Importance of the Nucleotide Loop Moiety Coordinated to the Cobalt Atom of Adenosylcobalamin for Coenzymic Function in the Diol Dehydrase Reaction

Atsuhiko ISHIDA,¹ Masakazu ICHIKAWA,¹ Kazuko KOBAYASHI,²
Torazo HITOMI,¹ Shinpei KOJIMA,^{1,*}
and Tetsuo TORAYA^{1,**}

¹*Department of Chemistry, College of Liberal Arts and Sciences,
Kyoto University, Sakyo-ku, Kyoto 606, Japan*

²*Department of Chemistry, Meiji College of Oriental Medicine,
Hiyoshi-cho, Funai-gun, Kyoto 629-03, Japan*

(Received October 23, 1992)

Summary Three analogs of adenosylcobalamin were synthesized and their coenzymic properties in the diol dehydrase reaction were studied. Neither adenosylcobinamide nor adenosylcobinamide phosphate was active as coenzyme and showed very low affinity for apoenzyme, irrespective of the presence of nucleotide loop fragments, such as 5,6-dimethylbenzimidazole, α -D-ribazole, or α -D-ribazole-3'-phosphate. The coordination of pyridine to the cobalt atom neither confers the coenzymic function upon adenosylcobinamide nor strengthens the inhibitory effect of cyanoaquacobinamide and methylcobinamide significantly. The analog of adenosylcobalamin in which the N-3 position of 5,6-dimethylbenzimidazole is methylated was also not active as coenzyme and showed very low affinity for apoenzyme. Since 3,5,6-trimethylbenzimidazole in this analog is no longer coordinated to the cobalt atom, these results show that at least a part of the nucleotide loop moiety coordinated to the cobalt atom of adenosylcobalamin is essential for tight binding to the apoenzyme

Abbreviations: AdoCbl, adenosylcobalamin; CN-Cbl, cyanocobalamin; aqCbl, aquacobalamin; MeCbl, methylcobalamin; Cbl, cobalamin; AdoCbi, adenosylcobinamide; MeCbi, methylcobinamide; (CN, aq) Cbi, cyanoaquacobinamide; Cbi, cobinamide; AdoCbi-P, adenosylcobinamide phosphate; (CN,aq)Cbi-P, cyanoaquacobinamide phosphate; AdoCbl(3-Me), *Coa*-[α -(3,5,6-trimethylbenzimidazolyl)]-*Co* β -adenosylcobamide; CN-Cbl(3-Me), *Coa*-[α -(3,5,6-trimethylbenzimidazolyl)]-*Co* β -cyanocobamide; DBI, 5,6-dimethylbenzimidazole; α -ribazole, α -D-ribazole or 5,6-dimethyl-1- α -D-ribofuranosyl-1H-benzimidazole; α -ribazole-P, α -D-ribazole-3'-phosphate.

* Present address: Department of Natural Environment Sciences, Faculty of Integrated Human Studies, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

** To whom correspondence should be addressed at the present address: Department of Biotechnology, Faculty of Engineering, Okayama University, Okayama 700, Japan.

and therefore for manifestation of coenzymic function.

Key Words adenosylcobalamin, vitamin B₁₂ coenzyme, diol dehydrase, adenosylcobinamide, adenosylcobinamide phosphate, *N*(3)-methylated adenosylcobalamin, α -ribazole, α -ribazole-3'-phosphate, nucleotide loop of adenosylcobalamin

