Evidence for Axial Coordination of 5,6-Dimethylbenzimidazole to the Cobalt Atom of Adenosylcobalamin Bound to Diol Dehydratase†

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ABSTRACT: It was demonstrated by electron paramagnetic resonance (EPR) spectroscopy that organic radical intermediates disappeared and cob(II)alamin accumulated upon suicide inactivation of diol dehydratase by 2-methyl-1,2-propanediol. The resulting EPR spectra showed that the eight hyperfine lines due to the divalent cobalt atom of cob(II)alamin further split into triplets by the superhyperfine coupling to the ^{14}N nucleus. Essentially the same superhyperfine splitting of the octet into triplets was observed with [^{14}N]- and [^{15}N]apoenzyme. When the adenosyl form of [$^{14}N_2$]- and [$^{15}N_2$]imidazolyl analogues of the coenzyme [Toraya, T., and Ishida, A. (1991) *J. Biol. Chem. 266*, 5430−5437] was used with unlabeled apoenzyme, the octet showed superhyperfine splitting into triplets and doublets, respectively. Therefore, it was concluded that cobalamin is bound to this enzyme with 5,6-dimethylbenzimidazole coordinating to the cobalt atom. This conclusion is consistent with the fact that the consensus sequence forming part of a cobalamin-binding motif, conserved in methionine synthase and some of the other cobalamin enzymes, was not found in the deduced amino acid sequences of the subunits of diol dehydratase. Adenosylcobinamide methyl phosphate, a coenzyme analogue lacking the nucleotide moiety, underwent cleavage of the cobalt−carbon bond upon binding to the enzyme in the presence of substrate, forming a cob(II)inamide derivative without nitrogenous base coordination, as judged by EPR and optical spectroscopy. Therefore, this analogue may be a useful probe for determining whether the replacement of the 5,6-dimethylbenzimidazole ligand by a histidine residue takes place upon binding of cobalamin to proteins.

Recent X-ray crystallographic studies of Drennan et al. (*1*) on a Cbl[1]-containing fragment of *Escherichia coli* methionine synthase and of Mancia et al. (*2*) on *Propionibacterium shermanii* methylmalonyl-CoA mutase revealed that Cbl is bound to these enzymes with imidazole of the histidine residue coordinating to the cobalt atom in the lower axial (Coα) position. The nucleotide moiety containing 5,6-dimethylbenzimidazole is accommodated to the binding pocket as an extended tail. A hydrogen bonding network connects this histidine residue with neighboring aspartic acid and serine or lysine residues. On the basis of such structural information and site-directed mutagenesis, it has been proposed that the reactivity of cobalamin is modulated by a proton relay through the Co-His-Asp-Ser quartet in methionine synthase (*1*,*3*) or by movement of the Co-linked histidine in methylmalonyl-CoA mutase (*2*). It is of great interest that the sequence Asp-x-His-x-x-Gly containing the coordinating histidine residue is conserved in the deduced amino acid sequences of not only methionine synthase and methylmalonyl-CoA mutase but also glutamate mutase and 2-methyleneglutarate mutase (*1*, *4*, *5*). This finding suggests that the enzymes catalyzing AdoCbl-dependent rearrangements of carbon skeletons also bind the coenzyme in a similar manner. The EPR spectroscopic evidence for the replacement of 5,6-dimethylbenzimidazole by a histidine residue was reported for methionine synthase (*1*), methylmalonyl-CoA mutase (*6*), and glutamate mutase (*7*). Coordination of a histidine residue to the cobalt atom of *p*-cresolylcobamide was found with a corrinoid protein from *Sporomusa ovata* (*8*). Therefore, the coordination of a histidine residue of proteins to the cobalt atom may be a feature common to several cobalamin-binding enzymes or proteins.

In contrast, it was reported that 5-methoxybenzimidazolylcobamide and 5-hydroxybenzimidazolycobamide in corrinoid/iron-sulfur proteins from *Clostridium thermoaceticum* (*9*) and *Methanosarcina thermophila* (*10*), respectively, lack histidine ligation although their benzimidazole bases are uncoordinated to the cobalt atom. Recently, it has also been

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[1] Abbreviations: Cbl, cobalamin; Cbl^{II}, cob(II)alamin or B_{12r}; Cbl^{I}, cob(I)alamin or B_{12s}; AdoCbl, adenosylcobalamin or coenzyme B_{12}; EtCbl, ethylcobalamin; CbiP-ImPr, cobinamide 3-imidazolylpropyl phosphate; AdoCbiP-ImPr, adenosylcobinamide 3-imidazolylpropyl phosphate; CbiP-Me, cobinamide methyl phosphate; Cbi^{II}P-Me, cob(II)inamide methyl phosphate; AdoCbiP-Me, adenosylcobinamide methyl phosphate; Cbi^{II}, cob(II)inamide; Cbi^{I}, cob(I)inamide; AdoCbi, adenosylcobinamide; EtCbi, ethylcobinamide; EPR, electron paramagnetic resonance.

