Adenosylcobinamide Methyl Phosphate as a Pseudocoenzyme for Diol Dehydrase†

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ABSTRACT: Adenosylcobinamide methyl phosphate, a novel analog of adenosylcobalamin lacking the nucleotide loop moiety, was synthesized. It did not show detectable coenzymic activity but behaved as a strong competitive inhibitor against AdoCbl with relatively high affinity (K_i = 2.5 μM). When apoenzyme was incubated at 37 °C with this analog in the presence of substrate, the Co–C bond of the analog was almost completely and irreversibly cleaved within 10 min, forming an enzyme-bound Co(II)-containing species. The cleavage was not observed in the absence of substrate. The Co–C bond cleavage in the presence of substrate was not catalytic but stoichiometric, implying that the Co–C bond of the analog undergoes activation when the analog binds to the active site of the enzyme. 5′-Deoxyadenosine was the only product derived from the adenosyl group of the analog upon the Co–C bond cleavage. Apoenzyme did not undergo modification during this process. Therefore, it seems likely that adenosylcobinamide methyl phosphate acts as a pseudocoenzyme or a potent suicide coenzyme. Since adenosylcobinamide neither functions as coenzyme nor binds tightly to apoenzyme, it can be concluded that the phosphodiester moiety of the nucleotide loop of adenosylcobalamin is essential for tight binding to apoenzyme and therefore for subsequent activation of the Co–C bond and catalysis. It is also evident that the nucleotide loop is obligatory for the normal progress of catalytic cycle.

Diol dehydrase is an AdoCbl[1]-dependent enzyme which catalyzes the conversion of 1,2-propanediol, 1,2-ethanediol, and glycerol to propionaldehyde, acetaldehyde, and β-hydroxypropionaldehyde, respectively (Lee & Abeles, 1963; Toraya et al., 1976). It is widely accepted that the homolytic cleavage of the Co–C bond of the coenzyme is an essential early event in the AdoCbl-dependent enzymatic reactions (Abeles & Dolpin, 1976; Babior, 1975). We have investigated the structure–function relationship of this coenzyme by synthesizing many analogs of AdoCbl (Toraya & Fukui, 1982). From the results obtained so far, it has been concluded that the specific interaction of the adenosyl group of the coenzyme with its binding site of the enzyme is one of the essential elements for activation (labilization) of the Co–C bond (Toraya, 1985; Toraya et al., 1977, 1986, 1987; Ichikawa & Toraya, 1988) and that basal labilization of the bond takes place through specific tight interaction of the cobalamin moiety with apoprotein (Toraya & Ishida, 1988; Toraya et al., 1979). Recently, we synthesized several nucleotide loop-modified analogs of AdoCbl and demonstrated that the D-ribose moiety is important as a spacer to keep DBI in the proper position (Toraya & Ishida, 1991). The dimethylbenzo moiety of DBI was shown to play an important role in preventing the intermediate complexes from inactivation during catalysis.

During the course of our studies, we have shown that AdoCbi neither functions as coenzyme nor binds tightly to the apoprotein of diol dehydrase. Therefore, in order to study the role of a phosphate group in the nucleotide loop, we synthesized AdoCbi-PMe, a new coenzyme analog which lacks the nucleotide loop moiety. In this paper, we report the synthesis and the coenzymic properties of this analog. The roles of the phosphate group of the nucleotide loop in the binding to apoenzyme and in manifestation of coenzymic function are discussed.

EXPERIMENTAL PROCEDURES

Materials. Crystalline AdoCbl was a gift from Eisai Co. Ltd., Tokyo, Japan. CN-Cbl was obtained from Glaxo Research Laboratories, Greenford, U.K. (CN,aq)Cbi was prepared by cerous hydroxide hydrolysis of CN-Cbl in the presence of excess KCN by a modification of a published procedure (Friedrich & Bernhauer, 1954). 5′-Chloro-5′-deoxyadenosine was prepared by reaction of adenosine with $SOCl_2$ as described by Kikugawa and Ichino (1971). [8-^{14}C]-Adenosine was purchased from Amersham. All other chemicals were reagent-grade commercial products and were used without further purification.

Diol Dehydrase. The apoenzyme of diol dehydrase was purified as described previously (Poznanskaja et al., 1979) from cells of *Klebsiella oxytoca* ATCC 8724 (formerly *Klebsiella pneumoniae* and *Aerobacter aerogenes*) grown without aeration in a glycerol/1,2-propanediol medium (Abeles, 1966), except that 0.2% polypeptone was used instead of 0.2% yeast extract (Toraya & Ishida, 1988).

Enzyme and Protein Assays. The activity of diol dehydrase was determined by the 3-methyl-2-benzothiazolinone hydrazone method (Toraya et al., 1977). One unit is defined as the amount of enzyme activity which catalyzes the formation of 1 μmol of propionaldehyde/min under the standard assay conditions. Protein concentration was determined either by the method of Lowry et al. (1951) or by measurement of the absorbance at 278 nm. An absorption coefficient of 5.27 for 10 mg of diol dehydrase/mL and for a 1-cm light path was used for the latter method (Poznanskaja et al., 1979).

