Dioldehydrase: An Essential Role for Potassium Ion in the Homolytic Cleavage of the Cobalt−Carbon Bond in Adenosylcobalamin†

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Received January 15, 2007; Revised Manuscript Received March 28, 2007

ABSTRACT: The complex of dioldehydrase with adenosylcobalamin (coenzyme B_{12}) and potassium ion reacts with molecular oxygen in the absence of a substrate to oxidize coenzyme and inactivate the complex. In this article, high performance liquid chromatography and mass spectral analysis are used to identify the nucleoside products resulting from oxygen inactivation. The product profile indicates that oxygen inactivation proceeds by direct reaction of molecular oxygen with the 5′-deoxyadenosyl radical and cob(II)alamin. Formation of 5′-peroxyadenosine as the initial nucleoside product chemically correlates this reaction with aerobic, aqueous photoinduced homolytic cleavage of adenosylcobalamin (Schwartz, P. A., and Frey, P. A., (2007) *Biochemistry*, in press), indicating that both reactions proceed through similar chemical intermediates. The oxygen inactivation of the enzyme−coenzyme complex shows an absolute requirement for the same monocations required in catalysis by dioldehydrase. Measurements of the dissociation constants for adenosylcobalamin from potassium-free ($K_d = 16 \pm 2$ μM) or potassium-bound dioldehydrase ($K_d = 0.8 \pm 0.2$ μM) reveal that the effect of the monocation in stimulating oxygen sensitivity cannot be explained by an effect on the binding of coenzyme to the enzyme. Cross-linking experiments suggest that the full quaternary structure is assembled in the absence of potassium ion under the experimental conditions. The results indicate that dioldehydrase likely harvests the binding energy of the activating monocation to stimulate the homolytic cleavage of the Co−C5′ bond in adenosylcobalamin.

Dioldehydrase (DL-1,2-propanediol hydro-lyase, EC 4.2.1.28) catalyzes the adenosylcobalamin (5′-deoxyadenosylcobalamin[1])-dependent dehydration of 1,2-propanediol to propionaldehyde (*1*). Similar to the reaction of other adenosylcobalamin-dependent enzymes, the reaction involves a hydrogen abstraction from substrate by the 5′-deoxyadenosyl radical generated through homolytic cleavage of the Co−C5′ bond in the coenzyme (Figure 1) (*2*). After substrate-radical generation by hydrogen abstraction, isomerization proceeds through the 1,2-migration of the hydroxyl moiety to the terminal carbon, where a hydroxyl group is subsequently eliminated to form the aldehyde. A minimal mechanism for the reaction of dioldehydrase with 1,2-propanediol is illustrated in Scheme 1.

Dioldehydrase is a large enzyme comprising six subunits (molecular mass ∼207,000 Da) (*3*) and exists as a dimer of heterotrimers $(\alpha\beta\gamma)_2$ (*4*). Contained within the α-subunit is an $(\alpha/\beta)_8$ β-barrel that houses the active site. Enzymatic activity of dioldehydrase absolutely requires the presence of a monocation with an ionic radius approximately that of potassium ion (*5*). These cations include K^+, Tl^+, and NH_4^+ and are hereafter referred to as activating monocations.

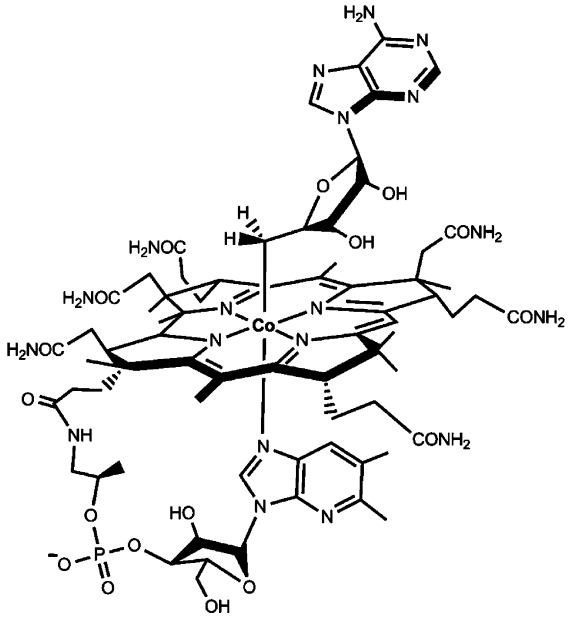

FIGURE 1: Structure of 5′-deoxyadenosylcobalamin.

Adenosylcobalamin supports the catalytic activity of a variety of enzymes through unique properties of the Co−C5′ bond. The Co−C5′ bond contains high covalent character, with a bond dissociation energy of 30−33 kcal mol^{-1},

† This research was supported by NIH Grant DK28607 from the National Institute of Diabetes and Digestive and Kidney Diseases.

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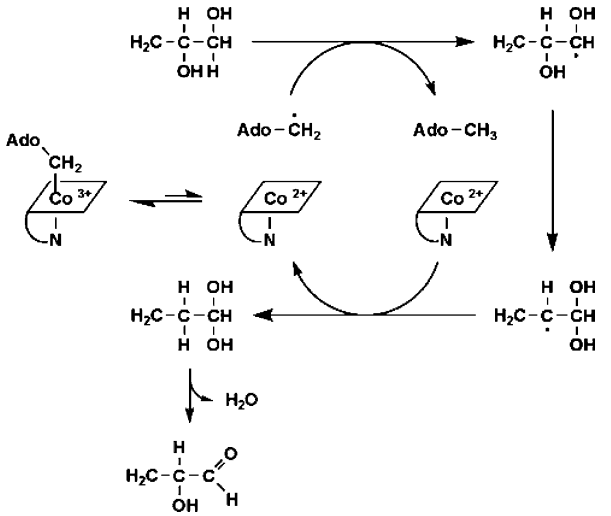
[1] Abbreviations: adenosylcobalamin, 5′-deoxyadenosylcobalamin; cob(III)alamin, hydroxocobalamin and/or aquocobalamin; 5′-deoxyadenosyl radical, 5′-deoxyadenosine-5′-yl; DSS, disuccinyl suberate; EPPS, *N*-[2-hydroxyethyl]piperazine-*N*′-3-propanesulfonic acid; ESI, electrospray ionization; FOX assay, ferrous oxidation in xylenol orange assay; HPLC, high performance liquid chromatography; K_d, equilibrium dissociation constant; LC, liquid chromatography; MS, mass spectrometry; NADH, reduced β-nicotinamide adenine dinucleotide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TOF, time-of-flight; TCA, trichloroacetic acid.