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reported that the base-coordinated Co(III) corrinoid in methanol:5-hydroxybenzimidazolylcobamide methyltransferase is converted to base-uncoordinated Co(II) corrinoid during the activation process (*11*). So far, however, there have been no papers which described conclusive evidence for coordination of 5,6-dimethylbenzimidazole to the cobalt atom of the protein-bound AdoCbl or other cobamides.

During the course of our structure−function studies on AdoCbl with nucleotide moiety-modified analogues (*12*−*14*), we have reported that their coenzymic function in the diol dehydratase reaction changes dramatically by substitution of imidazole or pyridine for 5,6-dimethylbenzimidazole. Such findings suggest that 5,6-dimethylbenzimidazole coordinates to the cobalt atom of AdoCbl in diol dehydratase. This paper presents direct EPR-spectroscopic evidence for the coordination of 5,6-dimethylbenzimidazole to the cobalt atom of the enzyme-bound AdoCbl. An attempt to use AdoCbiP-Me, a coenzyme analogue lacking the nucleotide moiety, as a probe for exploring the cobalamin binding sites of the enzyme is also described here.

EXPERIMENTAL PROCEDURES

Materials. Crystalline AdoCbl is a gift from Eisai Co., Ltd., Tokyo, Japan. AdoCbiP-Me was synthesized as described previously (*13*). EtCbl and EtCbi were prepared by reaction of Cbl^{I} and Cbi^{I}, respectively, with ethyl bromide (*15*). AdoCbiP-ImPr and AdoCbiP-[$^{15}N_2$]ImPr, the adenosyl form of unlabeled and [$^{15}N_2$]imidazolyl trimethylene analogues, in which the D-ribose moiety and 5,6-dimethylbenzimidazole of AdoCbl are replaced by the trimethylene group and imidazole, respectively, were synthesized as described before (*12*) using unlabeled and ^{15}N-labeled imidazole, respectively. Electrospray ionization (ESI)-mass spectra were measured on a Micromass AutoSpec-OA-TOF mass spectrometer. 2-Methyl-1,2-propanediol was synthesized by acid hydrolysis of isobutylene oxide. Other chemicals were reagent-grade commercial products. Apoenzyme of *Klebsiella oxytoca* diol dehydratase was purified to homogeneity from *E. coli* JM109 harboring expression plasmid pUSI2E-(DD) (*16*) which was grown aerobically in the LB medium containing 1,2-propanediol and induced by isopropyl-1-thio-β-D-galactopyranoside (*17*). ^{15}N-labeled apoenzyme was obtained from the recombinant *E. coli* strain grown in the M-9 minimal medium containing $^{15}NH_4Cl$ instead of $^{14}NH_4Cl$.

Enzyme and Protein Assays. Diol dehydratase activity was determined by the 3-methyl-2-benzothiazolinone hydrazone method (*18*). One unit is defined as the amount of enzyme activity that catalyzes the formation of 1 μmol of propionaldehyde per minute. Protein concentration of purified enzyme preparations was determined by measuring the absorbance at 278 nm. An extinction coefficient of 5.27 for 10 mg of diol dehydratase/mL and for a 1-cm light path was used (*19*).

EPR Measurements. Holoenzyme and the complexes of the enzyme with AdoCbiP-ImPr and AdoCbiP-Me were formed by incubating apoenzyme (ca. 100 units, 4.55 nmol unless otherwise indicated; specific activity more than 90 units/mg) at 25 °C for 5 min with 50 nmol of AdoCbl and the corresponding analogues, respectively, in 0.65 mL of 0.05 M potassium phosphate buffer (pH 8.0) and 13−18 mM octyl-β-D-thioglucoside or 5−10 mM sucrose monocaprate under a nitrogen atmosphere. 2-Methyl-1,2-propanediol (5 μmol) or 1,2-propanediol (50 μmol) was then added to start the reaction. After 1 min at 4 °C, the mixture was rapidly frozen in an isopentane bath (cooled to ca. −160 °C) and then in a liquid nitrogen bath. The sample transferred to the EPR cavity was cooled with a cold nitrogen gas flow controlled by a JEOL JES-VT 3A temperature controller. EPR spectra were taken at −130 °C on JEOL JES-RE 3X spectrometer modified with a Gunn diode X-band microwave unit. The mixture was then incubated at 25 °C for 3 min and frozen again as described above for the second EPR measurement. The mixture was incubated at 25 °C for an additional 30 min for the third EPR measurement.