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[1] Abbreviations: AdoCbl, adenosylcobalamin; CN-Cbl, cyanocobalamin; AdoCbi, adenosylcobinamide; (CN,aq)Cbi, cyanoaquacobinamide; AdoCbi-PMe, adenosylcobinamide methyl phosphate; (CN,aq)Cbi-PMe, cyanoaquacobinamide methyl phosphate; Cbl, cobalamin; Cbi, cobinamide; Cbi-PMe, cobinamide methyl phosphate; Cbl^{II}, cob(II)alamin; Cbi^{II}, cob(II)inamide; Cbi^{II}-PMe, cob(II)inamide methyl phosphate; Cbi^{I}-PMe, cob(I)inamide methyl phosphate; DBI, 5,6-dimethylbenzimidazole; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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Table I: Chromatographic and Electrophoretic Behaviors of Cbi-PMe

analog	$R_{CN\text{-}Cbl}$ in TLC[a]				relative mobility in paper electrophoresis[b]	
	solvent A	solvent B	solvent D	solvent E	pH 2.7	pH 7.0
(CN,aq)Cbi-PMe	0.81, 0.38	0.62, 0.20	1.34[c]	1.10[c]	0.03	0.00
AdoCbi-PMe	0.41	0.25			0.63	0.00
(CN,aq)Cbi	0.25, 0.14	0.36, 0.14	1.46[c]	2.27[c]	≡1	≡1
AdoCbi	0.13	0.16			1.38	0.80

[a] On Merck silica gel G-60 precoated plates. R_f values for CN-Cbl in solvents A, B, D, and E were 0.24, 0.07, 0.25, and 0.07, respectively. [b] At a voltage gradient of about 22 V/cm. Mobility of CN-Cbl and (CN,aq)Cbi was taken as 0 and 1, respectively. In 0.5 M acetic acid (pH 2.7) or in 0.01 M potassium phosphate buffer (pH 7.0). [c] (CN,aq)Cbi-PMe and (CN,aq)Cbi are converted to a dicyano form in this solvent.

Other Analytical Procedures. The concentration of corrinoids was determined spectrophotometrically after converting them to a dicyano form by reaction with 0.1 M KCN. Organocorrinoids were converted to a dicyano form by photolysis in the presence of KCN. ϵ_{367} of 30.4×10^3 M^{-1} cm^{-1} for dicyanoCbl (Barker et al., 1960) was employed. Visible and ultraviolet absorption spectra were measured on a Union Model SM-401 recording spectrophotometer. Thin-layer chromatography was performed on Merck silica gel G-60 precoated glass plates. Paper chromatography was carried out on Toyo filter paper No. 50. The solvent systems used are (A) 1-butanol/2-propanol/water (10:7:10 by vol); (B) water-saturated 2-butanol; (C) ethyl acetate/methanol (6:1 by vol); (D) solvent A containing 0.01 M KCN; (E) solvent B containing 0.01 M KCN; and (F) 1-butanol/water (86:14 by vol). Paper electrophoresis was carried out in 0.5 M acetic acid (pH 2.7) or 10 mM potassium phosphate buffer (pH 7.0) for analyzing and purifying corrinoids. ^{31}P-NMR spectra were obtained on a Varian VXR-200 NMR spectrometer operating in the Fourier transform mode. Mass spectra (FAB-MS) were measured on VG-70SE mass spectrometer.

Synthesis of (CN,aq)Cbi-PMe. To 400 mL of ice-cold 15 mM NaOH was added dropwise 0.2 mL of methyl dichlorophosphate with vigorous stirring. After the solution was stirred at 0 °C for 5 min, 2 mL of pyridine was added. The mixture containing pyridinium methyl phosphate was evaporated to dryness under reduced pressure, and 205 mg of (CN,aq)Cbi in 2 mL of H_2O was added to the mixture, followed by evaporation to dryness. After being dried overnight in vacuo, the residue was dissolved in a mixture of 10 mL of anhydrous pyridine and 15 mL of freshly distilled dimethylformamide. Dicyclohexylcarbodiimide (1.5 g) was then added to the solution, and the reaction mixture was stirred continuously at room temperature for 40 h. As judged by paper electrophoresis, the conversion to the desired product was ca. 70% at this time. The reaction was terminated by adding 50 mL of H_2O and 100 mg of KCN. After 30 min, pH of the mixture was adjusted to about 5 with acetic acid. Corrinoid products were desalted by phenol extraction, evaporated to dryness, and dissolved in 60 mL of H_2O. After being heated to 80 °C for 5 h, the solution was applied to a column (bed volume, 4.4 × 33 cm) of Amberlite XAD-2. The column was washed with 1.5 L of H_2O, and the corrinoids were eluted with 80% ethanol. The eluate was evaporated to dryness, dissolved into a small amount of H_2O, and passed through a CM-cellulose (H^+) column (3 × 18 cm). The effluent was concentrated to a small volume and applied to a column (3 × 18 cm) of DEAE-cellulose (acetate). Again, the through fraction was concentrated to a small volume. The desired product was then purified by preparative TLC on Merck silica gel G-60 precoated plates in solvent D. The dicyano form of Cbi-PMe thus obtained was converted to a monocyano form by adding a few drops of acetic acid, followed by evaporation to dryness under reduced pressure. The purity of this preparation of (CN,aq)Cbi-PMe was established by TLC in solvents D and E. Chromatographic and electrophoretic behaviors are summarized in Table I. Spectral data are given in Table II.