10.1021/bi700078z CCC: $37.00
Published on Web 05/22/2007

Scheme 1



and at 25 °C, it is stable in aqueous solution, exhibiting a slow rate of thermolysis ($t_{1/2}$ = 22 years) (*6*−*10*).

Adenosylcobalamin undergoes a marked change in its chemical properties when associated with dioldehydrase. In order to become catalytically competent, the Co−C5′ bond must be activated by shifting the equilibrium of the unreactive, covalently associated species toward cob(II)alamin and the 5′-deoxyadenosyl radical, the products of homolytic cleavage (Scheme 1) (*2*, *11*, *12*). This activation must account for a rate enhancement in thermolysis of approximately 10^{12} for the fully active complex (*7*, *13*). The mechanisms employed by adenosylcobalamin-dependent enzymes to achieve this rate enhancement are poorly understood and are a subject of ongoing research.

In the case of dioldehydrase, a clue to this strategy might be revealed by a side reaction of the enzyme-bound coenzyme with molecular oxygen. Dioldehydrase gradually loses activity in the absence of substrate through oxidation of the coenzyme (*1*, *14*). Oxidation leads to the formation of cob(III)alamin at the active site, a tight binding coenzyme analogue and inhibitor of dioldehydrase (*1*, *5*, *15*), and it requires O_2 (Figure 2) (*14*, *1*). It is postulated that oxygen sensitivity is a direct result of structural modifications to the coenzyme brought about by association with the enzyme, leading to the activated coenzyme (*1*). Interception of the activated coenzyme by oxygen leads to cob(III)alamin and products.

The results presented herein demonstrate that the oxidative inactivation of dioldehydrase by air results from the interception of the activated coenzyme by molecular oxygen. Furthermore, an activating monocation is required for oxygen inactivation, and it plays a key role in the activation of adenosylcobalamin in the dioldehydrase system.

MATERIALS AND METHODS

Materials. 1,2-propanediol, *N*,*N*′-(1,4-phenylenedimethylidyne)di-*o*-anisidine, and *N*-methylpiperidine were from Aldrich. EPPS buffer, Sephadex G-25, adenosylcobalamin, catalase, horseradish peroxidase, and cholic acid were from Sigma. Disuccinimidyl suberate, dialysis cassettes (0.5 mL Slide-A-Lyzer), and FOX assay reagents (Peroxoquant kit) were from Pierce. Adenosine related nucleosides resulting from the photolysis of adenosylcobalamin were prepared as

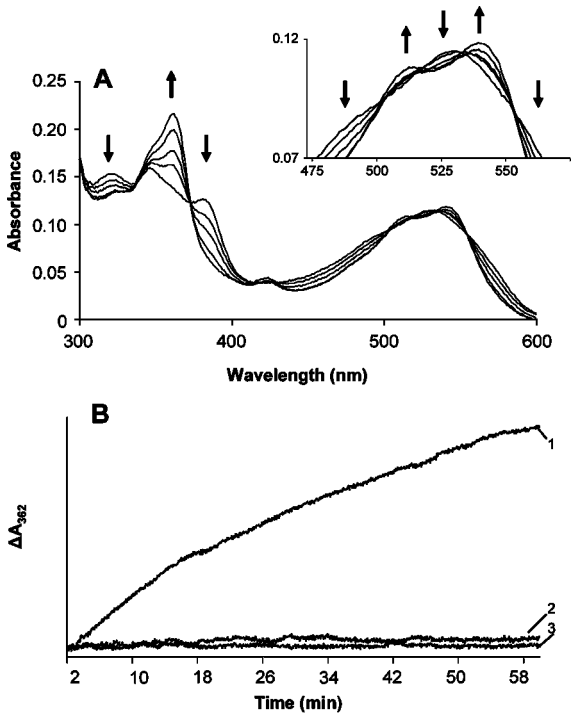


FIGURE 2: Time-dependent changes in the absorption spectrum of dioldehydrase−adenosylcobalamin complex. (A) Absorption spectra of aerobic dioldehydrase incubated with 10 mM potassium acetate and 13 μM adenosylcobalamin from 2 to 58 min. (B) Absorbance change at 362 nm for (1) the experiment in panel A and analogous experiments performed in the absence of (2) O_2 and (3) activating monocations.

described elsewhere (*16*). Laemmli SDS−PAGE sample buffer and high range molecular weight markers for SDS−PAGE were from Bio-Rad. Broad range molecular weight markers for SDS−PAGE were from New England Biolabs. Spin filters (YM-25, Centriprep) were from Millipore. The column used in HPLC analysis was filled with Altima-HP C_{18} reverse phase resin, purchased pre-packed from Alltech. The column used in LC/MS analysis was filled with Inertsil C_{18} reverse phase resin (GL Sciences), packed in-house.

The genes encoding the α-, β-, and γ-subunits of dioldehydrase in *Salmonella typhimurium* (*3*) were subcloned in the expression vector pT7.7 (*17*) and overexpressed in *E. coli*. The genes were a generous gift from Dr. T. A. Bobik, University of Florida, Gainesville, FL. The apoenzyme was purified as described elsewhere (*18*) and concentrated by ultrafiltration to 60 mg mL^{-1} as the stock enzyme, which displayed a specific activity of 40 U mg^{-1} at 25 °C.

Analytical Methods. Dioldehydrase activity was assayed by measurement of the rate of propionaldehyde formation in the presence of NADH and alcohol dehydrogenase as described (*19*). Nucleoside products were separated by HPLC and quantified as described (*16*). Dioldehydrase concentration was measured spectrophotometrically by using an extinction coefficient of 0.527 mL mg^{-1} at 278 nm.

