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Functions of the D-Ribosyl Moiety and the Lower Axial Ligand of the Nucleotide Loop of Coenzyme B_{12} in Diol Dehydratase and Ethanolamine Ammonia-lyase Reactions[1]

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The roles of the D-ribosyl moiety and the bulky axial ligand of the nucleotide loop of adenosylcobalamin in coenzymic function have been investigated using two series of coenzyme analogs bearing various artificial bases. The 2-methylbenzimidazolyl trimethylene analog that exists exclusively in the base-off form was a totally inactive coenzyme for diol dehydratase and served as a competitive inhibitor. The benzimidazolyl trimethylene analog and the benzimidazolylcobamide coenzyme were highly active for diol dehydratase and ethanolamine ammonia-lyase. The imidazolylcobamide coenzyme was 59 and 9% as active as the normal coenzyme for diol dehydratase and ethanolamine ammonia-lyase, respectively. The latter analog served as an effective suicide coenzyme for both enzymes, although the partition ratio (k_{cat}/k_{inact}) of 630 for ethanolamine ammonia-lyase is much lower than that for diol dehydratase. Suicide inactivation was accompanied by the accumulation of a cob(II)amide species, indicating irreversible cleavage of the coenzyme Co-C bond during the inactivation. It was thus concluded that the bulkiness of a *Co*-coordinating base of the nucleotide loop is essential for both the initial activity and continuous catalytic turnovers. Since the k_{cat}/k_{inact} value for the imidazolylcobamide in diol dehydratase was 27-times higher than that for the imidazolyl trimethylene analog, it is clear that the ribosyl moiety protects the reaction intermediates from suicide inactivation. Stopped-flow measurements indicated that the rate of Co-C bond homolysis is essentially unaffected by the bulkiness of the *Co*-coordinating base for diol dehydratase. Thus, it seems unlikely that the Co-C bond is labilized through a ground state mechanochemical triggering mechanism in diol dehydratase.

Key words: adenosylcobalamin, coenzyme B_{12}, diol dehydratase, ethanolamine ammonia-lyase, suicide inactivation.

An essential early event in all adenosylcobalamin (AdoCbl) (coenzyme B_{12})-dependent rearrangements is the generation of an adenosyl radical through homolytic cleavage of its Co-C bond. The mechanism underlying enzymatic activation (labilization) of the Co-C bond has therefore been an important subject of investigation for a long time. The recently solved structure of the diol dehydratase-adeninylpentylcobalamin complex and a modeling study based on it indicated that tight interactions between the enzyme and coenzyme at both the cobalamin moiety and the adenine ring of the adenosyl group produce angular strains and tensile force that likely contribute to labilization of the Co-C bond (*1*). Concerning the involvement of the lower axial base in the Co-C bond activation, enzyme-mediated compression of the Co-N bond of AdoCbl has been proposed to labilize the Co-C bond of the coenzyme (the so-called mechanochemical triggering mechanism) (*2–6*). This mechanism has been attractive for so-called "base-on" enzymes that bind AdoCbl in the base-on mode, such as diol dehyratase (*7–9*), ribonucleotide reductase (*10*), and ethanolamine ammonia-lyase (*11, 12*). The crystal structure of diol dehydratase, however, revealed that the Co-N bond of cobalamin is rather elongated upon binding to the enzyme (*9*).

So far, only a few papers have appeared on the role of a lower axial base of AdoCbl in enzymatic reactions. Toraya

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Abbreviations: AdoCbl, adenosylcobalamin; AdoCbiP-MBIPr, adenosylcobinamide 3-(2-methylbenzimidazolyl)propyl phosphate; AdoCbiP-DBIPr, adenosylcobinamide 3-(5,6-dimethylbenzimidazolyl)-propyl phosphate; AdoCbiP-BIPr, adenosylcobinamide 3-benzimidazolylpropyl phosphate; AdoCbiP-ImPr, adenosylcobinamide 3-imidazolylpropyl phosphate; AdoCbiP-PyPr, adenosylcobinamide 3-pyridylpropyl phosphate; Ado(BI)Cba, Coβ-adenosyl-Coα-benzimidazolylcobamide; Ado(Im)Cba, Coβ-adenosyl-Coα-imidazolylcobamide; BI, benzimidazole; (CN,aq)Cbi, cyanoaquacobinamide; CN-CbiP-BIPr, cyanocobinamide 3-benzimidazolylpropyl phosphate; CN-CbiP-MBIPr, cyanocobinamide 3-(2-methylbenzimidazolyl)propyl phosphate; CN-Cbl, cyanocobalamin; MBI, 2-methylbenzimidazole.

and coworkers synthesized coenzyme analogs in which the D-ribose and 5,6-dimethylbenzimidazole moieties of the nucleotide loop are replaced by a trimethylene group and imidazole (*13*) or pyridine (*14*), respectively (*15*) (Fig. 1). They synthesized cobinamide methyl phosphate (*16*), an analog lacking the entire nucleotide moiety, as well, and demonstrated with diol dehydratase that the steric bulk of the lower axial ligand is essential for preventing suicide inactivation during catalysis (*13–17*). Rétey and coworkers have shown that *p*-cresolylcobamide coenzyme, which exists exclusively in a base-off form, is inactive and rather inhibitory for diol and glycerol dehydratases, but highly active for methylmalonyl-CoA mutase (*18*), a so-called "base-off/His-on" enzyme. Banerjee and coworkers have proposed that in methylmalonyl-CoA mutase the conversion of a base-on form to a base-off form is assisted by the enzyme and followed by rapid docking of the cofactor in the active site (*19*). They reported that the cobinamide-GDP analog of the coenzyme supports catalysis by the enzyme without ligation of His610 to the cobalt atom (*20*).

Recently, Brown *et al.* (*21*) reported that the Co-C bond of Ado(Im)Cba, a coenzyme analog in which the natural 5,6-dimethylbenzimidazole axial base is replaced by imidazole, is cleaved 17 times more slowly than that of AdoCbl by ribonucleoside triphosphate reductase, although this analog supports steady state turnover at the same rate as AdoCbl. This observation is consistent with ground-state mechanochemical triggering based on molecular modeling studies (*6*) of this process.