AdoCbl (vitamin B₁₂ coenzyme) participates as coenzyme in more than ten kinds of enzymatic reactions. The structure-function relationship of this coenzyme has been extensively studied, but only a few papers appeared which deal with the roles of the nucleotide loop moiety in AdoCbl-dependent enzymatic catalysis. For diol dehydrase, for example, nucleotide-containing (so-called "complete") corrinoid coenzymes in which DBI is replaced with benzimidazole (1), adenine (1), 2-thioadenine (2), 6-methylmercaptapurine (3), or imidazole (4) were reported to serve as partially active coenzymes. In the preceding paper (5), we have shown that the analogs in which the D-ribose moiety of the nucleotide loop is replaced by tri- or tetramethylene group and a trimethylene analog containing imidazole instead of DBI are active coenzymes. The analog in which 2-amino-2-methyl-1-propanol is substituted for 1-amino-2-propanol was shown to be inactive (6). Kato *et al.* (7) reported that AdoCbi, an analog which lacks the entire nucleotide loop moiety, is partially active as coenzyme in the diol dehydrase reaction. However, they reached this conclusion using a microbial product tentatively identified as AdoCbi.

In contrast, we found that synthetic preparations of AdoCbi were completely inactive as coenzyme and showed very low affinity for the apoenzyme of diol dehydrase. In addition to AdoCbi, we synthesized AdoCbi-P, an incomplete corrinoid coenzyme, and AdoCbl(3-Me), a complete corrinoid coenzyme in which the *N*-3 position of DBI in the nucleotide loop is methylated and thus not coordinated to the cobalt atom. In this paper, we report the synthesis and coenzymic and binding properties of these analogs of AdoCbl in the diol dehydrase reaction. Importance of the nucleotide loop moiety coordinated to the cobalt atom is discussed here.

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. 5'-Iodo-5'-deoxyadenosine was a gift from Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan. 5'-Chloro-5'-deoxyadenosine was prepared by reaction of adenosine with SOCl₂, as described by Kikugawa and Ichino (8). 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 before (9) from cells of *Klebsiella oxytoca* ATCC 8724 (formerly *Klebsiella pneumoniae* and *Aerobacter aerogenes*) grown without aeration in a glycerol/1,2-pro-

panediol medium (10), except that 0.2% polypepton (Nihon Seiyaku) was used instead of 0.2% yeast extract (11).

Enzyme and protein assays. The activity of diol dehydrase was determined by the 3-methyl-2-benzothiazolinone hydrazone method (12). One unit is defined as the amount of enzyme activity catalyzing the formation of 1 μ mol of propionaldehyde per minute under the standard assay conditions.

Protein concentration was determined either by the method of Lowry *et al.* (13) or by measurement of the absorbance at 278 nm. An absorption coefficient of 5.27 for 10 mg of diol dehydrase per ml and for a 1 cm light path was used for the latter method (9).

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 the dicyano form by photolysis in the presence of 0.1 M KCN. ϵ_{367} of $30.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for dicyano Cbl was employed (14). Visible and ultraviolet spectra were taken on a Union model SM-401 recording spectrophotometer. Thin-layer chromatography (TLC) was performed on Merck silica gel G-60 plates. The solvent systems used are: A, 1-butanol/2-propanol/water (10:7:10, v/v); B, water-saturated 2-butanol; C, ethyl acetate/methanol (6:1, v/v); D, 1-butanol/2-propanol/water (10:7:10, v/v) containing 0.1 M KCN; E, water-saturated 2-butanol containing 0.1 M KCN; F, 1-butanol/water (86:14, v/v); G, water-saturated 2-butanol/acetic acid (99:1, v/v); H, water-saturated 2-butanol/28% aqueous ammonia (99:1, v/v); I, 1-butanol/acetic acid/water (4:1:1, v/v). Paper electrophoresis was carried out in 0.5 M acetic acid (pH 2.7) or 0.01 M potassium phosphate buffer (pH 7.0) for analyzing and purifying corrinoids. ^{31}P -NMR spectra were measured on a JEOL-FX-90Q (36.23

Fig. 1. Structures of the coenzyme analogs and the nucleotide loop fragments used in this study. [Co], corrin ring; Ado, adenosyl.

MHz) NMR spectrometer operating in the Fourier transform mode.