RESULTS AND DISCUSSION

Motif Analysis of Deduced Amino Acid Sequences of Diol Dehydratase. Diol dehydratase consists of the three subunits, α, β, and γ, which are encoded by the *pddA*, *pddB*, and *pddC* genes, respectively (*16*). We have reported primary structures of these subunits deduced from the nucleotide sequences of the above-mentioned genes encoding diol dehydratase. The consensus sequence Asp-x-His-x-x-Gly forming part of a Cbl-binding motif conserved in methionine synthase, methylmalonyl-CoA mutase, and some of the other cobalamin enzymes was not found in the deduced amino acid sequences of the subunits of *K. oxytoca* diol dehydratase. This sequence is not conserved in either *Klebsiella pneumoniae* or *Citrobacter freundii* glycerol dehydratases, whose genes have recently been cloned and analyzed for sequences in this and other laboratories (*20*, *21*), *Salmonella typhimurium* ethanolamine ammonia-lyase (*22*, *23*), or *Lactobacillus leichmannii* ribonucleotide reductase (*24*). These facts may indicate that these four enzymes bind cobalamin without displacement of the 5,6-dimethylbenzimidazole Coα-ligand by a histidine residue of proteins.

EPR Spectroscopic Evidence for Coordination of 5,6-Dimethylbenzimidazole to the Cobalt Atom of the Coenzyme in Holodiol Dehydratase. Diol dehydratase holoenzyme undergoes rapid suicide inactivation by 2-methyl-1,2-propanediol (*25*). The inactivation rate constant with this diol was 0.99 min^{-1} when determined by a dilution method as described by Bachovchin et al. (*25*). Figure 1 illustrates the time course of inactivation of the holoenzyme by 2-methyl-1,2-propanediol. Essentially all the activity was lost upon incubation of the holoenzyme with the diol at 37 °C for 15 min. The EPR spectrum obtained after reaction with this substrate at 4 °C for 1 min is shown in Figure 2A. No appreciable amount of organic radical intermediates from this substrate was detected as compared with that from 1,2-propanediol (Figure 2B). In Figure 2B, high-field doublet peaks and low-field broad signals are assigned to an organic radical intermediate and low-spin Co(II) of Cbl^{II}, respectively (*26*). The signals due to EPR-active species increased with time of incubation at 25 °C and reached maximum by 33 min (Figure 2A). Since the enzyme activity had been completely lost by this time, the spectrum must represent the one of the inactivated holoenzyme. The EPR spectrum obtained resembled that of Cbl^{II} (Figure 2C), indicating that the enzyme-bound AdoCbl was converted to Cbl^{II} upon inactivation of the holoenzyme by 2-methyl-1,2-propanediol. This conclusion is consistent with the previous observation by optical spectroscopy (*25*). A prominent resonance with

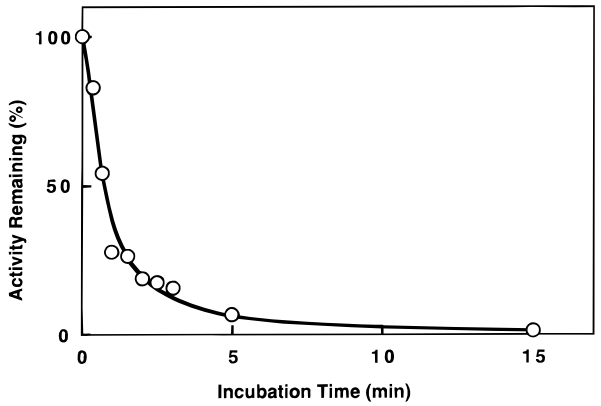

FIGURE 1: The time course of inactivation of holodiol dehydratase by 2-methyl-1,2-propanediol. Substrate-free apoenzyme (24 units) was incubated at 37 °C with 15 nmol of AdoCbl and 100 mmol of 2-methyl-1,2-propanediol in 0.04 M potassium phosphate buffer (pH 8.0). Ten-microliter aliquots were withdrawn at the indicated time and diluted in 0.99 mL of 0.05 M potassium phosphate buffer (pH 8.0) containing 0.1 M 1,2-propanediol for measurements of enzyme activity remaining. The amount of propionaldehyde formed by 10-min incubation at 37 °C was determined as described in the text.