It has been generally accepted that an adenosyl radical formed through homolysis of the coenzyme Co-C bond serves as a radical initiator but not as a catalytic radical by itself for ribonucleotide reductase (*22*), whereas the adenosyl radical serves as a catalytic radical for diol dehydratase and other AdoCbl-dependent enzymes (*23*). It is consequently possible that the mechanism underlying the Co-C bond homolysis in diol dehydratase is not the same as that in ribonucleotide reductase. To address this possibility, the coenzymic properties of four analogs [Ado(Im)Cba, Ado(BI)Cba, AdoCbiP-BIPr, and AdoCbiP-MBIPr in Fig. 1] were studied with the "base-on" enzymes, diol dehydratase, and ethanolamine ammonia-lyase, the results of which are reported here.

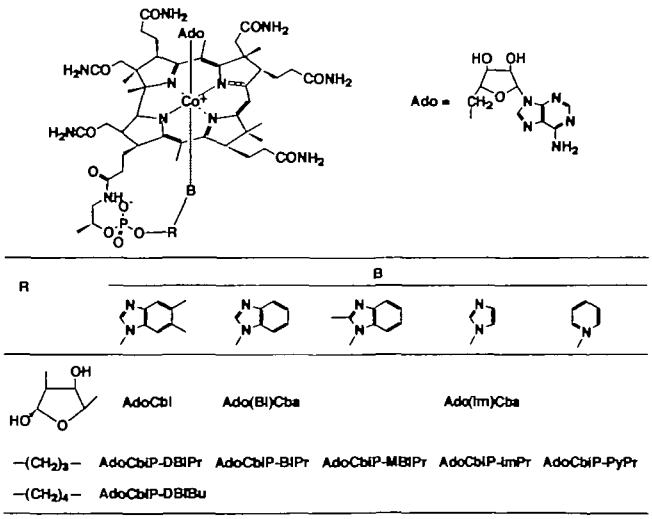

Fig. 1. **Structures of AdoCbl and its analogs with altered nucleotide bases used in this study.**

MATERIALS AND METHODS

Materials—Crystalline AdoCbl was a gift from Eisai, (Tokyo). Ado(BI)Cba and Ado(Im)Cba were prepared as described previously (*21*). All other chemicals were reagent-grade commercial products and were used without further purification. [1,1-D_2]1,2-Propanediol was synthesized by reduction of ethyl lactate with $LiAlD_4$ and purified by distillation under reduced pressure.

Apoenzymes of recombinant *Klebsiella oxytoca* diol dehydratase and *Escherichia coli* ethanolamine ammonia-lyase were purified to homogeneity from overexpressing *E. coli* JM109 cells harboring expression plasmids pUSI2E(DD) (*24, 25*) and pUSI2ENd (EAL) (Akita, K. and Toraya, T., to be published), respectively. Substrate-free apoenzyme was obtained by dialysis at 4°C for 2 days against 100 volumes of 0.05 M potassium phosphate buffer (pH 8.0) containing 20 mM sucrose monocaprate, with two buffer changes.

Synthesis of 1-(3-Hydroxypropyl)benzimidazole and 1-(3-Hydroxypropyl)2-methylbenzimidazole—To 1.2 g of BI or 1.3 g of MBI in 50 ml of dry dimethylformamide (DMF) was added 0.8 g of NaH in oil. After 30 min at room temperature, 3 ml of 3-chloropropyl tetrahydro-2-pyranyl ether, which was synthesized through reaction of 3,4-dihydropyran with 3-chloro-1-propanol, was added dropwise. After the mixture had been stirred for 10–23 h at room temperature, the reaction was terminated by adding 50 ml of water and the oil was removed by shaking with *n*-hexane. The mixture was then adjusted to pH 2.5–2.7 with HCl, diluted with copious water, and then applied to a Dowex 50 (H^+) column (bed volume, 200 ml). The column was washed successively with water and 30% ethanol, and the desired product was eluted with 30% ethanol containing 1 N NH_3. 1-(3-Hydroxypropyl) benzimidazole: R_f values on silica gel TLC, 0.59 in water-saturated 2-butanol, and 0.37 in ethyl acetate/methanol (6:1, v/v); ^1H NMR ($CDCl_3$), δ 2.02 (m, 2H, J = 6.6 Hz), 3.51 (t, 2H, J = 6.8 Hz), 4.25 (t, 2H, J = 7.0 Hz), 7.34 (m, 2H), 7.52 (m, 1H), 7.72 (m, 1H), and 8.09 ppm (s, 1H). 1-(3-Hydroxypropyl)-2-methylbenzimidazole: R_f values on silica gel TLC, 0.53 in water-saturated 2-butanol containing 1% NH_3 and 0.40 in ethyl acetate/methanol (6:1, v/v) containing 1% NH_3.

Synthesis of AdoCbiP-BIPr and AdoCbiP-MBIPr—1-(3-Hydroxypropyl)benzimidazole and 1-(3-hydroxypropyl)2-methylbenzimidazole were first converted to 3-benzimidazolypropyl phosphate and 3-(2-methylbenzimidazolyl)propyl phosphate, respectively, and then condensed with (CN,aq) Cbi in the presence of dicyclohexylcarbodiimide, as described previously for the synthesis of CN-CbiP-DBIPr (*13*). The conversion was more than 80% on the basis of (CN,aq) Cbi, as judged on paper electrophoresis. The desired products were purified as described for CN-CbiP-DBIPr (*13*). FAB-MS for CN-CbiP-BIPr: *m/e* 1,254 (MH^+), 1,228 (MH^+-KCN). The homogeneity of the purified products was established by TLC and HPLC. The chromatographic, electrophoretic and spectral properties of these new analogs are presented in Tables I and II, respectively.

The CN form of each analog (5 mg), in 1.5 ml of water, was reduced with 50 mg of $NaBH_4$ for 10–20 min. To the resulting solution was added 31 mg of 5′-tosyladenosine in

3 ml of ethanol. After 2 h at room temperature, the adenosylated analog formed was desalted by phenol extraction, and then purified to homogeneity by P-cellulose column chromatography or preparative paper electrophoresis. The isolation yields were 41% for AdoCbiP-BIPr and 33% for AdoCbiP-MBIPr on the basis of the CN form.

Enzyme and Protein Assays—The activities of diol dehydratase and ethanolamine ammonia-lyase were determined by the 3-methyl-2-benzothiazolinone hydrazone method (*26*). One unit is defined as the amount of enzyme activity that catalyzes the formation of 1 μmol of propionaldehyde or acetaldehyde per minute at 37°C under the standard assay conditions.