Mass spectral analysis of nucleosides was performed using an Agilent LC/MSD ESI-TOF spectrometer in the positive ion mode. A reaction sample for mass spectrometry was prepared as described (see Nucleoside Products Derived from the Oxidation of Dioldehydrase-Bound Adenosylcobalamin) and separated in-line on a 2.1 × 200 mm Inertsil C_{18} reverse phase column. Separation was performed isocratically at 95%

H_2O/5% CH_3CN. MS/MS analysis of tryptic peptides was performed on an Applied Biosystems 4800 matrix-assisted laser desorption/ionization time-of-flight-time-of-flight (MALDI TOF-TOF) mass spectrometer. Mass spectrometry was performed at the Mass Spectrometry Facility (Biotechnology Center, University of Wisconsin—Madison).

Absorption spectra were obtained on a Cary 50 UV/Vis spectrophotometer. Potassium analysis was performed at the Wisconsin State Laboratory of Hygiene by atomic absorption spectrophotometry.

Assay for Reaction of Oxygen with the Dioldehydrase−Adenosylcobalamin Complex. The progress of adenosylcobalamin oxidation at the active site of dioldehydrase was spectrophotometrically monitored. Stock dioldehydrase (250 μL, 60 mg mL^{-1}) was thawed on ice and inserted into a dialysis cassette, where the sample was dialyzed for 1 h against 300 mL of 20 mM *N*-methylpiperidinium-EPPS, 120 μM 1,2-propanediol, and 1 mM KCl at pH 8.0. The enzyme sample was removed from the dialysis cassette, diluted to 20 mg mL^{-1} with 20 mM *N*-methylpiperidinium-EPPS at pH 8.0 (column buffer), and immediately applied to a column (1 cm in diameter) containing 20 mL of Sephadex G-25 equilibrated with column buffer. Gel filtration proceeded at approximately 0.5 mL min^{-1}. Enzyme was collected and diluted to 12 μM (24 μM active sites). Rates in O_2-saturated solutions were pseudo first order in enzyme, with 12 μM enzyme and 0.5 mM O_2.

A stock sample of 20% cholic acid was prepared and brought to pH 8.0 with *N*-methylpiperidine, and appropriate amounts of either K^+- or NH_4^+-acetate at 1−20 mM were prepared in column buffer. In an Eppendorf tube, 400 μL of dioldehydrase and 23 μL of 20% *N*-methylpiperidinium-cholate (25 mM final) were added and mixed with thorough pipetting. To this mixture was added 10 μL of either column buffer or column buffer augmented K^+- or NH_4^+-acetate. Thorough mixing preceded transfer to a cuvette, where the baseline absorption spectrum was obtained. The assay was started with the addition of 5.5 μL of 1 mM adenosylcobalamin. The absorption spectrum from 250 to 600 nM was acquired every 10 s for 1 h. All steps of the protocol were performed in strictly dark conditions. In practice, the concentration of activating monocations was held below 20 mM to avoid precipitation of the enzyme. Absorbance at 362 nm was plotted versus time and fitted to a first-order rate equation to determine the rate constant for oxidation of the cobalt−carbon bond.

The above experiment was repeated under anaerobic conditions. After dilution of the enzyme to 12 μM, the sample was placed in a glass cuvette sealed with a stopcock, where it was subjected to partial evacuation for 20 s and flushed with argon. This procedure was repeated six times. The sample was sealed in a cuvette under a blanket of argon and passed into an anaerobic glove box. The remaining reaction components were made anaerobic by subjection to the same protocol. Reaction components were combined, sealed in an airtight cuvette, and brought to the spectrophotometer where they were observed by the method described. Subsequent reintroduction of air to the sample was done after 1 h by opening the sealed cuvette and pipetting air bubbles through the solution for 2 min using a Pasteur pipet.

Binding Affinity of Dioldehydrase for Adenosylcobalamin. Dissociation constants for the binding of adenosylcobalamin to dioldehydrase were measured in the presence of potassium ion and in its absence, with replacement by the *N*-methylpiperidinium ion, a nonactivating monocation. Stock dioldehydrase (250 μL, 60 mg mL^{-1}) was thawed on ice and inserted into dialysis cassettes, where samples were dialyzed for 1 h against 300 mL of either 20 mM K^+-EPPS, 120 μM 1,2-propanediol, and 12 mM K^+-cholate at pH 8.0 or against the same buffer with *N*-methylpiperidinium-EPPS in place of K^+-EPPs. Final concentrations of either *N*-methylpiperidinium or K^+ were approximately 25 mM in each buffer. Each enzyme sample was removed from the dialysis cassette, diluted to 20 mg mL^{-1} with column buffer, then immediately loaded onto a column (1 cm in diameter) containing 20 mL of Sephadex G-25 resin equilibrated either with 20 mM *N*-methylpiperidinium-EPPS and 12 mM *N*-methylpiperidinium-cholate at pH 8.0 or with the same buffer containing K^+ in place of *N*-methylpiperidinium ion. Gel filtration proceeded at 0.5 mL min^{-1}, and fractions containing dioldehydrase were collected and diluted to 15 μM (30 μM active sites). The sample was placed in a glass cuvette sealed with a stopcock, where it was subjected to partial evacuation for 20 s and flushed with argon. This procedure was repeated six times. The sample was sealed in a cuvette under a blanket of argon and passed into an anaerobic glove box. The same procedure to remove oxygen was repeated for a small volume of buffer, water, and 7.8 mM adenosylcobalamin.

In an anaerobic glove box, adenosylcobalamin was serially diluted with water to appropriate concentrations differing by 15 μM. Starting with undiluted adenosylcobalamin, 10 μL of each dilution was added to 490 μL of dioldehydrase. A series of controls was prepared using the same dilutions of adenosylcobalamin and buffer. Samples were incubated in the glove box for 15 min, sealed in Eppendorf tubes, and passed out of the glovebox. Samples were placed in microcentrifuge spin filters and spun on a table top centrifuge until approximately 200 μL of filtrate had been collected. Each filtrate was transferred to a small volume cuvette and the absorption spectrum obtained from 250 to 850 nm.