Preparation of (CN,aq)Cbi. (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 (15). The reaction mixture containing 1 g of CN-Cbl, 11.1 g of $\text{Ce}(\text{NO}_3)_3$, 2.1 g of NaOH and 0.18 g of KCN in 300 ml of H_2O was heated to 90–100°C for 1 h with stirring. After cooling to room temperature, the pH of the mixture was adjusted to 8.5 with aqueous ammonia. The mixture was kept overnight in a refrigerator and then filtered. The filtrate was desalted by phenol extraction and passed through a DEAE-cellulose (acetate) column. Dicyano Cbi in the through fraction was converted to the monocyano form by adding a small amount of acetic acid, followed by evaporation to dryness. The resulting (CN,aq)Cbi was further purified to homogeneity by column chromatography on phosphocellulose (adjusted to pH 6.0). The isolation yield was *ca.* 70–80%.

The purified preparation of (CN,aq)Cbi gave two orange-red spots in approximately equal amounts upon TLC in the absence of KCN (Table 1), although it was

Table 1. Chromatographic and electrophoretic behaviors of the analogs.

Corrinoid	R_f in TLC ¹				Relative mobility in paper electrophoresis ²	
	Solvent A	Solvent B	Solvent G	Solvent H	pH 2.7	pH 7.0
(CN,aq)Cbi	0.12	0.10	0.11	0.05	($\equiv$ 1)	($\equiv$ 1)
	0.07	0.06	0.06	0.02		
(CN,aq)Cbi-P			0.05	0.03	0.00	–1.00
CN-Cbl(3-Me)	0.08	0.07			0.84	0.78
	0.03	0.04				
AdoCbi	0.05	0.06	0.07	0.03	1.26	0.63
AdoCbi-P			0.04	0.05	0.64	–0.74
AdoCbl(3-Me)	0.01	0.01			1.25	0.53
CN-Cbl	0.22	0.07	0.12		(0)	(0)

¹On Merck silica gel G-60 precoated plates. ²At 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).

Table 2. Absorption spectra of the analogs.

Corrinoid	λ_{max} ($\epsilon \times 10^{-3}$) (nm ($\text{M}^{-1}\text{cm}^{-1}$))
(CN,aq)Cbi ¹	275 (11.4), 319 (10.5), 354 (26.8), 405 (4.2), 498 (7.8), 531 (7.3)
(CN,aq)Cbi-P ²	275 (10.0), 320 (10.1), 355 (27.7), 405 (4.44), 495 (8.2), 525 (7.9)
CN-Cbl(3-Me) ¹	277 (17.3), 320 (10.3), 354 (26.2), 406 (4.5), 498 (8.2), 531 (7.8)
AdoCbi ¹	263 (36.0), 304 (22.2), 380 (8.0), 459 (8.6)
AdoCbi-P ²	265 (46.9), 306 (26.8), 380 (8.7), 460 (9.0)
AdoCbl(3-Me) ¹	263 (40.8), 287 (22.6), 304 (22.2), 380 (8.1), 459 (8.8)

¹In 0.01 M potassium phosphate buffer (pH 7). ²In water.

established to be homogeneous in the presence of KCN. These correspond to the coordination isomers (*a*- and *b*-isomers), in which coordination positions of CN^- and H_2O are reversed (16). As shown in Table 2, the absorption spectrum of (CN,aq)Cbi coincided with that reported previously (17). Positive-ion fast atom bombardment-mass spectrometry (FAB-MS) indicated that neither the molecular ion nor *quasi*-molecular ions of (CN,aq)Cbi (MW 1,034) appear. Instead, three prominent peaks were observed at m/e 989, 991, and 992 with relative intensities of 1:0.81:0.39, respectively. The most intense peak of the three (m/e 989) can be assigned to the fragment ion formed by loss of HCN and H_2O .