Table II: Absorption Spectra of Cbi-PMe[a]

analog	pH	λ_{max} (nm) ($\epsilon \times 10^{-3}$ (M^{-1} cm^{-1}))
(CN,aq)Cbi-PMe	7	275 (11.3), 304 s, 321 (11.0), 354 (27.0), 388 s, 407 (4.8), 468 s, 498 (8.6), 529 (8.0)
	1	identical to those in pH 7
AdoCbi-PMe	7	264 (37.2), 305 (23.2), 317 s, 330 s, 382 (8.6), 460 (9.4)
	1	264 (35.7), 305 (22.2), 317 s, 330 s, 382 (8.1), 461 (9.2)

[a] In 0.01 M potassium phosphate buffer (pH 7) or in 0.1 N HCl (pH 1).

Conversion to Adenosyl Form. To 14 mg of (CN,aq)-Cbi-PMe in 5 mL of H_2O was added 50 mg of $NaBH_4$. All the following operations were carried out in the dark. After 15 min at room temperature, 40 mg of 5′-chloro-5′-deoxyadenosine in 5 mL of dimethylformamide was added, and the mixture was allowed to stand for 30 min at room temperature. As judged by paper electrophoresis in 0.5 M acetic acid, the conversion to the adenosyl form was ca. 40%. The reaction mixture was then diluted by adding 40 mL of 0.06 M potassium phosphate buffer (pH 5). The corrinoids were desalted by phenol extraction, and AdoCbi-PMe was purified by phosphocellulose (adjusted to pH 3 with NaOH) column chromatography. The slowly-moving corrinoid fraction was collected. Crude AdoCbi-PMe thus obtained was further purified to homogeneity by preparative TLC in solvent A. The behaviors in TLC and paper electrophoresis and spectral data of AdoCbi-PMe are shown in Tables I and II, respectively.

Synthesis of [8-^{14}C]Adenosyl Form of Cbi-PMe. [8-^{14}C]-5′-Chloro-5′-deoxyadenosine was synthesized from [8-^{14}C]-adenosine (8 mg, 10 μCi) and purified as described previously (Toraya & Abeles, 1980; Toraya & Ishida, 1991). (CN,aq)-Cbi-PMe was reduced to Cbi^{I}-PMe with Zn/10% NH_4Cl. [8-^{14}C]AdoCbi-PMe was obtained by reaction of resulting Cbi^{I}-PMe with [8-^{14}C]-5′-chloro-5′-deoxyadenosine and purified according to the methods described previously for the synthesis of [8-^{14}C]AdoCbl *e*-carboxylic acid (Toraya & Abeles, 1980). The radioactive AdoCbi-PMe thus obtained was confirmed to be homogeneous and radiochemically pure by criteria of TLC in solvent A and paper chromatography in solvent B.

RESULTS

Chemical Properties of Cbi-PMe. (CN,aq)Cbi-PMe exhibited a sharp singlet peak with a chemical shift of 3.81, 3.57, or 3.76 ppm when ^{31}P-NMR spectra were taken in H_2O,

0.1 M HEPES buffer (pH 7.8), or 0.1 M HEPES buffer/75 mM KCN (pH 7.8), respectively, with 85% H_3PO_4 as external standard (data not shown). All of the samples contained about 15–25% D_2O. CN-Cbl also showed a sharp singlet peak with a chemical shift of 2.10 ppm in H_2O/D_2O under the same conditions. It is therefore concluded that the synthetic compound possesses only one kind of phosphate group, i.e., phosphodiester. When this compound was subjected to cerous hydroxide hydrolysis in the presence of KCN under the conditions employed for preparation of Cbi from CN-Cbl, dicyanoCbi was the only corrinoid species formed, as analyzed by TLC in solvents D and E. Thus, it is evident that the Cbi moiety of this compound was not modified during the condensation reaction with pyridinium methyl phosphate.