The alcohol dehydrogenase-NADH coupled assay method (*27*) was also used to assay both diol dehydratase and ethanolamine ammonia-lyase. The reaction mixture contained an appropriate amount of apoenzyme, 10 μM AdoCbl, 0.1 M 1,2-propanediol or ethanolamine, 120 μg of yeast alcohol dehydrogenase, 0.4 mM NADH, and 50 mM potassium phosphate buffer (pH 8.0), in a total volume of 1.0 ml. Reactions were initiated by adding the coenzyme, and then the change in absorbance at 340 nm was recorded.

The protein concentrations of purified preparations of the enzymes were determined by measuring the absorbance at 280 nm. The molar absorption coefficients at 280 nm ($\varepsilon_{M,280}$) calculated by the method of Gill and von Hippel (*28*) from the deduced amino acid compositions and subunit structures were 120,500 and 302,400 M^{-1} cm^{-1} for diol dehydratase and ethanolamine ammonia-lyase, respectively. Based on the predicted molecular weights, $\varepsilon_{1\%,280}$ was calculated to be 5.81 and 6.21, respectively.

Stopped-Flow Spectrophotometry—A RA-401 stopped-flow spectrophotometer (Otsuka Electronics, Osaka) and a flow cell of 2-mm light path were used for measurements. Substrate-free apodiol dehydratase (1,000 units) was mixed on ice with 2.0 molar equivalents of coenzyme in 3 ml of 0.05 M potassium phosphate buffer (pH 8.0) containing 20 mM sucrose monocaprate. After replacement of the air in the apparatus with argon by repeated evacuation/argon-introduction three times, the mixture was incubated anaerobically at 37°C for 10 min, cooled, and then kept on ice. The holoenzyme was placed in one reservoir, and a substrate solution (0.1 M) in the same buffer containing sucrose monocaprate and coenzyme at the same concentrations was placed in the other reservoir. After incubating both reservoirs at 4°C, equal volumes of the holoenzyme and substrate solutions were rapidly mixed. The change in absorbance at 525 nm was recorded with a DL708E digital oscilloscope (Yokogawa, Tokyo) to monitor the conversion of the coenzyme to cob(II)alamin or cob(II)amide. Five to ten traces were accumulated and averaged for curve fitting. After a dead-time, the absorbance changes followed first-order kinetics and fitted well to a double exponential function.

Other Analytical Procedures—The concentration of adenosylcobamides was determined spectrophotometrically after converting it to a dicyano form by photolysis in the presence of 0.1 M KCN, using $\varepsilon_{367} = 30.4 \times 10^3$ M^{-1} cm^{-1} for dicyanocobalamin (*29*). ^1H NMR spectra were obtained with a Varian VXR-200 (200 MHz) NMR spectrometer operating in the Fourier transform mode. Fast atom bombardment (FAB)-mass spectra were obtained with a VG-70SE mass spectrometer.

RESULTS

Chemical Properties of the Analogs—The spectrum of CN-CbiP-BIPr at pH 7 was essentially identical to that of CN-Cbl (Table II), indicating that in the neutral solution it exists in the "base-on" form—that is, with the benzimidazole moiety coordinating to the cobalt atom. At pH 1, however, it gave a spectrum that was quite similar to that of (CN,aq) Cbi. The p*K* (pK_a) value for the "base-on/base-off" conversion was 3.2 for CN-CbiP-BIPr. This value is much higher than that for CN-Cbl ($pK_a = 0.1$) (*30*), indicating that the base coordination to the cobalt atom in this analog is much weaker than that in CN-Cbl. This tendency was clearer for the adenosyl form. The spectrum of AdoCbiP-

TABLE I. **Chromatographic and electrophoretic behaviors of new analogs.**

Form[a]	Analog	$R_{CN\text{-}Cbl}$ in TLC[b]				Relative mobility on paper electrophoresis[c]	
		Solv. A	Solv. B	Solv. C	Solv. D	pH 2.7	pH 7.0
CN	CbiP-BIPr	0.6	0.54	1.6	1.9	0.6	0
	CbiP-MBIPr	0.6, 1.2	0.45, 1.2	1.52	1.24	0.8	0.6
Ado	CbiP-BIPr	0.44	0.36	0.36	0.25	1.2	0
	CbiP-MBIPr	0.52	0.45	0.48	0.25	1.2	0.5

[a]Upper axial ligand. Ado, adenosyl. [b]On Merck Silica Gel G-60 precoated plates. The R_f values for CN-Cbl in A–D were 0.25, 0.089, 0.25, and 0.63, respectively. [c]In 0.5 M acetic acid (pH 2.7) or 0.01 M potassium phosphate buffer (pH 7.0) at a voltage gradient of 22 V/cm. The mobilities of CN-Cbl and (CN,aq)Cbi were taken as 0 and 1, respectively.

TABLE II. **Absorption spectra of analogs.**

Form	Analog	pH[a]	λ_{max} (nm) ($\varepsilon \times 10^{-3}$, M^{-1} cm^{-1})
CN	CbiP-BIPr	7	275 (16.5), 306 (9.4), 325 (7.8), 363 (28.2), 411s (4.0), 520 (8.0), 550 (9.0)
		1	269 (18.4), 276 (18.7), 322 (11.7), 357 (26.7), 405 (5.3), 495 (8.6), 530 (8.3)
	CbiP-MBIPr	7	275 (16.6), 281 (14.9), 322 (11.1), 356 (26.4), 406 (5.0), 465-467s, 497 (8.8), 528 (8.3)
		1	270 (17.0), 277 (17.8), 304s (7.9), 322 (10.5), 355 (25.2), 388s (4.4), 405 (4.7), 465-467s, 497 (8.3), 527 (7.8)
Ado	CbiP-BIPr	7	264 (46.9), 306 (22.6), 315s (20.7), 381 (9.0), 460 (8.9)
		1	264 (48.1), 305 (23.2), 318s (20.8), 381 (8.2), 460 (9.3)
	CbiP-MBIPr	7	305s (32.6), 317s (25.5), 330s (20.4), 382 (10.2), 457 (10.2)
		1	305s (32.0), 317s (25.5), 330s (21.8), 382s (10.3), 461 (10.2)