Preparation of (CN,aq)Cbi-P. To 35 mg of (CN,aq)Cbi was added 0.3 ml of POCl_3 . After stirring for 3 days at room temperature, 3 ml of water were added dropwise to the mixture with stirring in an ice-water bath. Corrinoids were desalted by phenol extraction and applied to a DEAE-cellulose (acetate) column. The column was developed with water. The slowly-moving corrinoid fraction was collected and evaporated to dryness. (CN,aq)Cbi-P thus obtained in a yield of *ca.* 43% was essentially homogeneous as judged by TLC and paper electrophoresis. When this preparation was hydrolyzed with cerous hydroxide, the corrinoid product co-migrated with (CN,aq)Cbi upon paper electrophoresis in 0.5 M acetic acid (pH 2.7).

The presence of a phosphate group in the preparation of Cbi-P was confirmed by ^{31}P -NMR spectroscopy. When ^{31}P -NMR spectra were measured in $\text{H}_2\text{O}/\text{D}_2\text{O}$, (CN,aq)Cbi-P did not show detectable peaks (data not shown). On the other hand, when spectra were taken after adding a trace of KCN, the resulting dicyano form of Cbi-P exhibited a sharp singlet peak with a chemical shift of 5.24 ppm (85% H_3PO_4 as external standard). CN-Cbl also showed a sharp singlet peak with a chemical shift of 1.16 ppm in $\text{H}_2\text{O}/\text{D}_2\text{O}$ under the same conditions. These results suggest that the phosphate group of Cbi-P is coordinated to the cobalt atom in the absence of KCN.

Preparation of CN-Cbl(3-Me). The *N*-3 position of DBI of CN-Cbl was methylated with methyl iodide by a modification of a published procedure (18). To 100 mg of CN-Cbl in 20 ml of water were added 100 mg of KCN and 2 ml of methyl iodide. After stirring at room temperature for 12 days, 200 mg of KCN were supplemented every day, and 1 ml of methyl iodide was supplemented on the 14th day. The reaction was terminated on the 19th day by phenol extraction of the corrinoids. The conversion to the methylated derivative was *ca.* 90%. The corrinoids were converted to monocyano forms as described above and applied to a column of phosphocellulose (adjusted to pH 6). After washing the column with copious water, CN-Cbl(3-Me) was eluted with 50 mM NaCl. The purity of this corrinoid was established by paper electrophoresis in 0.5 M acetic acid and TLC in solvents D and E.

Conversion to adenosyl form. The adenosyl form of analogs (Fig. 1) was obtained by the reaction of NaBH_4 -reduced analogs with 5'-chloro-5'-deoxyadenosine or 5'-iodo-5'-deoxyadenosine. MeCbi was prepared by the similar

procedure using methyl iodide. The products were desalted by phenol extraction and purified by paper electrophoresis in 0.5 M acetic acid and preparative paper chromatography. Chromatographic and electrophoretic behaviors of adenosyl and monocyano forms of analogs are summarized in Table 1. Spectral data are shown in Table 2.

Preparation of α -ribazole-P. α -Ribazole-P was prepared essentially according to the procedure of Brown and Hakimi (19). CN-Cbl (1.0 g) was partially hydrolyzed at 25°C for 24 h in 75 ml of 2 M H₂SO₄. After cooling on ice, the pH of the mixture was adjusted to 3.2 with NaOH. The mixture was desalted by phenol extraction and applied to a XAD-2 column. The column was washed sequentially with water and 10% EtOH and then developed with 20% EtOH. α -Ribazole-P in the eluate was further purified by HPLC on a reversed-phase column (Cosmosil 5 C₁₈) using 30% methanol as solvent and preparative TLC in solvent I (R_f =0.22).

Preparation of α -ribazole. CN-Cbl (1.0 g) was hydrolyzed with cerous hydroxide, as described above. After removal of cerous hydroxide by filtration, the filtrate was diluted fivefold with water and applied to a Dowex 50 (H⁺) column. The column was washed with water and then developed with 30% EtOH containing 2 N pyridine. The eluate was concentrated to a small volume, and α -ribazole was purified by paper chromatography in solvent F and subsequent TLC in solvent B. R_f values of α -ribazole in TLC in solvents A, B, and F were 0.66, 0.61, and 0.55, respectively.