Cross-Linking of Dioldehydrase Subunits. Cross-linking experiments were performed using DSS, a homobifunctional *N*-hydroxysuccinimide ester containing a noncleavable 8 carbon spacer used for linking primary amines. In each experiment, dioldehydrase was made free of potassium and substrate (see Determination of Adenosylcobalamin Binding Affinity for Potassium-Bound and Potassium-Free Dioldehydrase) and diluted to 4 mg mL^{-1} ($\sim$ 20 μM dimer) with 20 mM *N*-methylpiperidinium-EPPS. Cross-linking reactions were performed with samples of enzyme in the presence of 10 mM KCl, 100 μM adenosylcobalamin, or 1% 1,2-propanediol. Another sample was kept free of any additions. Reactions were initiated by the addition DSS to 500 μM. Every 5 min, aliquots were diluted 1:2 into Laemmli SDS−PAGE sample buffer to stop the reaction. Reaction times ranged from 5 to 30 min. Samples were boiled for 10 min, loaded onto a 7.5% SDS−PAGE gel, and run against a series of molecular weight markers. Peptide fingerprinting mass spectral analysis was performed on bands from SDS−PAGE gels in order to confirm subunit composition (Supporting Information).

Nucleoside Products in the Reaction of Oxygen with the Dioldehydrase−Adenosylcobalamin Complex. A protocol was developed to isolate nucleoside products from oxygen

inactivated dioldehydrase for identification by the standard HPLC method. Stock dioldehydrase (250 μL, 60 mg mL^{-1}) was thawed on ice and inserted into a dialysis cassette, where the sample was dialyzed for 4 h against 300 mL of 20 mM K^+-EPPS and 12 mM K^+-cholate at pH 8.0 with five buffer changes.

The reaction mixture consisted of 200 μM dioldehydrase and 200 μM adenosylcobalamin in 250 μL of 20 mM K^+-EPPS and 12 mM K^+-cholate at pH 8.0. The reaction proceeded at room temperature for 2 h in the dark and was quenched by 0.75% TCA. The resultant precipitate was removed by 3 × 20 min spins on a table top centrifuge. The bulk of the TCA was removed by five equal volume extractions into water-saturated ether, with subsequent removal of ether from the aqueous layer by a stream of wet argon across the surface. The pH was then brought to neutrality with dilute ammonium hydroxide. Detection of nucleoside products was performed by the standard HPLC method.

Assays for Peroxide Production in the Oxidation of the Dioldehydrase−Adenosylcobalamin Complex. The method of ferrous oxidation in xylenol orange (FOX assay) (*20*) was used to detect the formation of any peroxide, hydroperoxide, or hydrogen peroxide formed during the potassium- and oxygen-activated dark oxidation of adenosylcobalamin. Stock dioldehydrase (250 μL, 60 mg mL^{-1}) was thawed on ice and inserted into a dialysis cassette, where the sample was dialyzed for 4 h against 300 mL of 25 mM K^+-EPPS and 12 mM K^+-cholate at pH 8.0, with five buffer changes. Dialyzed dioldehydrase was diluted to 15 mg mL^{-1} (70 μM dimer) by addition of dialysis buffer. A reaction mixture was prepared by the addition of 10 μL of 2.8 mM adenosylcobalamin to 260 μL of dialyzed dioldehydrase. Approximately every 9 min, a 20 μL aliquot was withdrawn and added to 200 μL of FOX assay reagent, incubated for 15 min, and centrifuged in a table top centrifuge for 5 min. The absorption spectra were acquired and values at 590 nm compiled. An identical experiment, excluding adenosylcobalamin, was repeated as a negative control. An identical experiment was also repeated in the presence of ~500 U mL^{-1} catalase. A control was performed in which ~500 U mL^{-1} of catalase was incubated with 16 mM H_2O_2 and subjected to the FOX assay.

The above incubation of adenosylcobalamin with dioldehydrase was repeated and the resultant reaction mixture placed in a cuvette where its absorption spectrum from 300 to 850 nm was acquired every 10 s for 1 h. Absorbance at 355 nm was plotted versus time, and a first-order rate equation was fitted to the data to determine the rate constant for oxidation of the cobalt−carbon bond.

In a separate experiment, each assay component was placed in a glass cuvette sealed with a stopcock, where it was subjected to partial evacuation for 20 s and flushing with argon six times. The samples were sealed in a cuvette under a blanket of argon and passed into an anaerobic glove box. The adenosylcobalamin oxidation and FOX assays were repeated in the anaerobic glovebox as described. After centrifugation, the samples were brought out of the glove box and the absorbance measured.

Assays for Hydrogen Peroxide. Dioldehydrase was dialyzed as described (see Assays for Peroxide Production in the Reaction of Oxygen with the Dioldehydrase-Adenosylcobalamin Complex). The enzyme was incubated with 100 μM adenosylcobalamin for 45 min, horseradish peroxidase added (500 U mL^{-1}) , and the absorption spectrum from 250 to 600 nm acquired. The sample was then incubated in 100 μM *N*,*N*′-(1,4-phenylenedimethylidyne)di-*o*-anisidine and the absorption spectrum re-acquired. As a positive control, H_2O_2 was added to ~10 mM.