Mass spectra (FAB-MS) of (CN,aq)Cbi-PMe (MW 1128) were taken, but neither the molecular ion nor quasimolecular ions appeared. Instead, three prominent peaks were noted at *m/e* 1083, 1085, and 1086 with relative intensities of 1:0.73:0.33, respectively (data not shown). (CN,aq)Cbi (MW 1034) also did not give the molecular ion or quasimolecular ions, but very similar prominent peaks were observed at *m/e* 989, 991, and 992 with relative intensities of 1:0.81:0.39, respectively. The masses of the three peaks of (CN,aq)Cbi-PMe are larger than those of the respective peaks of (CN,aq)Cbi by 94, which coincides with the difference of their molecular weights. This offers strong evidence for the presence of an additional PO_3CH_3 group in the synthetic AdoCbi-PMe. The most intense peak of the three (*m/e* 1083 of (CN,aq)Cbi-PMe and 989 of (CN,aq)Cbi) may be assigned to the fragment ion formed by loss of HCN and H_2O.

As shown in Table II, the absorption spectra of (CN,aq)-Cbi-PMe and AdoCbi-PMe were essentially the same as those of the corresponding Cbi. Like (CN,aq)Cbi, the purified preparation of (CN,aq)Cbi-PMe gave two orange-red spots in approximately equal amounts upon TLC in the absence of KCN, although it was established to be homogeneous in the presence of KCN (Table I). These corresponds to the coordination isomers (a- and b-isomers), in which coordination positions of CN^- and H_2O are reversed (Friedrich, 1966).

Coenzymic Activity and Binding Properties of Cbi-PMe in the Diol Dehydrase System. AdoCbi-PMe did not show detectable coenzymic activity even at a concentration of 20 μM in the diol dehydrase reaction (less than 0.02% that of AdoCbl). This was also confirmed in the alcohol dehydrogenase–NADH coupled assay (Toraya et al., 1979), by which we were not able to see any rapid initial phase of the reaction. AdoCbi-PMe and (CN,aq)Cbi-PMe rather behaved as competitive inhibitors with respect to AdoCbl (K_m = 0.80 μM), with apparent K_i values of 2.5 and 3.5 μM, respectively. Therefore, it is evident that both forms of Cbi-PMe bind to apoenzyme with fairly high affinity.

The coenzymic and binding properties of AdoCbi-PMe were not improved by the addition of DBI, α-D-ribazole, and/or α-D-ribazole phosphate, fragments of the nucleotide loop, in agreement with the results with AdoCbi (Ishida et al., 1993).

Upon preincubation of apoenzyme with 5 μM AdoCbi-PMe for 10 min in the presence of substrate, the enzyme activity was almost completely lost when assayed with added AdoCbl. In the absence of substrate, an AdoCbi-PMe-dependent loss of activity was much less significant (data not shown).

Spectral Changes of AdoCbi-PMe upon Binding to Apoenzyme. When AdoCbi-PMe was incubated with excess apoenzyme in the presence of substrate, distinct spectral changes were observed (Figure 1B). The most prominent changes are