RESULTS

Coenzymic and binding properties of incomplete corrinoid coenzymes

AdoCbi and AdoCbi-P were completely inactive coenzymes in the diol dehydrase reaction (coenzymic activity less than 0.02% that of AdoCbl). These analogs inhibit this enzyme only partially even when they are preincubated with apoenzyme at a concentration 13–20-fold higher than that of AdoCbl prior to addition of AdoCbl. CN-Cbl and other Cbl completely inhibited the enzyme under the same conditions. Therefore, it is clear that the apoenzyme shows very low affinity for AdoCbi and AdoCbi-P. The K_i value for AdoCbi was determined to be 0.02–0.03 mM, whereas the K_m for AdoCbl was 0.80 μ M. These results indicate that the nucleotide loop moiety of AdoCbl plays an essential role in the binding to the apoenzyme and therefore in manifestation of coenzymic function. Introduction of a phosphate group to AdoCbi did not confer upon it either the coenzymic function or high affinity for the apoenzyme.

Effect of the nucleotide loop fragments on coenzymic and binding properties of incomplete corrinoid coenzymes

If apoprotein of diol dehydrase binds AdoCbl by so-called induced-fit mechanism, addition of the nucleotide loop fragments to the assay mixture might affect the coenzymic or binding property of the incomplete corrinoid coenzymes. The

Table 3. Inhibitory action of the analogs in the presence and absence of the nucleotide loop fragments or bases.¹

Analog ²	Addition ³	Inhibition (%)	
		With preincubation ⁴	Without preincubation ⁵
AdoCbi	None	39	33
AdoCbi	DBI	42	32
AdoCbi	α -ribazole	32	30
AdoCbi	α -ribazole-P	34	33
AdoCbi-P	None	52	
AdoCbi-P	α -ribazole	50	
AdoCbl(3-Me)	None	56	0
None	DBI	0	0
None	α -ribazole	0	0
None	α -ribazole-P	0	0
(CN,aq)Cbi	None	36	
(CN,aq)Cbi	Pyridine ⁶	35	
MeCbi	None	55	
MeCbi	Pyridine ⁷	64	
None	Pyridine ⁷	13	
CN-Cbl	None	100	70
aqCbl	None	100	
MeCbl	None	100	

¹ Inhibitory effect was determined with respect to 1 μ M AdoCbl. ² 20 μ M AdoCbi, 13 μ M AdoCbi-P or MeCbi, 11 μ M AdoCbl(3-Me), or 10 μ M CN-Cbl, aqCbl or MeCbl. ³ 0.2 mM unless otherwise indicated. ⁴ Apoenzyme was preincubated with each analog at 37°C for 30 min prior to addition of AdoCbl. ⁵ Each analog and AdoCbl were added to apoenzyme at the same time. ⁶ 0.2 M. ⁷ 0.1 M.

addition of DBI, α -ribazole or α -ribazole-P neither made them coenzymically active (data not shown) nor enhanced the extent of inhibition markedly (Table 3). Therefore, it can be concluded that the addition of the nucleotide loop fragments does not change the coenzymic or binding property of incomplete corrinoid coenzymes, that is, components of the nucleotide loop must be covalently linked to the corrinoid to exert their effects.