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

BIPr at pH 7 or 8 resembled that at pH 1, indicating that only a small part (~10% or less) of this analog exists in a base-on form even at pH 7 or 8 (Table II and also Fig. 4A). As reported previously (*13*), AdoCbiP-DBIPr and AdoCbiP-ImPr also exist as equilibrium mixtures of their base-on and base-off forms in the ratio of <1:1, although AdoCbiP-DBIBu exists chiefly in a base-on form (~85%). The higher stability of a base-on form of AdoCbiP-DBIPr than that of AdoCbiP-BIPr at pH 7 or 8 may be explained by the higher basicity of DBI than that of BI. When the spectra of free Ado(BI)Cba and Ado(Im)Cba in the assay mixture for diol dehydratase (pH 8.0) were compared with that of AdoCbl (see Fig. 3, A–C), it is evident that, like AdoCbl, Ado(BI)Cba exists predominantly in the base-on form. A major portion of Ado(Im)Cba also exists in the base-on form at pH 8.0, although a small part of the "base-off" form may also be present in the solution of this analog. These results indicate that the presence of the α-D-ribofuranose moiety in the nucleotide loop affects coordination of the base to the cobalt atom from the lower axial position.

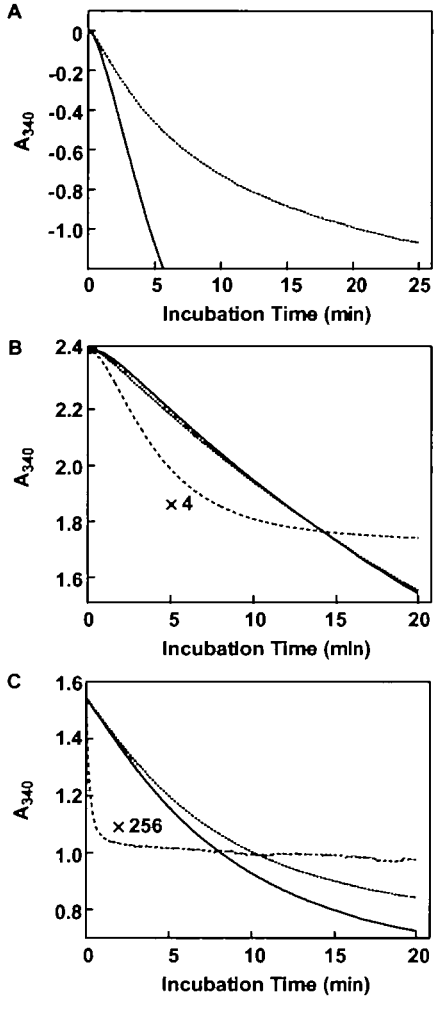

Fig. 2. **Time courses of reactions catalyzed by diol dehydratase (A, B) and ethanolamine ammonia-lyase (C).** Propionaldehyde and acetaldehyde formed by the enzymes were assayed by the alcohol dehydrogenase-NADH coupled method, as described in the text. Reactions were initiated by adding each coenzyme. A: AdoCbl (solid line) and AdoCbiP-BIPr (dotted line). B and C: AdoCbl (solid line), Ado(BI)Cba (dotted line), and Ado(Im)Cba (dashed line). "×4" in B and "×256" in C indicate that 4 and 256 times higher concentrations of apoenzyme were used, respectively.

The spectra of CN-CbiP-MBIPr and Ado-CbiP-MBIPr at both pH 7 and 1 were essentially the same as those of (CN,aq) Cbi and AdoCbi, respectively (Table II). On TLC on silica gel, purified CN-CbiP-MBIPr gave two spots of coordination isomers (cyanoaqua and aquacyano forms) with CN^--free solvents, and gave a single spot with CN^--containing solvents (Table I). These facts indicate that this analog exists exclusively in the base-off form even at pH 7 or 8 (see also Fig. 4B), probably because of the steric repulsion between the corrin ring and the methyl group on C-2 of MBI.

Coenzymic Activity of the Coenzyme Analogs in the Diol Dehydratase and Ethanolamine Ammonia-lyase Reactions—AdoCbiP-MBIPr, a trimethylene analog containing 2-methylbenzimidazole, was a totally inactive coenzyme for diol dehydratase and behaved as a competitive inhibitor with respect to AdoCbl. It was fairly tightly bound to the apoenzyme (K_i = 2.7 μM), although the affinity of the enzyme for the cyanoaqua form was much lower (data not shown). As mentioned above, this analog exists exclusively in a base-off form. Therefore, it is evident that axial coordination of a base to the cobalt atom is essential for manifestation of the coenzymic activity, in accordance with the previous finding with adenosyl-(3,5,6-trimethylbenzimidazolyl)cobamide (*31*).

AdoCbiP-BIPr, Ado(BI)Cba, and Ado(Im)Cba served as active coenzymes for diol dehydratase (Fig. 2). However, as shown in Fig. 2B, the diol dehyratase reaction with Ado(Im)Cba as the coenzyme was accompanied by concomitant irreversible inactivation of the enzyme during catalysis, catalysis ceasing completely within 12 min. This tendency is essentially the same as that observed with the imidazolyl trimethylene analog (AdoCbiP-ImPr in Fig. 1) (*13*) in which the D-ribose and 5,6-dimethylbenzimidazole moieties are

TABLE III. **Coenzyme activity and kinetic parameters for the analogs in the diol dehydratase and ethanolamine ammonia-lyase reactions.**[a]

Coenzyme	Diol dehydratase							Ethanolamine ammonia-lyase					
	k_{cat} (s^{-1})	(%)	k_{inact} (min^{-1})	k_{cat}/k_{inact} $\times10^{-4}$	K_m (μM)	$k_{cat}/K_m\times10^{-6}$ (M^{-1} s^{-1})	K_i (μM)	k_{cat} (s^{-1})	(%)	k_{inact} (min^{-1})	k_{cat}/k_{inact} $\times10^{-4}$	K_m (μM)	$k_{cat}/K_m\times10^{-6}$ (M^{-1} s^{-1})
AdoCbl[b]	366	(100)	0.014	157	0.80	458		281	(100)	0.110	16.2	0.070	4010
Ado(BI)Cba	348	(95)	0.028	75	0.96	363		256	(91)	0.122	12.8	0.074	3460
Ado(Im)Cba	216[e]	(59)	0.145	8.9	1.04	208		27[e]	(9)	2.58	0.063	0.95	280
AdoCbiP-DBIPr[c]	216	(59)	0.017	76	0.82	263							
AdoCbiP-BIPr	146[e]	(40)	0.091	9.6	2.7	54							
AdoCbiP-PyPr[d]	92[e]	(25)	0.37	1.5	0.29	317							
AdoCbiP-ImPr[c]	29[e]	(8)	0.50	0.35	0.99	29							
AdoCbiP-MBIPr	~0	(0)					2.7						

[a]Determined at 37°C. [b]From Ref. *27*. [c]From Ref. *13*. [d]From Ref. *14*. [e]From the initial velocity.