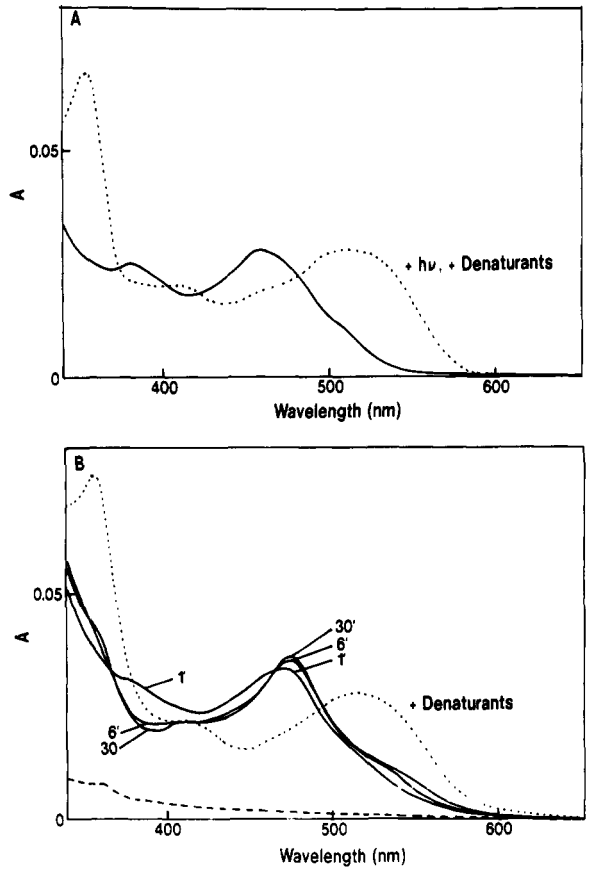


FIGURE 1: Spectral changes observed during incubation of AdoCbi-PMe with apodiol dehydrase in the presence of substrate. Spectra are corrected for dilution. (A) Free AdoCbi-PMe (minus apoenzyme control) (—) and diaqua Cbi-PMe (···) (photolyzed for 10 min with a 300-W tungsten light bulb at a distance of 20 cm after addition of 6 M guanidine hydrochloride/0.06 M citric acid followed by neutralization. (B) Apoenzyme (60 units, 3.7 nmol) (– –) was incubated at 37 °C for the indicated time periods with 3.0 nmol of AdoCbi-PMe in 0.04 M potassium phosphate buffer (pH 8.0) containing 0.2 M 1,2-propanediol (—), and then the enzyme was denatured by adding 6 M guanidine hydrochloride/0.06 M citric acid. The pH of the mixture was 2.6. After 10 min at 37 °C, the mixture was neutralized to pH 8 with 200 μL of 1 M potassium phosphate buffer (pH 8.0) and 63 μL of 5 N KOH, and the spectrum was taken (···).

the disappearance of an absorption maximum at 460 nm and the appearance of a new peak at 474 nm with isosbestic points at 369 and 466 nm. This shift in the absorption spectrum is suggestive of the conversion of a base-off form of coenzyme (AdoCbi-PMe) to the enzyme-bound Cbi^{II}-PMe. The diol dehydrase-bound Cbl^{II} shows an absorption maximum at 478 nm (Toraya, 1985). It has been reported that the base-on form of free Cbl^{II} absorbs light at 473 nm (Brady & Barker, 1961) and the base-off form of Cbl^{II} and Cbi^{II} absorb at 470 nm (Pratt, 1972).

The spectrum obtained was quite stable until the enzyme was denatured, indicating that the enzyme-bound Cbi^{II}-PMe-like species was protected against oxidation by atmospheric oxygen. When 6 M guanidine hydrochloride/0.06 M citric acid was added to the mixture as a denaturant, marked changes of the spectrum were brought about. The spectrum obtained after neutralization coincided with that of a diaqua form of Cbi-PMe (Figure 1A). Addition of 6 M urea to the mixture instead of guanidine hydrochloride/citric acid did not alter the absorption spectrum, but the incubation of the resulting mixture at 54 °C for 3 h resulted in similar changes of the spectrum. A control experiment without enzyme showed that AdoCbi-PMe was quite stable under the same conditions.

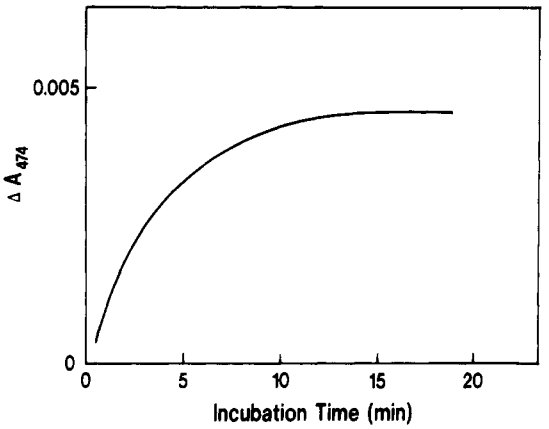


FIGURE 2: Time course change of the absorbance at 474 nm upon incubation of AdoCbi-PMe with apodiol dehydrase in the presence of substrate. The experimental conditions are the same as those in the legend for Figure 1B.

Thus, it can be concluded that the Co–C bond of AdoCbi-PMe was irreversibly cleaved by incubation with apodiol dehydrase in the presence of substrate (1,2-propanediol), forming Cbi^{II}-PMe-like species. In the absence of substrate, no spectral changes were observed with similar treatments (data not shown). Therefore, it is evident that cleavage of the Co–C bond of AdoCbi-PMe by apoenzyme is dependent upon substrate.

Time Course and Stoichiometry of the Co–C Bond Cleavage of AdoCbi-PMe by Apoenzyme. The time course of the Co–C bond cleavage of AdoCbi-PMe by slightly excess apoenzyme was monitored by measuring an increase of the absorbance at 474 nm. As shown in Figure 2, the spectral change almost ended within 10 min of incubation. The extent of the Co–C bond cleavage after a 10-min incubation was about 90–100%, as determined from an increase in absorbance at 516 nm after denaturation by 6 M guanidine hydrochloride/0.06 M citric acid followed by neutralization.

Stoichiometry of the reaction is illustrated in Figure 3. AdoCbi-PMe (3.0 nmol) was incubated at 37 °C for 10 min with the indicated concentrations of apoenzyme. After denaturation with 6 M guanidine hydrochloride/0.06 M citric acid followed by neutralization, the extent of the Co–C bond cleavage was determined spectrophotometrically as described above. The extent of the Co–C bond cleavage was essentially proportional to an [enzyme]/[AdoCbi-PMe] ratio in the range from 0 to 0.75 and reached a maximum (90–100%) when the molar concentration of apoenzyme was equal to that of AdoCbi-PMe. It is therefore clear that the apoenzyme cleaves the Co–C bond of AdoCbi-PMe not catalytically but stoichiometrically. Since diol dehydrase possesses one coenzyme-binding site per molecule (Essenberg et al., 1971; Toraya, 1985), this result suggests strongly that activation and cleavage of the Co–C bond of AdoCbi-PMe take place by binding to the coenzyme-binding site of the enzyme.