Effect of axial ligand coordination on the coenzymic and binding properties of incomplete corrinoids

Effects of coordination of an axial ligand to the cobalt atom of Cbi on coenzymic and binding properties were studied. As judged from absorption spectra, exogenously added α -ribazole or α -ribazole-P (0.2 mM) was not coordinated to the cobalt atom of AdoCbi or AdoCbi-P (data not shown). Since these compounds were not soluble at high concentrations, several bases were tested for their ability to be coordinated to the cobalt atom. The spectrum of AdoCbi did not significantly change in the presence of imidazole (10 mM), benzimidazole (1 mM),

Fig. 2. Effects of pyridine on the spectra of MeCbi and AdoCbi. A: MeCbi in 0.05 M potassium phosphate buffer (pH 8) in the absence (—) and presence of 0.1 M (---) or 0.5 M (— · —) pyridine. B: AdoCbi in 0.05 M potassium phosphate buffer (pH 8) in the absence (—) and presence (---) of 0.1 M pyridine.

or DBI (0.4 mM). As shown in Fig. 2B, however, AdoCbi was at least partially converted to AdoCbi-pyridine complex in the presence of 0.1 M pyridine. When apodiol dehydrase and substrate were added to this solution, the diol dehydrase reaction did not take place at all (data not shown). Since 0.1 M pyridine did not significantly inhibit the diol dehydrase reaction with AdoCbi as coenzyme (Table 3), it can be concluded that the formation of the six-coordinated complex itself is not sufficient for manifestation of coenzymic function.

As shown in Fig. 2A, the spectrum of MeCbi was markedly changed by the addition of pyridine (18). The spectrum obtained in the presence of 0.5 M pyridine was the same as that in the presence of 0.8 M pyridine, indicating that essentially all MeCbi forms the MeCbi-pyridine complex at these concentrations of pyridine. About 50% of MeCbi seems to exist as the complex with pyridine in the presence of 0.1 M pyridine. The coordination of pyridine to the cobalt atom of (CN,aq)Cbi was also confirmed spectroscopically (data not shown). These results indicate that

pyridine is readily coordinated to the cobalt atom of Cbi having a less bulky upper ligand than the adenosyl group. However, the extent of inhibition of diol dehydrase reaction by MeCbi or (CN,aq)Cbi with respect to AdoCbl was not potentiated by the presence of 0.1–0.2 M pyridine (Table 3). This indicates that the affinity of MeCbi or (CN,aq)Cbi for the apoenzyme was not increased by the coordination of pyridine to the cobalt atom. Thus, it is also likely that the formation of a six-coordinated complex is not sufficient for tight binding of the incomplete corrinoid to the apoenzyme.

Coenzymic and binding properties of AdoCbl(3-Me)

AdoCbl(3-Me) is a complete corrinoid coenzyme in which DBI is methylated in the *N*-3 position and thus not coordinated to the cobalt atom. This was confirmed by the fact that a visible absorption spectrum of this analog was almost identical to that of AdoCbi. That is, the analog exists exclusively as a base-off form. AdoCbl(3-Me) was totally inactive as coenzyme in the diol dehydrase reaction (coenzymic activity less than 0.02% that of AdoCbl). As shown in Table 3, the inhibitory effect of the analog was almost negligible when the analog and AdoCbl were added to apoenzyme at the same time. Precise determination of the K_i value for AdoCbl(3-Me) by the conventional method was difficult, because the inhibition was too weak. When 20 μ M AdoCbl(3-Me) was preincubated with apoenzyme prior to the addition of 1 μ M AdoCbl, the inhibition was still partial. These results are suggestive of its very low affinity for the apoenzyme. Therefore, it is evident that the presence of phosphate, ribose or DBI moiety in the nucleotide loop is necessary, but not sufficient, for coenzymic activity. At least a part of the nucleotide loop moiety coordinated to the cobalt atom of AdoCbl must be required for manifestation of coenzymic function and for tight binding to the apoenzyme.

DISCUSSION

From the data presented in this paper, we concluded that AdoCbi is totally inactive as coenzyme for diol dehydrase. This conclusion is in clear contrast to that of Kato *et al.* (7). They reported that a microbial product tentatively identified as AdoCbi is a partially active coenzyme with the affinity 20-fold lower than that of AdoCbl for the apoenzyme. The reason for this discrepancy is not clear, but our results were verified with several preparations of AdoCbi which were synthesized by well-established chemical methods using various reducing systems and adenosylating agents. A recent paper of Eberhard *et al.* (4) has reported the same conclusion as ours.