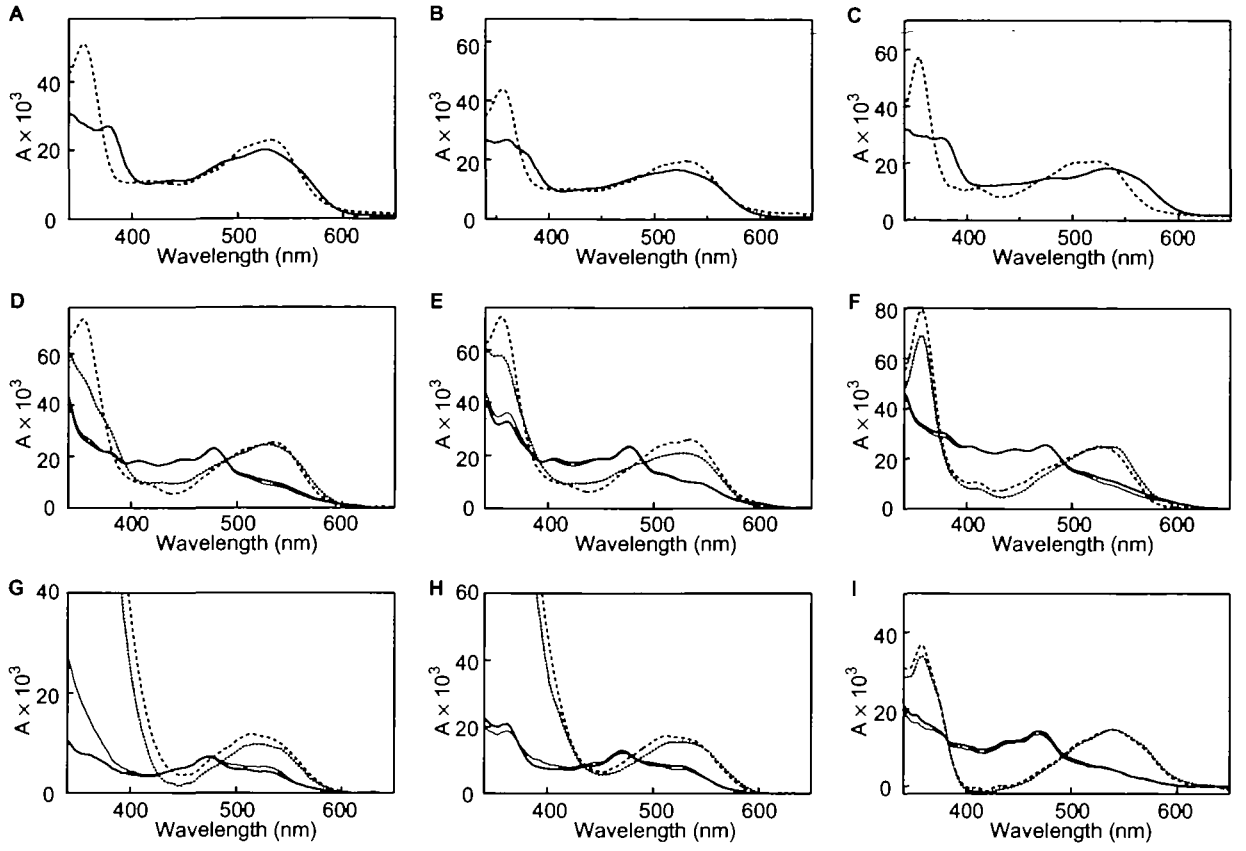

Fig. 3. **Spectral changes of coenzyme analogs upon incubation with apoenzymes of diol dehydratase and ethanolamine ammonia-lyase.** A–C: Free AdoCbl (A), Ado(BI)Cba (B), or Ado(Im)Cba (C) (3.5 μM) in 0.05 M potassium phosphate buffer (pH 8.0) containing 1 M 1,2-propanediol (solid lines). Spectra of the illuminated analogs were also taken (broken lines). D–F: Apodiol dehydratase (100 units/ml, 6.4 μM) was incubated with 3.5 μM AdoCbl (D), Ado(BI)Cba (E), or Ado(Im)Cba (F) in 0.05 M potassium phosphate buffer (pH 8.0) containing 1 M 1,2-propanediol, in a final volume of 1.0 ml. Spectra were taken at 5-min (thick solid lines) and 10-min (thin solid lines) incubation. After 10 min, the enzyme was denatured by adding 6 M guanidine-HCl/0.06 M citric acid. The pH of the mixture was 2.6. After 10 min at 37°C, the mixture was neutralized by adding 200 μl of 1 M potassium phosphate buffer (pH 8.0) and 70 μl of 5 N KOH, and then the spectrum was taken (dotted lines). Samples were finally illuminated at 0°C for 10 min with a 200-W tungsten light bulb at a distance of 15 cm (broken lines). G–I: Apoethanolamine ammonia-lyase (40 units/ml, 6.4 μM) was incubated with 3.5 μM AdoCbl (G), Ado(BI)Cba (H), or Ado(Im)Cba (I) in 0.05 M potassium phosphate buffer (pH 8.0) containing 0.2 M ethanolamine, in a final volume of 1.0 ml. Spectra were measured at 1-min (thin solid lines in H and I), 5-min (thick solid lines), and 10-min (thin solid line in G) incubation. Spectra of the denatured and neutralized (dotted lines) and illuminated (broken lines) samples were measured as described in D–F.