RESULTS

Reactivity of Adenosylcobalamin with Oxygen at the Enzymatic Site. In the absence of a substrate, the dioldehydrase−adenosylcobalamin complex undergoes slow decomposition to cob(III)alamin, which inactivates the enzyme. Previous studies suggest that cob(III)alamin formation depends upon the presence of both O_2 and potassium ion (*1*, *14*). A spectrophotometric assay was developed to monitor this process under a variety of conditions by measuring the formation of cob(III)alamin. Figure 2 illustrates the time-dependent changes in the visible absorption spectrum of dioldehydrase incubated with adenosylcobalamin and potassium ion in the presence of air as well as the requirement for both potassium ion and molecular oxygen in the spectral change. The distinct isosbestic points indicate a clean conversion of adenosylcobalamin to cob(III)alamin without observable intermediates or side reactions (Figure 2A). Anaerobically, very little formation of cob(III)alamin is observed, and what is observed likely arises from oxygen contamination (Figure 2B). Reverse phase HPLC of the anaerobic sample reveals detectable but trace amounts of nucleoside products derived from adenosylcobalamin. Aerobically and in the absence of potassium and other activating monocations, absolutely no cob(III)alamin could be detected within the duration of an experiment (2 h, Figure 2B). In the control, *N*-methylpiperidinium ion, which does not activate dioldehydrase, replaced all activating monocations.

The rate of oxidation of the coenzyme exhibits a saturable dependence on the concentration of an activating monocation. The reaction progress curve is observed by monitoring the absorbance change for λ_{max} at 362 nm in the cob(III)alamin absorption spectrum, and it exhibits first-order kinetic behavior. Figure S1 (Supporting Information) shows the effect of varying activating monocation concentrations on the observed first-order rate constant for cob(III)alamin formation with ammonium or potassium ion as the activator. The kinetic model in Scheme 2 describes the behavior of adenosylcobalamin oxidation by potassium-activated dioldehydrase. Here M^+, E, AdoCbl, and Cbl(III) symbolize activating monocation, dioldehydrase, adenosylcobalamin, and oxidized adenosylcobalamin, respectively. In this model, the assumptions that binding of M^+ is reversible and that the reaction with O_2 is irreversible are made. Data were obtained for a range of concentrations, and eq 1 was fitted to the data as follows

$$k_{obs} = \frac{k_3[M^+]}{\left(\frac{k_2 + k_3}{k_1}\right) + [M^+]} \quad (1)$$

where k_{obs} is the observed first-order rate constant at a given activating monocation concentration, k_3 is the pseudo-first-order rate constant[2] for oxidation of adenosylcobalamin, the

Scheme 2

$$M^+ + E\bullet AdoCbl \underset{k_2}{\overset{k_1}{\rightleftharpoons}} M^+\bullet E\bullet AdoCbl$$

$$M^+\bullet E\bullet AdoCbl + O_2 \xrightarrow{k_3} M^+\bullet E\bullet Cbl(III)$$

ratio of rate constants $(k_2 + k_3)/k_1$ describes the concentration of activating monocation needed to achieve half-maximal k_{obs}, and $[M^+]$ is the concentration of activating monocation.

For potassium ion, values of $k_3 = (8.1 \pm 0.1) \times 10^{-4}$ s^{-1} and $(k_2 + k_3)/k_1 = 7.6 \pm 0.2$ mM were obtained, whereas for ammonium ion, the values were $k_3 = (5 \pm 1) \times 10^{-3}$ s^{-1} and $(k_2+k_3)/k_1 = 20 \pm 7$ mM. The apparent second-order rate constants are $k_1k_3/(k_2+k_3)$, and the values for ammonium and potassium activation are 0.25 M^{-1} s^{-1} and 0.1 M^{-1} s^{-1}, respectively. At saturation, ammonium ion is nearly 7-fold more activating than potassium ion.

It was demonstrated that for a series of activating monocations, the rate of oxidation of the coenzyme in the active site was proportional to the overall effectiveness of that cation in supporting the normal enzymatic process, in agreement with earlier reports (*5*). This trend in the rate of inactivation and its relationship to activating monocation identity were also observed with sodium ion, which provided the slowest rate; the concentration dependence could not be evaluated because of the low enzyme solubility at higher sodium concentrations. In addition, if one could make the assumption that equilibrium is established between the enzyme and activating monocation, that is, $k_2 \gg k_3$, one sees that the term describing concentration of activating monocation at half-maximal k_{obs} becomes the dissociation constant (K_d) for the M^+·E·AdoCbl complex, with respect to M^+. It is possible that the values obtained reflect this K_d, when the very small values for k_3 are considered. Limitations to the solubility of dioldehydrase in the presence of high solute concentration, especially that of adenosylcobalamin and activating monocation, prevented measurement at higher concentrations of activating monocation and contributed to significant error in some results.

Effect of Potassium Ion on the Binding of Adenosylcobalamin to Dioldehydrase. Early reports indicated that the effect of activating monocations on the rate of inactivation was rooted in it being the essential factor for both the binding of coenzyme and subunit association (*5*, *15*, *21*). Experiments were performed to determine the effect of potassium ion on the binding of adenosylcobalamin to dioldehydrase. Samples of stock dioldehydrase were prepared by gel filtration chromatography in order to remove both substrate and potassium ion, incubated with varying concentrations of adenosylcobalamin, and subjected to ultrafiltration assays, where the extent of binding was measured as the residual adenosylcobalamin in the filtrate. Figure 3 illustrates the binding affinity of apodioldehydrase for adenosylcobalamin in the presence and absence of potassium ion. Equation 2 is fitted to the data in Figure 3

$$\alpha = \frac{C[B_{12}]_F}{K_d + [B_{12}]_F} + S[B_{12}]_F \qquad (2)$$

where α is the fractional saturation, C is the ratio describing moles of binding sites per mole of dimer, K_d is the dissociation constant, $[B_{12}]_F$ is the concentration of free adenosylcobalamin, and S is a correction factor to the fractional saturation for the contribution of nonspecific binding between adenosylcobalamin and dioldehydrase. The fitted values of parameters are $K_d = 0.8 \pm 0.2$ μM for potassium-activated dioldehydrase and $K_d = 16 \pm 2$ μM for dioldehydrase freed of activating monocations.

