoCbl (*20*).

DISCUSSION

Reactivity of DDH with anAdoCbl. The data presented in this paper show a much lower catalytic activity of DDH with *an*AdoCbl as coenzyme compared to that with AdoCbl. The overall reaction with AdoCbl as coenzyme displays a kinetic isotope effect on V_{max} of 13.6 ± 0.5, showing that a hydrogen

[3] The pK_a for H_2O−Cbl is 7.8. OH−Cbl and H_2O−Cbl in aqueous solution have different absorption spectra.

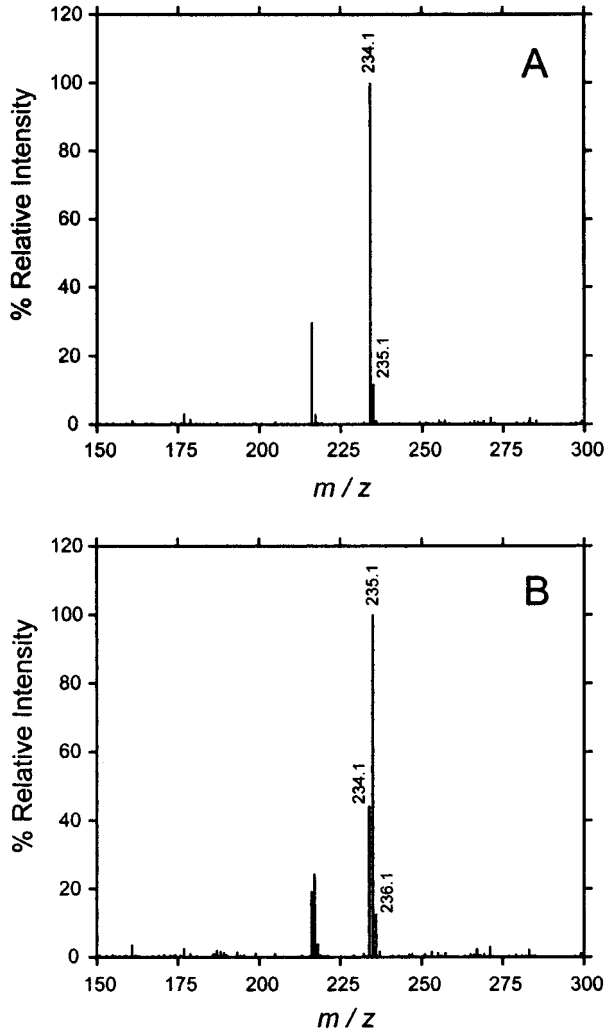
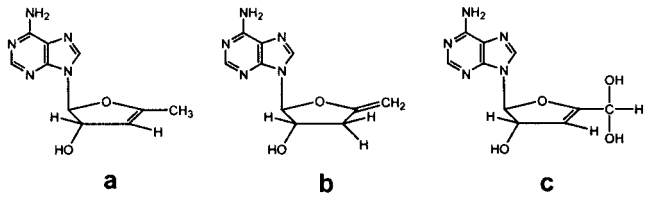


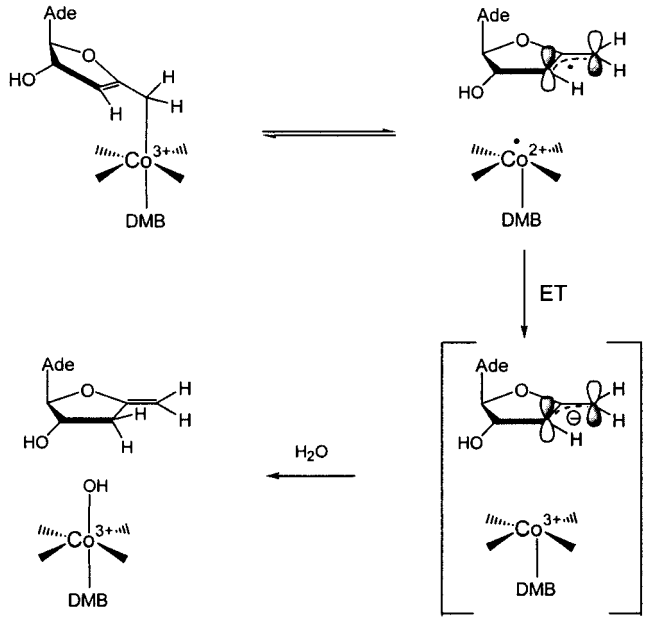
FIGURE 4: ESI-MS of a nucleoside product formed upon the inactivation of DDH in the presence of *an*AdoCbl. The compound eluted after 20 min from a reversed-phase HPLC column under the conditions described in Experimental Procedures. (A) Spectrum obtained from sample in a H_2O buffer. (B) Spectrum obtained from sample in an ~80% D_2O buffer.