replaced by a trimethylene group and imidazole, respectively. AdoCbiP-BIPr also caused inactivation, but to a lesser extent. In contrast, the reaction with Ado(BI)Cba proceeded almost linearly with the incubation time. In Table III, the coenzymic activities and kinetic parameters of these coenzyme analogs for diol dehydratase are summarized. Ado(BI)Cba and Ado(Im)Cba showed essentially the same binding affinity for diol dehydratase, and were 95 and 59%, respectively, as active as the normal coenzyme, as judged from the initial activity. However, Ado(Im)Cba caused suicide inactivation of diol dehydratase during catalysis similar to AdoCbiP-ImPr. When the k_{cat}/k_{inact} values for Ado(BI)Cba and Ado(Im)Cba are compared with those for the corresponding trimethylene analogs, it is evident that the probability of suicide inactivation during catalysis with Ado(Im)Cba and Ado(BI)Cba is much lower than that with the respective trimethylene counterparts (Table III). This indicates that one of the roles of the ribosyl moiety in the nucleotide ligand is protection of the reaction intermediates from irreversible inactivation during catalysis. The apparent lower affinity of diol dehydratase for AdoCbiP-BIPr than for AdoCbiP-DBIPr (Table III) may be accounted for by the smaller contribution of the base-on form with the former analog.

For ethanolamine ammonia-lyase, another AdoCbl-dependent "base-on" enzyme, Ado(BI)Cba, was a highly effective coenzyme, whereas Ado(Im)Cba functioned as a very poor coenzyme. Figure 2C indicates that the ethanolamine ammonia-lyase reaction with the latter as the coenzyme was accompanied by rapid irreversible inactivation of the enzyme and ceased completely after 2 min, while the reaction with Ado(BI)Cba was essentially linear within about 5 min. This is similar to the results with diol dehydratase, but suicide inactivation takes place much more rapidly with ethanolamine ammonia-lyase. The coenzymic activity and kinetic parameters of the analogs for ethanolmine ammonia-lyase are also summarized in Table III. Ado(BI)Cba exhibited essentially the same K_m and k_{cat} values as the normal coenzyme. On the other hand, Ado(Im)Cba showed less than one-tenth of the initial activity of AdoCbl and

caused marked suicide inactivation of the enzyme. The k_{cat}/k_{inact} value for Ado(Im)Cba indicates that the holoenzyme with this coenzyme analog becomes inactivated after approximately 630 turnovers on average. This probability of inactivation is 14- and 5-times higher than that with Ado(Im)Cba and AdoCbiP-ImPr, respectively, in the diol dehydratase reaction. Thus, it is clear that Ado(Im)Cba is a much stronger suicide inactivator for ethanolamine ammonia-lyase than for diol dehydratase.

From these results, it was concluded that the 5,6-dimethylbenzimidazole moiety of the natural coenzyme is important for both the initial activity and continuous catalytic turnovers, possibly due to the bulkiness of the *Co*-coordinating base of the nucleotide loop.

Spectroscopic Studies—Figure 3, D–F, shows the spectral changes of AdoCbl, Ado(BI)Cba, and Ado(Im)Cba upon incubation with diol dehydratase in the presence of the substrate 1,2-propanediol. When compared with the spectra of free cobamide coenzyme or analogs (Fig. 3, A–C), in all cases, a new peak appeared at 478 nm, and a peak at ~525 nm disappeared within 5 min. The spectra obtained with Ado(BI)Cba and Ado(Im)Cba were quite similar to that seen with the normal coenzyme, and are thus assigned to that of the diol dehydratase-bound, base-on cob(II)-alamin (*32*). However, when the mixture was subsequently illuminated after denaturation of the enzyme by adding 6 M guanidine·HCl, further spectral changes were observed with the complex of diol dehydratase with AdoCbl and Ado(BI)Cba, but essentially not with the complex with Ado-(Im)Cba. These results indicated that at least a proportion of the Co-C bond cleavage of AdoCbl and Ado(BI)Cba was reversible before the denaturation, whereas the Co-C bond of Ado (Im)Cba had already been cleaved fully and irreversibly by the enzyme before denaturation. It should be noted that the diol dehydratase reaction proceeds almost linearly with time with AdoCbl and Ado(BI)Cba as coenzymes, whereas the majority of the enzyme activity is lost within 10 min with Ado(Im)Cba. Thus, it is very likely that the cob(II) alamin-like spectrum obtained with AdoCbl and Ado(BI) Cba should be assigned mainly to a steady-state concentration of Co(II)-containing intermediates, whereas the similar spectrum obtained with Ado(Im)Cba is likely due to a Co(II)-containing inactivated species. Similar spectra were previously observed for the inactivated holoenzyme with AdoCbiP-ImPr as well (*13*).

Figure 4 shows spectral changes of AdoCbiP-BIPr and AdoCbiP-MBIPr upon incubation with diol dehydratase in the presence of a substrate. It is suggested that the Co(II)-containing species was formed as an intermediate when AdoCbiP-BIPr was used as the coenzyme. In contrast, no spectral change was observed with AdoCbiP-MBIPr, at least within 120 min, although the binding affinity of diol dehydratase for this analog was pretty high (Table III).

The spectral changes of AdoCbl, Ado(BI)Cba, and Ado-(Im)Cba upon incubation with ethanolamine ammonia-lyase in the presence of ethanolamine are shown in Fig. 3, G–I. Again, a new peak appeared at 478 nm, and a peak at ~525 nm disappeared within 5 min with either coenzyme, indicating the formation of enzyme-bound Co(II)-containing cobamide from each coenzyme. In this case, accumulation of a large amount of product disturbed the spectral observations at wavelengths shorter than 450 nm. However, since the time courses of the ethanolamine ammonia-lyase reaction with AdoCbl and Ado(BI)Cba as coenzymes proceed linearly with incubation time, the spectra with these

TABLE IV. **Rate constants for the Co-C bond homolysis of the coenzymes by diol dehydratase with a deuterated substrate.**[a]

Coenzyme	$k_{h,app}$ at 4°C (s^{-1})
AdoCbl	2.3±0.2[b]
Ado(BI)Cba	3.7±0.3[c]
Ado(Im)Cba	2.0±0.2[d]

[a]Apparent rate constants for the Co-C bond homolysis were obtained from the average of 5–10 traces by curve fitting. [1,1-D_2]1,2-Propanediol was used as the substrate. [b]From two experiments. [c]From two experiments. [d]Means ± SD (n=5).