Fate of [8-^{14}C]Adenosyl Group of AdoCbi-PMe after the Co–C Bond Cleavage. Apoenzyme (200 units, 12.4 nmol) was incubated at 37 °C for 40 min with 20 nmol of the [8-^{14}C]-adenosyl form of Cbi-PMe (specific activity, 727 dpm/nmol) in 1.0 mL of 0.05 M potassium phosphate buffer (pH 8.0) containing 0.4 M 1,2-propanediol. The enzyme activity was completely lost by this treatment. Ethanol was then added to a concentration of 80% (v/v). Unlabeled 5′-deoxyadenosine (40 nmol) was also added to the mixture as a carrier. The mixture was heated at 90 °C for 3 min, and the denatured protein was removed by filtration. After evaporation of the

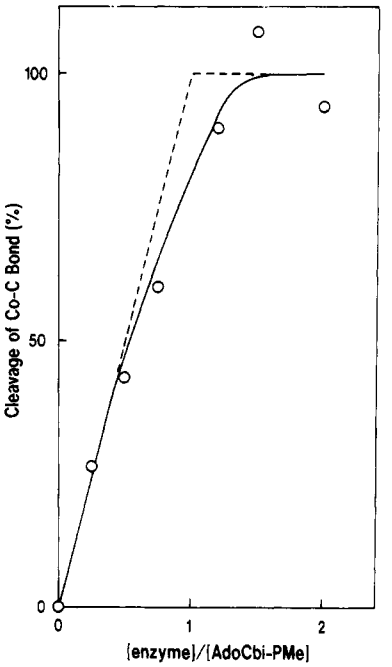


FIGURE 3: Effect of enzyme concentration on the extent of the Co–C bond cleavage of AdoCbi-PMe. The experimental conditions are the same as those described in Figure 1B, except that the indicated concentrations of apoenzyme were used. The extent of the Co–C bond cleavage was determined from an increase in absorbance at 516 nm after denaturation of enzyme with 6 M guanidine hydrochloride/0.06 M citric acid followed by neutralization.

filtrate to dryness under reduced pressure, the residue was taken up into a small amount of 50% ethanol and chromatographed on Toyo filter paper No. 50 in solvent F. Two radioactive areas were detected on the paper chromatogram. One radioactive compound remaining at the origin most likely represents excess [8-^{14}C]AdoCbi-PMe, whereas the other coincided with the 5′-deoxyadenosine marker. The latter area was cut out and eluted with water. From the radioactivity in the eluate (8 613 dpm), the amount of the nucleoside formed by the Co–C bond cleavage was calculated to be 11.8 nmol. This value was in good agreement with the amount of apoenzyme used (12.4 nmol). Hence, the recovery of the radioactivity in the nucleoside was essentially quantitative (95%). The eluate was concentrated to a small volume, and the radioactive compound(s) in this fraction was(were) analyzed by TLC on silica gel with solvents B, C, and F. 5′-Deoxyadenosine, 5′,8-cyclic adenosine, 4′,5′-anhydroadenosine, adenosine 5′-aldehyde, adenosine, and adenine were used as markers. In all the solvent systems used, essentially all the radioactivity comigrated with 5′-deoxyadenosine (data not shown). Therefore, it can be concluded that 5′-deoxyadenosine is the only product derived from the adenosyl group of AdoCbi-PMe upon cleavage of the Co–C bond.

Resolution of Enzyme–AdoCbi-PMe Complex after the Co–C Bond Cleavage. The complex whose Co–C bond had been cleaved was obtained by incubation of apoenzyme (117 units, 7.3 nmol, specific activity of 80 units/mg of protein) at 37 °C for 30 min with 20 nmol of AdoCbi-PMe in 1.0 mL of 0.05 M potassium phosphate buffer (pH 8.0) containing 1 M 1,2-propanediol, followed by dialysis. The remaining activity of the resulting complex was 7% (specific activity of 5.6 units/mg of protein) of that of the original apoenzyme when assayed in the presence of added AdoCbl. The amount of corrinoid bound to this complex was 1.3 mol/mol of enzyme. The complex was then resolved into apoprotein and a corrinoid species by acid ammonium sulfate treatment, according to

the procedure described previously (Toraya & Abeles, 1980). From the spectrophotometric determination of corrinoid, the "resolved enzyme" still contained 0.77 mol of corrinoid/mol of enzyme; that is, only 0.53 mol of corrinoid/mol of enzyme was removed by the resolution procedure. Thus, the extent of resolution was calculated to be ca. 41% on the basis of the corrinoid contents. The spectrum of the "resolved corrinoid" which was recovered from the supernatant solution coincided with that of a diaqua form of Cbi-PMe.