Scheme 3



transfer step is at least partially rate-limiting. A kinetic isotope effect of 12 on V_{max} was reported in the reaction of (*R*)-[(*R*)-1-^{2}H]propanediol (*21*). Which step(s) contribute mainly to rate limitation has not been elucidated. However, given that a substrate-derived radical builds up in the steady-state of the reaction (*22*) and that all of the coenzyme builds up as Cbl^{II} in the steady-state (*10*), a reabstraction of a hydrogen atom from 5′-deoxyadenosine (**b** in Scheme 1) seems likely to limit the rate. A kinetic isotope effect could not be accurately determined with *an*AdoCbl as coenzyme, because the initial rate was so slow with deuterated substrate that the competing inactivation process became problematic. The isotope effect can be estimated to be >10, on the basis of the limited amount of data.

Scheme 4



A slow hydrogen-atom abstraction step by the anhydroadenosyl radical from the substrate can account for the lower enzymatic activity of DDH when *an*AdoCbl serves as the coenzyme, a view that is supported by an appreciable kinetic isotope effect. The decrease in reaction rate can arise from the stability of the allylic anhydroadenosyl radical formed upon Co−C bond homolysis. The lower rate is quantitatively in accord with the interpretation as follows. The DDH activity is lowered by 5900-fold by the substitution of *an*AdoCbl for AdoCbl. This corresponds to an increase in activation energy for H transfer of 5.1 kcal mol^{-1}. The Co−C5′ bond in *an*AdoCbl is weaker than that in AdoCbl by about 6 kcal mol^{-1}, owing to the stability of the anhydroadenosyl radical relative to the 5′-deoxyadenosyl radical (*11*). The 6 kcal mol^{-1} greater stability of the anhydroadenosyl radical would make it correspondingly less reactive as a hydrogen abstracting agent. Allylic stabilization can provide ~11 kcal mol^{-1} in energy, as reflected in the difference in bond dissociation energy between a general alkyl C−H bond and an allylic C−H bond (*23*). The full effect is not observed for *an*AdoCbl, presumably because of the strain imposed on the five-membered cyclic anhydroribosyl moiety (*11*).

Electron Transfer and Inactivation of DDH. A mechanism for the inactivation of DDH caused by interaction with *an*AdoCbl is presented in Scheme 4. Absorption spectra show that the Co−C bond of the enzyme bound coenzyme undergoes homolytic scission to produce Cbl^{II}. Subsequent one-electron oxidation of Cbl^{II} is apparent upon prolonged incubation, both in the absence and presence of substrate. Reaction progress curves also show that the enzyme undergoes inactivation at a rate similar to that of Cbl^{III} formation. The observed Cbl^{III} species is most likely OH−Cbl on the basis of the spectral characteristics, especially the appearance of the γ band at 358 nm. Also, a bathochromic shift of λ_{max} for the α band of free OH−Cbl from 535 to 542 nm is in accordance with the spectrum of OH−Cbl bound to DDH (*24*).

The rate of Cbl^{II} formation is different in the absence or presence of substrate. Complete cleavage of the Co−C bond

has occurred before the first recorded timepoint in the absence of substrate. However, in the presence of 1,2-propanediol, the appearance of Cbl^{II} is slower, and an apparent rate constant of 0.33 min^{-1} is obtained (Figure 3B). Apparently, the interactions in the enzyme−coenzyme−substrate complex result in reduced rate of cleavage of the Co−C bond as compared to the enzyme−coenzyme complex. This observation is surprising, because no net formation of Cbl^{II} can be observed with AdoCbl as coenzyme in the absence of substrate (*18*). However, with saturating substrate, Cbl^{II} builds up to a steady-state level during the progress of the reaction with AdoCbl as coenzyme (*18*).

For AdoCbl-dependent enzymes such as glutamate mutase (*25*), methylmalonyl-CoA mutase (*26*), and ethanolamine ammonia lyase (*27*), the Co−C bond cleavage is kinetically coupled with hydrogen atom abstraction from the substrate, as evidenced by a substrate-dependent kinetic isotope effect on the rate of Cbl^{II} formation. Experiments such as these demonstrate that the 5′-deoxyadenosyl radical does not buildup to a significant amount during catalysis by these enzymes. Analogous experiments have not been performed with DDH. None of the experiments has, however, addressed whether Co−C bond cleavage occurs in the absence of substrate. That is, a rapid pre-equilibrium could exist strongly in favor of the intact coenzyme, which would not lead to a measurable formation of Cbl^{II}. The presence of substrate would then serve to shift the equilibrium toward homolysis. The finding that *an*AdoCbl can be cleaved by DDH in the absence of substrate suggests that substrate binding is not providing much energy for inducing Co−C bond homolysis. The observation of slower Co−C bond homolysis of *an*AdoCbl implies that substrate slightly impedes cleavage of the Co−C bond in *an*AdoCbl. This does not mean, however, that substrate would exert the same effect with AdoCbl. The coenzyme and the analogue are different and may interact differently with DDH.