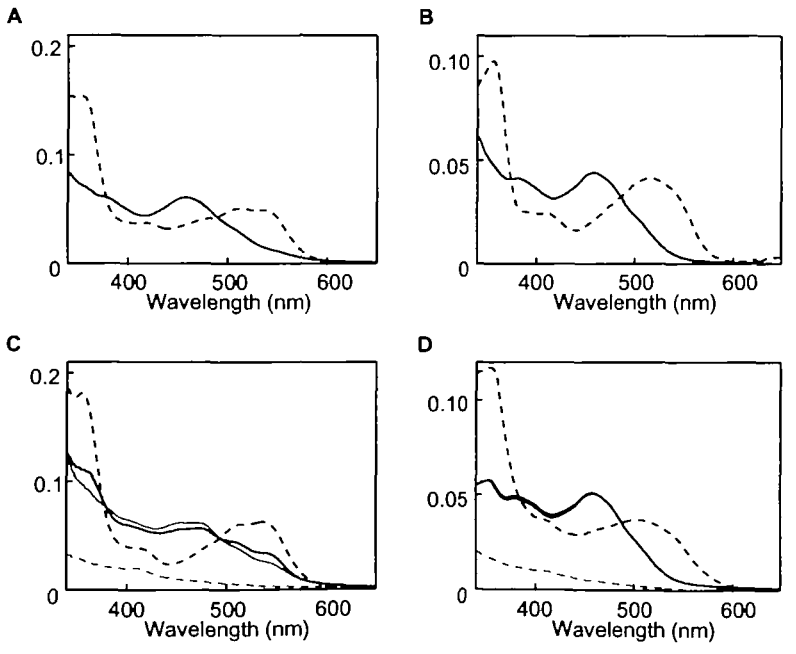

Fig. 4. **Spectral changes of coenzyme analogs upon incubation with apoenzyme of diol dehydratase.** A, B: Free AdoCbiP-BIPr (A) or AdoCbiP-MBIPr (B) (6.3 μM) in 0.05 M potassium phosphate buffer (pH 8.0) containing 1 M 1,2-propanediol (solid lines). Spectra of the illuminated analogs were also taken (broken lines). C, D: Apodiol dehydratase (185 units/ml, 8.0 μM) (thin broken lines) was incubated with 6.3 μM AdoCbiP-BIPr (C) or AdoCbiP-MBIPr (D) in 0.05 M potassium phosphate buffer (pH 8.0) containing 1 M 1,2-propanediol, in a final volume of 1.0 ml. Spectra were taken at 1-min (thin solid lines) and 5-min (thick solid lines) incubation. The enzyme was denatured, neutralized, and illuminated (broken lines), as described in the legend to Fig. 3.

TABLE V. **Correlation between k_{cat}/k_{inact} in the diol dehydratase reaction and the bulkiness or basicity of the *Co*-coordinating base.**

Analog	Group connecting phosphate and base	$k_{cat}/k_{inact}\times10^{-4}$	Base moiety			Ref.
			Co-coordinating base	Bulkiness	Basicity (pK_a)	
AdoCbl	Ribose	157	5,6-Dimethylbenzimidazole	Very large	6.09	*27*
Ado(BI)Cba	Ribose	75	Benzimidazole	Large	5.48	This study
Ado(Im)Cba	Ribose	8.9	Imidazole	Small	6.95	This study
AdoCbiP-DBIPr	$(CH_2)_3$	76	5,6-Dimethylbenzimidazole	Very large	6.09	*13*
AdoCbiP-BIPr	$(CH_2)_3$	9.6	Benzimidazole	Large	5.48	This study
AdoCbiP-PyPr	$(CH_2)_3$	1.5	Pyridine	Medium	5.19	*14*
AdoCbiP-ImPr	$(CH_2)_3$	0.35	Imidazole	Small	6.95	*13*
AdoCbiP-Me		~0	None	No	–	*16*

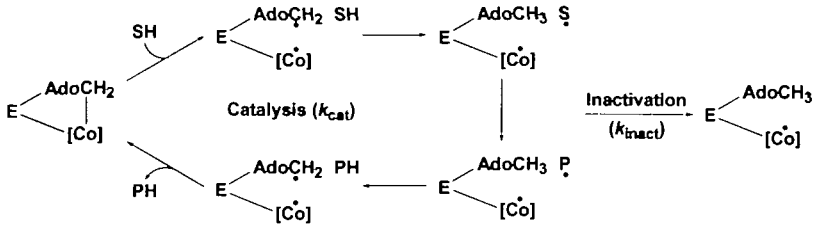

Fig. 5. **Catalysis and suicide inactivation of diol dehydratase. $AdoCH_2$, adenosyl; [Co], cobamide; SH, substrate; PH, product.**

coenzymes reflect a steady-state concentration of Co(II)-containing intermediates. In the case of Ado(Im)Cba, when the reaction mixture was subsequently illuminated after denaturation of the enzyme by adding 6 M guanidine·HCl, further spectral changes were not observed (Fig. 3I). This indicates that Ado(Im)Cba had already undergone complete and irreversible cleavage of the Co-C bond before the denaturation. This result, together with the finding that this analog causes complete suicide inactivation of ethanolamine ammonia-lyase within 2 min, strongly suggests that the cob(II)alamin-like spectrum obtained with Ado(Im)Cba is that of the completely inactivated species. This conclusion is very similar to that reached with diol dehydratase.

Stopped-flow Analysis of the Rate of Co-C Bond Cleavage of Coenzyme Analogs by Diol Dehydratase—To investigate the effect of the bulkiness of a lower axial base on the rate of Co-C bond cleavage of the coenzyme analogs, stopped-flow analysis was carried out with diol dehydratase. Since the binding of cobalamin to apodiol dehydratase is rather slow, the holoenzyme was preformed through anaerobic incubation of the apoenzyme with the coenzyme. When the holoenzyme was mixed with [1,1-D_2]1,2-propanediol in the presence of 2.0 molar equivalents of a coenzyme, a very rapid decrease in the absorbance at 525 nm was observed, indicating the conversion of the enzyme-bound coenzyme to cob (II) alamin. To minimize the error, the mixing was repeated at least 10 times at 0.5-s intervals. The rate constants for each coenzyme were obtained by curve fitting the average of five to ten traces. [1,1-D_2]1,2-propanediol was used as the substrate for accurate measurement of the rate constants, because the reaction with unlabeled substrate was too fast to be accurately measured (dead-time, *ca.* 3–4 ms). The rate constants for Co-C bond homolysis ($k_{h,app}$) summarized in Table IV indicate that the values are not very different among AdoCbl, Ado(BI)Cba, and Ado(Im)-Cba. Thus, it can be concluded that the rate of Co-C bond homolysis is essentially unaffected by the bulkiness of the *Co*-coordinating base of the nucleotide ligand.