AdoCbi did not serve as coenzyme and showed very low affinity for the apoenzyme of diol dehydrase. It is clear that the nucleotide loop moiety is important for tight binding to the apoenzyme and therefore for catalysis. Introduction of a phosphate group to AdoCbi was apparently not effective for improving its coenzymic and binding properties. This may be because the phosphate group of

Cbi-P is coordinated to the cobalt atom, as suggested by ^{31}P -NMR measurements. In order to study the role of the phosphate group more properly, a new analog containing a phosphodiester rather than a phosphomonoester group would be required.

Addition of a nucleotide loop fragment to incomplete corrinoid coenzymes did not confer coenzymic activity upon them or increase their affinity for the apoenzyme. Added DBI, α -ribazole or α -ribazole-P was shown spectroscopically not to be coordinated to the cobalt atom of AdoCbi. Among the bases tested, pyridine, less bulky base than DBI, was significantly coordinated to the cobalt atom at a concentration of 0.1 M, although its coordination did not confer coenzymic activity on AdoCbi or increase the binding affinity for MeCbi or (CN,aq)Cbi. These results indicate that components of the nucleotide loop must be covalently bound to the coenzyme molecule. This may be due to the effect of approximation or so-called high "effective concentration" of the nucleotide loop in AdoCbl. In the complete corrinoids, the coordination of DBI to the cobalt atom is entropically much favored, and each component of the nucleotide loop is properly positioned by the coordination of DBI to the cobalt atom. Apoprotein of diol dehydrase seems to show high affinity for such a base-on form by interacting with at least a part of the nucleotide loop moiety coordinated to the cobalt atom. This idea is supported by the finding that AdoCbl(3-Me) was a totally inactive coenzyme and showed very low affinity for the apoenzyme. This analog did not inhibit the reaction at all when added together with AdoCbl at the same time, although the significant inhibition was observed when it was preincubated with the apoenzyme prior to the addition of AdoCbl. This may be suggestive of the slower rate of binding of AdoCbl(3-Me) to the apoenzyme than that of AdoCbl. Although this analog belongs to a complete corrinoid coenzyme, the coordination of DBI to the cobalt atom is prevented by quaternization of N-3. We have previously reported that the coenzyme analog in which the ribose moiety of the nucleotide loop is replaced by the trimethylene group is an efficient coenzyme and binds to the apoenzyme ($K_m=0.82\mu\text{M}$), as strongly as AdoCbl does ($K_m=0.80\mu\text{M}$) (5). The trimethylene analog containing imidazole instead of DBI also binds to the apoenzyme fairly tightly ($K_m=0.99\mu\text{M}$), although it serves as a poor coenzyme (5). Hence, it is likely that a component of the nucleotide loop interacting with apoenzyme is the phosphate group. This is consistent with the results of our recent study on the role of the phosphate group in the coenzymic function and in the tight binding to the apoenzyme using methyl ester of AdoCbi-P, which will be published elsewhere (20).

REFERENCES

- 1) Abeles, R. H., and Lee, H. A., Jr. (1961): An intramolecular oxidation-reduction requiring a cobamide coenzyme. *J. Biol. Chem.*, **236**, 2347-2350.
- 2) Hayashi, M., and Kamikubo, T. (1971): Biosynthesis and properties of 2-thioadenyl cobamide and its coenzyme form. *FEBS Lett.*, **15**, 213-216.