Product analyses by LC−ESI-MS in conjunction with experiments performed in D_2O show that the anhydroadenosyl radical formed upon Co−C bond homolysis serves as an oxidizing agent. The resulting allylic anion suffers protonation by a solvent molecule or a solvent exchangeable site on the protein. The site of protonation was established by NMR characterization of the product and shown to be at the 3′ position, yielding 3′,5′-dideoxyadenosine-4′,5′-ene. Selective protonation at C3′ can be attributed, at least partly, to higher reactivity at a secondary position as compared to a primary carbon for a carbanion. Alternatively, a suitable proton donor from an enzyme side chain could be more accessible to C3′ than to C5′. According to Scheme 4, a single water molecule is involved in the inactivation process to produce HO−Cbl. The remaining proton could potentially react with the allylic anion, either directly or through a proton relay system.

Why electron transfer occurs between Cbl^{II} and the anhydroadenosyl radical is an interesting question. The redox potential in solution for the $Cbl^{II} + H_2O \rightarrow OH{-}Cbl^{III} + H^+ + e^-$ reaction at pH 8 is −0.18 (*28*), which shows that oxidation of Cbl^{II} is unfavorable. However, if this process is directly coupled to the reduction of the radical ($R\bullet + H^+ + e^- \rightarrow R{-}H$), which has a reduction potential that is at least greater than +0.5 V, the overall reaction is thermodynamically favorable. Electron transfer does not occur during the reaction with AdoCbl, presumably because the 5′-deoxyadenosyl radical is a transiently formed species which reacts quickly with 1,2-propanediol and does not accumulate during steady-state turnover. With *an*AdoCbl, the main complex that is formed either in the absence or presence of substrate is that of Cbl^{II} and the allylic anhydroadenosyl radical. These two paramagnets have been shown to be separated by less than 4 Å and are, therefore, ideally primed for electron transfer (Magnusson et al., manuscript in preparation). A precedent for electron transfer is the analogous reaction recently reported of the AdoCbl-dependent enzyme lysine 5,6-aminomutase. This enzyme undergoes rapid, substrate-induced inactivation by a mechanism that involves one-electron oxidation of Cbl^{II} by a substrate-derived radical (*29*).

A substrate-derived radical builds up in the steady state to approximately 75% of the active sites of DDH (*30*). Although this radical has not been structurally characterized, the distance separating the radical from Co(II) has been estimated to be ∼10 Å (*31*). The crystal structure of DDH has recently become available (*5*). In this structure, which has cyanocob(III)alamin bound in lieu of AdoCbl, the distance between Co and C1 and C2 of (*S*)-1,2-propanediol are 8.4 and 9.0 Å, respectively. It is, therefore, likely that rearrangement of substrate radicals occur distant from the cobalt. This may in fact be advantageous, because it would diminish the possibility of electron transfer between substrate radicals and Cbl^{II}. It is tempting to speculate that such an active site structure has evolved in response to the detrimental effects of electron transfer.

It should, however, be pointed out that diol dehydrase does undergo inactivation with various substrates (*18*). In fact, glycerol is a better substrate for the DDH than 1,2-propanediol, but the former also causes rapid inactivation of the enzyme, which results in the formation of an alkylcobalamin or a thiolcobalamin species, although a thorough characterization has not been conducted (*24*). Other good substrates such as 1,2-ethanediol also function as inactivators of DDH (*18*). Whether electron transfer is involved in any of these inactivation processes is not known, but presumably the nature of the reactions will vary. A different example is the inactivation by glycolaldehyde, which results in the formation of Cbl^{II} (*10*). This is a mechanism-based inactivation where the formation of a stable semidione radical terminates the reaction and leaves the enzyme trapped (*32*). In this case, an electron transfer does not occur, possibly because the distance of ∼11 Å between Co(II) and the radical is too great for that to happen.

Rétey has introduced the term of "negative catalysis", which postulates that enzyme-bound radical intermediates can take reaction pathways that would not be expected on the basis of solution-based chemistry and that the role of the protein is mainly to prevent side reactions (*33*). The study described in this paper provides an example of how an enzyme-catalyzed radical reaction can go wrong. In this case, a functional change in the coenzyme is responsible, but in other instances, the use of alternative substrates or substrate analogues can lead to enzyme inactivation. A third possibility would be through the mutation of important amino acid residues, a case that has recently been reported for the AdoCbl-dependent enzyme methylmalonyl-CoA mutase (*34*).

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