DISCUSSION

We have previously studied the roles of the base moiety of the nucleotide loop using a series of coenzyme analogs in which the D-ribose and 5,6-dimethylbenzimidazole moieties were replaced by a trimethylene group and a base, respectively (*13–17*). In this study, more exact information about the structure-function relationship of the D-ribose and base moieties of the coenzyme was obtained by using two series of coenzyme analogs—cobamides and trimethylene analogs bearing artificial bases. The chromatographic and electrophoretic behaviors as well as absorption spectra indicated that, among the analogs tested in this study, only CbiP-MBIPr exists exclusively in the base-off form, irrespective of the kind of upper axial ligand. This is probably because of the steric repulsion between the corrin ring and the methyl group on C-2 of MBI.

The data obtained so far with various coenzyme analogs in the diol dehydratase system are summarized in Table V. These results demonstrate that coenzyme analogs having imidazole or pyridine each serve as a strong suicide coenzyme for both diol dehydratase and ethanolamine ammonia-lyase. The nature of this type of inactivation by coenzyme analogs has been analyzed in detail with AdoCbiP-ImPr (*13*) and AdoCbiP-Me (*16*). The inactivation is triggered by an undesired side reaction of the reactive radical intermediate(s) in the normal catalytic process or their escape from the active site, resulting in irreversible cleavage of the Co-C bond of the coenzymes (*13–17*) (Fig. 5). As regeneration of a coenzyme is impossible, the modification is irreversible. The fact that the modified coenzyme remains tightly bound to the cobalamin-binding site results in suicide inactivation of the enzymes. In the case of AdoCbiP-Me, the k_{cat}/k_{inact} value was almost zero (*16*). We proposed to call such an analog a "pseudocoenzyme." Here, we reached the important conclusion that the base moiety is obligatory for continuous catalytic turnovers. In the radical catalyzed reactions, one of the most important roles of enzymes is to protect highly-reactive radical intermediates

from undesired side reactions (*15, 17*). This idea is consistent with the concept of negative catalysis proposed by Rétey (*33*). In the cases of diol dehydratase and ethanolamine ammonia-lyase, at least a part of this pivotal role is played by the base moiety of the lower axial ligand. In Table V, the correlation between the k_{cat}/k_{inact} values and the bulkiness or basicity of the bases is compared. It is likely that the "stabilizing" effect of the base correlates well with its steric bulk rather than basicity in both series of coenzyme analogs containing D-α-ribose and trimethylene as a connecting group between the phosphate and a base. Therefore, the steric effect of the base seems more important than the electronic one for stabilizing radical intermediates during catalysis. That is to say, the bulkiness of the base may be essential for continuous catalytic turnover as well as for the initial activity. The same tendency was observed with cobamide analogs and trimethylene analogs, although the probability of inactivation with the cobamide analogs is much lower than that with the corresponding trimethylene analogs having the same base. This suggests an important role of the ribose moiety in the nucleotide ligand for protecting the enzyme from irreversible inactivation during catalysis. Such an effect of the α-D-ribofuranose moiety might be exerted through stabilization of the base-on form of cobamide coenzymes, because the presence of the ribose moiety in the nucleotide loop effects *Co*-coordination of the base from the lower axial position. The ribose moiety has been shown not to be essential for the activity but to be important as a spacer keeping the base 5,6-dimethylbenzimidazole in the proper position (*13*).

The rates of Co-C bond cleavage of adenosyl cobamide with different *Co*-coordinating bases were determined by stopped-flow kinetics with diol dehydratase. The data in Table IV show that the rate constants for the Co-C bond homolysis of Ado(Im)Cba and AdoCbl are the same. This rules out ground state mechanochemical triggering in diol dehydratase, since the enzyme would have to be less efficient at homolyzing Ado(Im)Cba than AdoCbl if this mechanism were in play (*6*). This is in contrast to the results for ribonucleotide reductase (*21*), where Brown *et al.* found that the enzyme-catalyzed Co-C bond homolysis was 17 times slower for Ado(Im)Cba than for AdoCbl (which means that ground state mechanochemical triggering is still possible).

These conclusions may suggest that the activating mechanism for Co-C bond cleavage differs between these two enzymes. The crystal structure of diol dehydratase reveals that it binds cobalamin in the base-on mode with the corrin ring almost planar (*9*). For diol dehydratase, we previously concluded, on the basis of the three-dimensional structure, that the Co-C bond of the coenzyme becomes activated through angular strains and tensile force that are produced through tight interactions between the enzyme and coenzyme at both the cobalamin moiety and the adenine ring of the adenosyl group (*1*). In this mechanism, a *Co*-coordinating base would not be directly involved in the Co-C bond activation. This is consistent with the data reported here and with our previous finding that the adenosyl form of cobinamide methyl phosphate also undergoes Co-C bond cleavage by diol dehydratase (*16*). At present, it is not clear why the bulky base 5,6-dimethylbenzimidazole in the lower axial position is important for continuous catalytic turnovers, but it might be reasonable to assume that the steric bulk of the *Co*-coordinating base affects the reactivity of the cobalt atom by altering the length of the Co-N bond. To examine this possibility, we are currently attempting the crystallization of the complex of diol dehydratase with the imidazolyl trimethylene analog of the coenzyme for X-ray analysis.

It is clear from the k_{cat}/k_{inact} values that the ethanolamine ammonia-lyase holoenzyme is more susceptible to inactivation during catalysis than diol dehydratase, even when AdoCbl is used as the coenzyme. When the imidazolyl cobamide was used as the coenzyme, the resulting holoenzyme underwent suicide inactivation after 630 turnovers on average. This number of catalytic turnovers before inactivation is much smaller than that with the same analog for diol dehydratase. This suggests that the stabilizing effect of a *Co*-coordinating base on the radical intermediate(s) in the ethanolamine ammonia-lyase reaction is much more sensitive to the bulkiness of the axial base than in the diol dehydratase reaction. We must await X-ray crystallographic analysis of the former enzyme to account for such a difference between the two enzymes.

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