- 3) Uchida, U., Hayashi, M., and Kamikubo, T. (1973): *Propionibacterium arabinosum* ni yoru 6-methylmercaptapurine donyu korinoido no seishitsu. *Vitamins (Kyoto)*, **47**, 27–32.
- 4) Eberhard, G., Schlayer, H., Joseph, H., Fridrich, E., Utz, B., and Müller, O. (1988): Untersuchungen zur biologischen Funktion der Nucleotidbase von Vitamin B₁₂. *Biol. Chem. Hoppe-Seyler*, **369**, 1091–1098.
- 5) Toraya, T., and Ishida, A. (1991): Roles of the D-ribose and 5,6-dimethylbenzimidazole moieties of the nucleotide loop of adenosylcobalamin in manifestation of coenzymic function in the diol dehydrase reaction. *J. Biol. Chem.*, **266**, 5430–5437.
- 6) Friedrich, W., Heinrich, H. C., König, E., and Schulze, P. (1964): Chemical synthesis and some biological properties of the coenzyme forms of an alkanolamine-type B₁₂ antivitamin. *Ann. N. Y. Acad. Sci.*, **112**, 601–614.
- 7) Kato, T., Shimizu, S., and Fukui, S. (1964): Chemical studies on vitamin B₁₂ and its related compounds. IX. Formation of cobinamide coenzyme by cultivation of *Propionibacterium shermanii* in the presence of cobinamide. *J. Vitaminol.*, **10**, 89–98.
- 8) Kikugawa, K., and Ichino, M. (1971): Direct halogenation of sugar moiety of nucleoside. *Tetrahedron Lett.*, **2**, 87–90.
- 9) Poznanskaja, A. A., Tanizawa, K., Soda, K., Toraya, T., and Fukui, S. (1979): Coenzyme B₁₂-dependent diol dehydrase: Purification, subunit heterogeneity, and reversible association. *Arch. Biochem. Biophys.*, **194**, 379–386.
- 10) Abeles, R. H. (1966): Dioldehydrase. *Methods Enzymol.*, **9**, 686–689.
- 11) Toraya, T., and Ishida, A. (1988): Acceleration of cleavage of the carbon-cobalt bond of sterically hindered alkylcobalamins by binding to apoprotein of diol dehydrase. *Biochemistry*, **27**, 7677–7681.
- 12) Toraya, T., Ushio, K., Fukui, S., and Hogenkamp, H. P. C. (1977): Studies on the mechanism of the adenosylcobalamin-dependent diol dehydrase reaction by the use of analogs of the coenzyme. *J. Biol. Chem.*, **252**, 963–970.
- 13) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951): Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
- 14) Barker, H. A., Smyth, R. D., Weissbach, H., Munch-Petersen, A., Toohey, J. I., Ladd, J. N., Volcani, B. E., and Wilson, M. R. (1960): Assay, purification, and properties of the adenylobamide coenzyme. *J. Biol. Chem.*, **235**, 181–190.
- 15) Renz, P. (1971): Some intermediates in the biosynthesis of vitamin B₁₂. *Methods Enzymol.*, **18C**, 82–92.
- 16) Friedrich, W. (1966): Zur Struktur der Cyano-Aquo-Komplexe der Corrinoiden. *Z. Naturforsch.*, **21b**, 138–144.
- 17) Friedrich, W. (1966): Über einige Eigenschaften der Cyano-Aquo-Corrinoiden. *Z. Naturforsch.*, **21b**, 595–596.
- 18) Brodie, J. D. (1969): On the mechanism of catalysis by vitamin B₁₂. *Proc. Natl. Acad. Sci. U.S.A.*, **62**, 461–467.
- 19) Brown, K. L., and Hakimi, J. M. (1986): Heteronuclear NMR studies of cobalamins. 4. α -Ribazole-3'-phosphate and the nucleotide loop of base-on cobalamins. *J. Am. Chem. Soc.*, **108**, 496–503.
- 20) Ishida, A., and Toraya, T. (1993): Adenosylcobinamide methyl phosphate as a pseudocoenzyme for diol dehydrase. *Biochemistry*, **32**, 1535–1540.