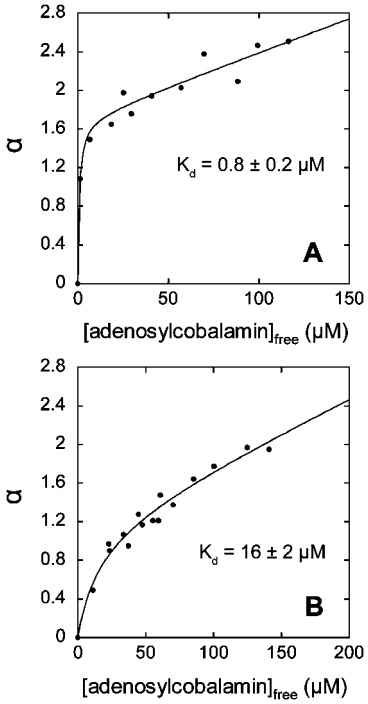


FIGURE 3: Binding affinity (K_d) of adenosylcobalamin for dioldehydrase. (A) Binding in the presence of 10 mM potassium ion and (B) in the absence of an activating monocation. α is the fractional saturation of dioldehydrase with adenosylcobalamin and is defined as $([B_{12}]_T - [B_{12}]_F)$/[dioldehydrase]. The binding model is discussed in the text.

Nonspecific binding due to peripheral interactions of adenosylcobalamin with dioldehydrase was observed, presumably at a site or sites remote from the binding pocket for the coenzyme. Nonspecific binding interactions between cobalamins and enzymes have also been observed with methylmalonyl-CoA mutase (*22*), methionine synthase (5-methyltetrahydrofolate-homocysteine cobalamin methyltransferase) (*23*), and ethanolamine ammonia-lyase (*24*). The value obtained for S in Figure 4 was 0.0070 ± 0.0005 μM^{-1}, and it was independent of the presence or absence of potassium ion, as expected for nonspecific binding.

Because of a varying activity loss due to the process of gel filtration, the number of total binding sites could not be definitiely determined. The gel filtration of dioldehydrase caused some activity loss, the worst case being when all activating monocations were removed from dioldehydrase. Activity loss was never more than 40% after gel filtration and remained largely consistent from one experiment to another within each data set (data not shown). The constant describing the number of binding sites per dimer (the asymptote for the first hyperbolic term) was approximated

[2] The constant k_3 is pseudo first order because the reaction requires O_2, which is 0.3 mM in air-equilibrated water, much higher in concentration than the enzyme. Consumption of O_2 is insignificant relative to the enzyme−coenzyme complex.

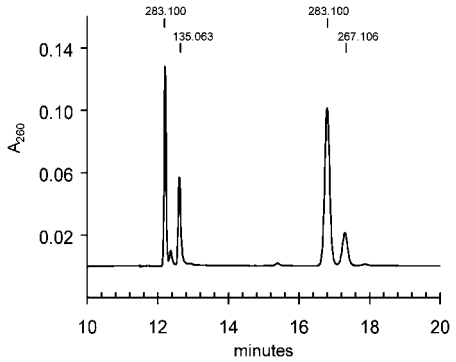


FIGURE 4: HPLC elution profile of nucleoside oxidation products from the complex of dioldehydrase and adenosylcobalamin. Samples were subjected to TCA precipitation, and the oxidized adenosylcobalamin-derived nucleoside products remaining in the supernatant fluid were analyzed by C_{18} reverse phase chromatography. Above each peak is the mass (amu) as acquired by ESI-TOF mass spectrometry. Retention times were adenosine-5′-aldehyde, 12.2 min (*m*/*z* 283.1); adenine, 12.5 min (*m*/*z* 136.06) ; 5′-peroxyadenosine, 16.8 min (283.1); and adenosine, 17.3 min (*m*/*z* 267.1).

by first fitting the data to eq 2, obtaining a value, then refitting the data using the approximated value for *C*. In the dioldehydrase dimer, the theoretical limit for this ratio is 2. The value used for *C* in potassium-free dioldehydrase was 1.2, and the value used for potassium-bound dioldehydrase was 1.7, representing an approximate loss of 40% and 15% of the total number of cofactor binding sites, respectively, and was approximately in agreement with the activity loss observed.

Cross-Linking of Dioldehydrase Subunits in the Presence and Absence of Potassium Ion, Substrate, and Adenosylcobalamin. The binding of adenosylcobalamin occurs at the interface between the α- and β-subunits. Evidence suggests that the binding of substrate in the presence of potassium ion to adenosylcobalamin-free dioldehydrase triggers subunit association as observed by electrophoretic studies (*25*).

To investigate whether the binding of adenosylcobalamin in potassium-free dioldehydrase somehow fails to promote subunit association, subunit cross-linking studies were performed. Cross-linking experiments were performed using DSS, a homo-bifunctional *N*-hydroxysuccinimide ester containing a noncleavable 8 carbon spacer used for linking primary amines. Cross-linked subunits were visualized by SDS−PAGE. In each study, the addition of potassium ion, adenosylcobalamin, or substrate failed to change the observed cross-linking patterns (Figure S2, Supporting Information). Indeed, the absence of everything but apoenzyme also gave rise to identical cross-linking patterns, both early and late in the cross-linking reaction (Figure S2, Supporting Information). Visualization of the reaction by SDS−PAGE revealed two important bands, at ca. 80 and 85 kDa, indicating cross-linked components of dioldehydrase consisting of the large−small (α, γ) and large−medium (α, β) subunits, respectively. MS/MS analysis of tryptic peptides from these bands confirmed the identities of the subunits. The results indicated that the association of subunits, under all conditions studied, occurred in the absence or presence of adenosylcobalamin, a substrate, or K^+.

Nucleoside Products Derived from the Enzymatic Oxidation of Adenosylcobalamin. The nucleoside products in supernatants of TCA-precipitated samples of oxidized holodioldehydrase were analyzed by C_{18} reverse phase chromatography. Analysis showed the formation of four principal nucleoside products, as determined by the presence of an adenine moiety spectrophotometrically identified from the absorbance output of the HPLC detector. Figure 4 shows the HPLC elution profile for nucleoside products derived from the reaction of oxygen with the dioldehydrase−adenosylcobalamin complex.