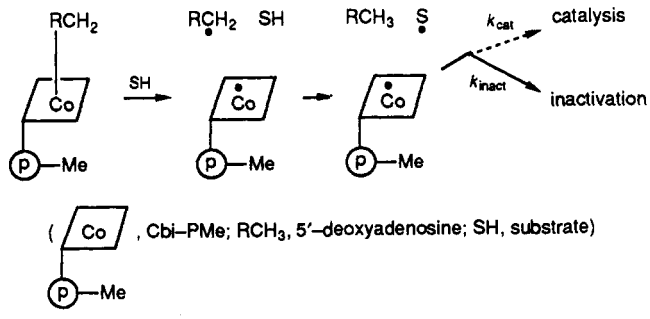
About 59% of the "resolved enzyme" was successfully reconstituted into catalytically active holoenzyme upon addition of AdoCbl (specific activity of 47 units/mg of protein). Since the complex retained 7% of the activity before resolution, the extent of resolution was ca. 52% on the basis of the recovered activity. The extent of resolution based on the recovery of enzymatic activity (52%) was in reasonable agreement with that based on the removal of corrinoid (41%). Therefore, it is likely that the apoenzyme recovered from the complex by acid ammonium sulfate treatment was fully active. As described above, no 5′-deoxyadenosine remained covalently bound to the enzyme after the cleavage of the Co–C bond. These lines of evidence suggest that the apoenzyme itself was not modified in the process of the Co–C bond cleavage.

DISCUSSION

Previously, we demonstrated that AdoCbi is neither active as coenzyme nor bound tightly to apodiol dehydrase. In this paper, the synthesis of adenosyl and cyanoaqua forms of Cbi-PMe is first reported. The role of a phosphate group of the nucleotide loop in manifestation of coenzymic function was investigated by comparing the coenzymic function and binding properties of this analog with those of AdoCbi. AdoCbi-PMe did not show detectable coenzymic activity but bound to apoenzyme with fairly high affinity. Therefore, it can be concluded that a phosphodiester moiety of the nucleotide loop is indispensable for tight binding to the apoenzyme. It seems unlikely that the introduction of a methyl group brought about such a drastic change in the affinity for the apoenzyme, because the hydrophobic interaction through a methyl group is not so strong.

The data presented in this paper demonstrated clearly that the Co–C bond of AdoCbi-PMe is cleaved upon binding to apoenzyme. This is the only analog lacking the nucleotide loop reported so far that undergoes cleavage of its Co–C bond. Therefore, it is clear that this analog fulfills the minimum of the structural requirement for activation of the Co–C bond by apoenzyme but not for catalytic activity. However, this fact does not necessarily eliminate the possibility that the coordination of DBI of the nucleotide loop to the cobalt atom is important for cleavage of the Co–C bond at a faster rate.

Spectral changes of the enzyme–AdoCbi-PMe complex in the presence of substrate showed that the Co–C bond of AdoCbi-PMe was cleaved by the apoenzyme forming a tightly bound Co(II)-containing corrinoid species. Such changes were observed only in the presence of substrate, indicating the involvement of substrate in the process of the Co–C bond cleavage. The time course change of the spectra shows that the Co–C bond cleavage is almost completed within 10 min. It was demonstrated that 5′-deoxyadenosine is the only product derived from the adenosyl group of AdoCbi-PMe upon cleavage of the Co–C bond. Resolution experiments with acid ammonium sulfate showed that the apoprotein itself is not damaged in this process. From these lines of evidence, the following scheme could be drawn for the mechanism of cleavage of the Co–C bond of AdoCbi-PMe by apodiol dehydrase:



It should be noted that AdoCbi-PMe undergoes activation of its Co–C bond upon binding to apoenzyme, although it is inactive as coenzyme. In the presence of substrate, initial steps of the normal catalytic cycle proceed. That is, the Co–C bond is cleaved forming an adenosyl radical and Co(II)-containing corrinoid species, and the adenosyl radical abstracts a hydrogen atom from substrate forming 5′-deoxyadenosine and a substrate radical. However, at least one of the radical intermediates is unstable and undergoes undesired side reactions with high probability. As a result, the organic radical(s) disappears, and its recombination with the Co(II)-corrinoid becomes impossible. A resulting Co(II)-containing corrinoid species and 5′-deoxyadenosine remain tightly bound to the active site and are not exchanged for AdoCbl, which leads to total inactivation of the enzyme when assayed with added AdoCbl. The coenzymic activity of AdoCbi-PMe was less than 0.02% that of AdoCbl ($k_{cat} < 4.0$ min^{-1}). The first-order rate constant for the Co–C bond cleavage of enzyme-bound AdoCbi-PMe obtained from Figure 2 was 0.22 min^{-1}. A k_{cat}/k_{inact} ratio was thus calculated to be less than 18 for AdoCbi-PMe, which is much lower than the value for AdoCbl (1.4×10^6) (Toraya et al., 1979). This ratio might be nearly zero if coenzymic activity of AdoCbi-PMe is determined by a highly sensitive assay method. Therefore, it is likely that this is an extreme case of suicide inactivation and that AdoCbi-PMe behaves as a "pseudocoenzyme" or a very efficient "suicide coenzyme". Some analogs of AdoCbl have been shown to induce suicidal inactivation of diol dehydrase during catalysis, but their efficiency of inactivation was much lower. For example, k_{cat}/k_{inact} values for 3′-deoxyAdoCbl, 2′-deoxyAdoCbl, AdoCbl *e*-carboxylic acid, and the imidazolyl trimethylene analog were reported to be 7×10^4 (Ichikawa & Toraya, 1988), 6×10^4 (Ichikawa & Toraya, 1988), 1×10^4 (Toraya & Abeles, 1980), and 3.3×10^3 (Toraya & Ishida, 1991), respectively. It is evident that the nucleotide loop of AdoCbl plays a pivotal role in preventing the highly reactive radical intermediate(s) from unfruitful side reactions. In general, undesired side reactions of the reactive radical intermediates lead to cessation of the enzymatic reactions involving free radicals. Therefore, such a negative control of the reactivity of radical intermediates by enzymes would be absolutely important for radical-dependent enzymatic catalysis. This effect of enzymes might be exerted by spatial isolation of a radical species from nearby reactive groups of the active site.

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REFERENCES

Abeles, R. H. (1966) *Methods Enzymol. 9*, 686–689.
Abeles, R. H., & Dolphin, D. (1976) *Acc. Chem. Res. 9*, 114–120.

Babior, B. M. (1975) *Acc. Chem. Res. 8*, 376–384.
Barker, H. A., Smyth, R. D., Weissbach, H., Munch-Petersen, A., Toohey, J. I., Ladd, J. N., Volcani, B. E., & Wilson, M. R. (1960) *J. Biol. Chem. 235*, 181–190.
Brady, R. O., & Barker, H. A. (1961) *Biochem. Biophys. Res. Commun. 4*, 373–378.
Essenberg, M. K., Frey, P. A., & Abeles, R. H. (1971) *J. Am. Chem. Soc. 93*, 1242–1251.
Friedrich, W. (1966) *Z. Naturforsch. 21b*, 138–144.
Friedrich, W., & Bernhauer, K. (1954) *Z. Naturforsch. 9b*, 685–694.
Ichikawa, M., & Toraya, T. (1988) *Biochim. Biophys. Acta 952*, 191–200.
Ishida, A., Ichikawa, M., Kobayashi, K., Hitomi, T., Kojima, S., & Toraya, T. (1993) *J. Nutr. Sci. Vitaminol.* (in press).
Kikugawa, K., & Ichino, M. (1971) *Tetrahedron Lett. 2*, 87–90.
Lee, H. A., Jr., & Abeles, R. H. (1963) *J. Biol. Chem. 238*, 2367–2373.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Poznanskaja, A. A., Tanizawa, K., Soda, K., Toraya, T., & Fukui, S. (1979) *Arch. Biochem. Biophys. 194*, 379–386.
Pratt, J. M. (1972) Inorganic Chemistry of Vitamin B_{12}, p 104, Academic Press, London.
Toraya, T. (1985) *Arch. Biochem. Biophys. 242*, 470–477.
Toraya, T., & Abeles, R. H. (1980) *Arch. Biochem. Biophys. 203*, 174–180.
Toraya, T., & Fukui, S. (1982) in *B_{12}* (Dolphin, D., Ed.) Vol. 2, pp 233–262, John Wiley & Sons, New York.
Toraya, T., & Ishida, A. (1988) *Biochemistry 27*, 7677–7681.
Toraya, T., & Ishida, A. (1991) *J. Biol. Chem. 266*, 5430–5437.
Toraya, T., Shirakashi, T., Kosuga, T., & Fukui, S. (1976) *Biochem. Biophys. Res. Commun. 69*, 475–480.
Toraya, T., Ushio, K., Fukui, S., & Hogenkamp, H. P. C. (1977) *J. Biol. Chem. 252*, 963–970.
Toraya, T., Krodel, E., Mildvan, A. S., & Abeles, R. H. (1979) *Biochemistry 18*, 417–426.
Toraya, T., Matsumoto, T., Ichikawa, M., Itoh, T., Sugawara, T., & Mizuno, Y. (1986) *J. Biol. Chem. 261*, 9289–9293.
Toraya, T., Watanabe, N., Ichikawa, M., Matsumoto, T., Ushio, K., & Fukui, S. (1987) *J. Biol. Chem. 262*, 8544–8550.