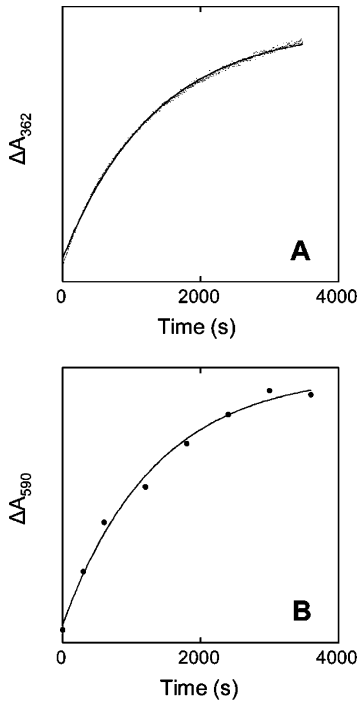


FIGURE 5: Rate of enzymatic oxidation of adenosylcobalamin by potassium-activated dioldehydrase. Dioldehydrase was incubated with 30 mM potassium ion and 100 μM adenosylcobalamin. Rate of reaction was monitored for the production of (A) cob(III)alamin by change in absorption spectra at 362 nm and (B) peroxides by the FOX assay. The values for the fitted curves are indicated in the text.

The products were identified by their masses and by co-injection and coelution with standards in HPLC. Commercial adenine and adenosine comigrated with the two products that displayed retention times of 12.6 and 17.3 min, respectively, as shown in Figure 5. Mass spectral analysis of the two peaks confirmed the identities of these products as adenine (135.063 amu) and adenosine (267.106 amu). In addition, detectable but minor amounts of 5′-deoxyadenosine were identified by HPLC and mass spectral analysis (data not shown). The remaining two reaction products emerged with the same retention times as those of the two major reaction products arising in the photolysis of adenosylcobalamin in aerobic or O_2-saturated water. These two products were identified as adenosine-5′-aldehyde (*26*) and 5′-peroxyadenosine (*16*). Samples of photolyzed adenosylcobalamin were combined with samples of adenosylcobalamin oxidized by potassium-activated dioldehydrase and assayed by the standard HPLC method. Adenosine-5′-aldehyde and 5′-peroxyadenosine from the photolysis reaction comigrated with the peaks that had retention times of 12.2 and 16.8 min, respectively, as shown in Figure 4. HPLC analysis revealed that the nucleoside identified as 5′-peroxyadenosine (retention time 16.8 min and mass 283.100 amu in Figure 4) decomposed to the other three nucleoside products (nonenzymatically) at a rate comparable

to that of the same process observed for the photolysis-derived peroxide in the presence of cob(III)alamin (*16*). Mass spectrometry supported the conclusion that the major nucleoside products of these two reactions were the same.

A discontinuous assay for the formation of 5′-peroxyadenosine was performed by the FOX method, a colorimetric assay sensitive to peroxides. Matched samples of potassium-activated holodioldehydrase were incubated in an aerobic aqueous solution. One sample was monitored by the oxygen sensitivity assay and the other by the FOX assay. Reaction curves from both assays exhibited the same first-order rate and suggest that 5′-peroxyadenosine production was kinetically linked with cob(III)alamin formation (Figure 5). Pseudo-first-order rate constants obtained from the fitted curves were $(7.50 \pm 0.03) \times 10^{-4}$ s^{-1} for cob(III)alamin formation (Figure 5A) and $(7 \pm 1) \times 10^{-4}$ s^{-1} for 5′-peroxyadenosine production (Figure 5B).

In order to determine if the results of the FOX assay were, in part or in whole, due to H_2O_2 production, a series of enzymatically coupled assays were performed. In the first assay, the dioldehydrase−adenosylcobalamin complex kept for 45 min under aerobic conditions was and then incubated in 100 μM *N*,*N*′-(1,4-phenylenedimethylidyne)di-*o*-anisidine and horseradish peroxidase. No reaction of the peroxidase with its electron donor, *N*,*N*′-(1,4-phenylenedimethylidyne)-di-*o*-anisidine, was detected until an addition of 10 mM H_2O_2 was made. In a separate experiment, the FOX assay, as described above, was repeated in the presence of catalase to test if the compound giving a positive response to the colorimetric assay could be decomposed by that enzyme. The FOX assay still gave positive response in the presence of catalase. In a control, 16 mM H_2O_2 was incubated with catalase and subjected to the FOX assay, yielding negative results.

DISCUSSION

Oxygen Sensitivity in Substrate-Free Dioldehydrase. Catalysis by dioldehydrase requires an activating monocation (*1*), with maximal catalytic activity correlated to the ionic radius of the cation (*5*). The same requirement for an activating monocation is observed for the oxidation of adenosylcobalamin at the active site (Figure 2) (*1*, *14*).

The postulate has been put forward that an activating monocation was essential for the association of adenosylcobalamin with dioldehydrase (*5*, *15*, *21*, *28*). These workers resolved the apoenzyme from the coenzyme by gel filtration chromatography of dioldehydrase in the absence of potassium ion. The same experiment in the presence of potassium ion failed to separate the coenzyme. The conclusion drawn was that activating monocations were the essential factor facilitating the binding of adenosylcobalamin to dioldehydrase, and this would also explain why potassium- and substrate-free dioldehydrase fails to promote the cleavage of adenosylcobalamin to cob(III)alamin. The results reported herein do not sustain the latter conclusion.

The present work demonstrates that potassium ion provides an approximately 20-fold increase in binding affinity between dioldehydrase and adenosylcobalamin (Figure 3). The binding of adenosylcobalamin to apodioldehydrase in the absence of potassium ion remains strong ($K_d = 16$ μM). The difference in binding affinities between potassium-bound or potassium-free dioldehydrase and adenosylcobalamin likely accounts for the observed difference in resolution of the coenzyme and apoenzyme by gel filtration chromatography (*25*). However, the effect of potassium on binding adenosylcobalamin cannot account for the >2500-fold enhancement to the rate of adenosylcobalamin oxidation. Moreover, cross-linking experiments indicate that the absence of potassium ion does not lead to the dissociation of subunits under the experimental conditions used here. An alternative role for the activating monocation in catalysis and oxidative cleavage of adenosylcobalamin is required.

Evidence for the Formation of Activated Coenzyme in Dioldehydrase. Recently, evidence for the formation of a geminate, triplet radical pair produced from the enzyme-mediated thermolysis of the Co−C5′ bond has been observed through electron paramagnetic resonance spectroscopy of dioldehydrase incubated with an analogue of coenzyme B_{12}, 3′,4′-anhydroadenosylcobalamin. A strongly spin-correlated triplet radical pair arises between Co(II) of cob(II)alamin and the resonance stabilized analogue of the 5′-deoxyadenosyl radical, 5′-deoxy-3′,4′-anydroadenosine-5′-yl (*19*, *28*, *29*). Other evidence had previously been reported in studies of the adenosylcobalamin-dependent enzyme ethanolamine ammonia-lyase, where Harkins and Grissom discovered a magnetic field induced effect on V_{max}/K_m, possibly caused by a change in the intersystem crossing rates between the singlet and the triplet spin states in the [Ado• + cob(II)alamin] spin-correlated radical pair, leading to an increase in the rate of radical recombination (where V_{max} is the maximal enzyme velocity, and K_M is the Michaelis constant) (*30*). Similar effects were seen in the photolysis of adenosylcobalamin (*31*).

Experiments designed to test the existence of an equilibrium between adenosylcobalamin and a discrete radical pair resulting from homolytic scission of the Co−C5′ bond have been performed in both methylmalonyl-CoA mutase (*32*) and mutants of ribonucleotide reductase (*33*) and involved chirally deuterated 5′-deoxyadenosylcobalamin, with isoptopic substitution at the C5′. In the absence of the target of hydrogen atom abstraction by the 5′-deoxyadenosyl radical, both enzymatic systems showed epimerization of the chiral label. These results indicated discrete equilibria involving homolysis at the Co−C5′ bond.

The studies herein provide chemical proof for the formation of a similarly activated coenzyme through identification of the products resulting from the oxygen inactivation of dioldehydrase. Most notably, the product profile for nucleoside products from oxygen-inactivated dioldehydrase is very similar to that obtained from aerobically photolyzed adenosylcobalamin (*16*).

The initial principal nucleoside product in the photolysis of adenosylcobalamin is 5′-peroxyadenosine (*16*). This is also the case in the oxidative cleavage of adenosylcobalamin at the active site of dioldehydrase. However, quantitative differences in the product profiles between the two reactions provide additional insight into the coenzyme−enzyme interaction. The formation of adenosine-5′-aldehyde proceeds at least 15-times faster at the enzymatic site than in solution (subsequent to photolysis). The difference may be explained by the proximity of cob(III)alamin and 5′-peroxyadenosine in the active site: in solution the two are diffusible and must collide to react.

A striking phenomenon in the product profile for adenosylcobalamin oxidation at the active site of dioldehydrase is the larger amount of adenine formed compared to the photolytic process (Figure 4) (*16*). Conceivably, under increased strain from binding interactions, adenine might arise from hydrolysis of the N-glycosidic linkage. Alternatively, adenine might arise from electron transfer between cob(II)alamin and the 5′-deoxyadenosyl radical, yielding cob(III)alamin and a carbanion at C5′ of the nucleoside. This carbanion would either eliminate adenine, similar to heterolysis of the Co−C5′ bond (*34*−*37*), or acquire a proton to form 5′-deoxyadenosine. Several precedents for the inactivation of adenosylcobalamin-dependent enzymes by suicide electron transfer in the coenzyme or its analogue have been published (*19*, *38*−*40*). 5′-Deoxyadenosine in the enzymatic product profile implicates electron transfer as involved in product formation. 5′-Deoxyadenosine is not a product in the aqueous photolysis of adenosylcobalamin (*16*).

Activating Stimulates the Homolytic Cleavage of the Co-C5′ Bond. Crystallographic studies of dioldehydrase have yielded much information regarding the structure of the active site, including the binding sites for potassium and substrate deep within the β-barrel of the α-subunit. Several possible roles for the activating monocation have been put forward, including that it in some way affects the structure of the β-barrel (*4*), that it plays a role in binding the substrate or facilitating catalysis (*4*), that it stimulates product release (*41*), or that it might participate directly in catalysis as a Lewis acid (*4*).

The present results indicate that dioldehydrase likely employs the binding energy of an activating monocation to induce a conformational change that stimulates homolytic cleavage in the coenzyme. It is likely that the same potassium-stimulated conformational change that leads to a moderate increase in coenzyme binding affinity is related to the potassium-dependent activation of adenosylcobalamin. Exactly how the conformational effects in dioldehydrase induce the coenzyme to undergo homolytic cleavage of the Co−C5′ bond requires further investigation.

Independent evidence for induction of strain has been obtained in crystallographic studies involving the coenzyme analogue adeninylpentylcobalamin. This coenzyme analogue was used to identify the adenine binding pocket (*41*) and provided the bases for a comparative study of the available crystal structures to determine the steric effects induced on adenosylcobalamin upon binding (*42*). It was concluded from modeling adenosylcobalamin into the adenine ring and cyanocobalamin binding pockets, that in potassium-bound dioldehydrase, a distortion was imposed on the Co−C5′ bond in the coenzyme. It was further concluded that the majority of this strain already exists in the substrate-free form, with a much smaller effect resulting from the binding of substrate. The present work, in light of structural data, suggests that potassium ion is an essential factor for the activation of adenosylcobalamin in dioldehydrase.

ACKNOWLEDGMENT

We thank Jim Brown for his assistance in LC/MS analysis and Gregorz Sabat for his assistance with peptide fingerprint MS at the Mass Spectrometry Facility (Biotechnology Center, University of Wisconsin—Madison).

SUPPORTING INFORMATION AVAILABLE

Figure S1 shows the potassium and ammonium concentration dependence of the pseudo-first-order rate constants for oxidation of adenosylcobalamin at the active site of dioldehydrase. Figure S2 shows SDS−PAGE gels of dioldehydrase chemically cross-linked in the absence or presence of adenosylcobalamin, substrate, or potassium ions. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI700078Z