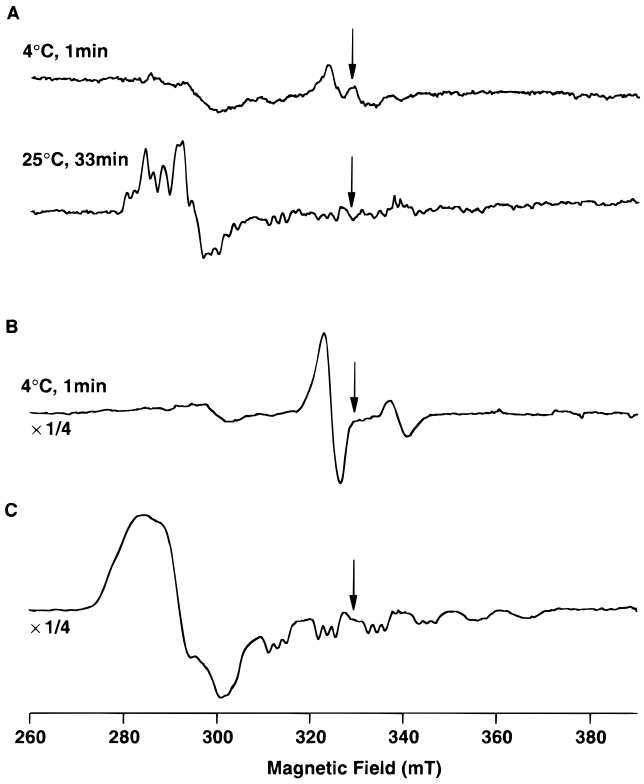

FIGURE 2: EPR spectra obtained upon incubation of holodiol dehydratase with 2-methyl-1,2-propanediol. Holoenzyme was incubated with 2-methyl-1,2-propanediol at 4 °C for 1 min and then at 25 °C for an additional 33 min (A). The spectra of reacting holoenzyme with 1,2-propanediol at 4 °C for 1 min (B) and Cbl^{II} (70 μM) (C) were measured as controls. Cbl^{II} was formed by photolysis of EtCbl. EPR spectra were taken as described in the text at −130 °C. The arrows correspond to $g = 2.0$. EPR microwave frequency, 9.174−9.176 GHz. Microwave power, 10 mW. Modulation amplitude, 1 mT; modulation frequency, 100 kHz.

a $g_\perp$ value of 2.2−2.3 and eight broad signals centered at a $g_\parallel$ value of 2.0 can be assigned to an unpaired electron of Co(II) of Cbl^{II}. Hyperfine splitting into an octet is due to the interaction of the unpaired spin of Co(II) with the cobalt

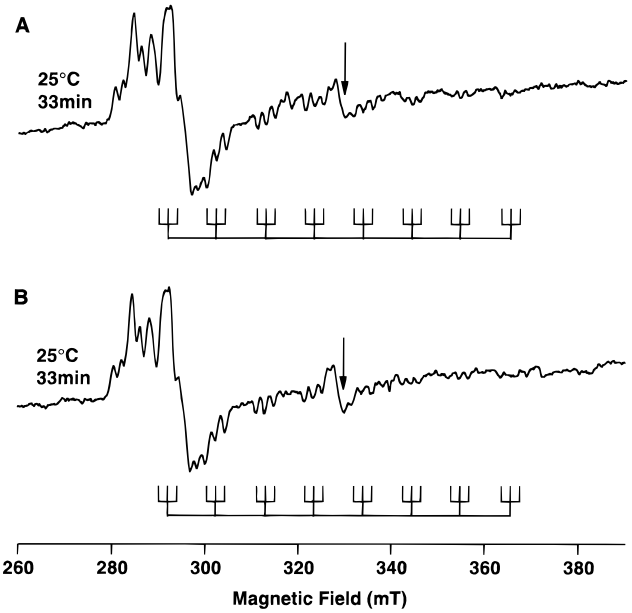

FIGURE 3: EPR spectra of 2-methyl-1,2-propanediol-inactivated holoenzyme obtained with [^{14}N]- and [^{15}N]apoenzyme. Unlabeled apoenzyme (A) and ^{15}N-labeled apoenzyme (B) were purified from *E. coli* JM109 carrying pUSI2E(DD) grown in M-9 minimal medium containing $^{14}NH_4Cl$ and $^{15}NH_4Cl$, respectively. Holoenzyme formed by incubation with unlabeled AdoCbl was further incubated at 25 °C for 33 min with 7 mM 2-methyl-1,2-propanediol. EPR spectra were taken at −130 °C as described in the legend to Figure 2. The arrows correspond to $g = 2.0$.

nucleus ($I = 7/2$) (coupling constant, 10.5 mT). Superhyperfine splitting of the hyperfine lines (peaks in the $g_\parallel$ region) into triplets (coupling constant, 2.0 mT) is due to the interaction with the ^{14}N nucleus ($I = 1$) of a nitrogenous base coordinating to Co(II) in the lower axial position.

To determine whether the coordinating nitrogen atom belongs to 5,6-dimethylbenzimidazole of the coenzyme or imidazole of a histidine residue of the enzyme, the same experiment was performed with ^{15}N-labeled apoenzyme which was purified from *E. coli* JM109 carrying expression plasmid pUSI2E(DD) grown in the M-9 minimal medium containing $^{15}NH_4Cl$ instead of $^{14}NH_4Cl$. As illustrated in Figure 3B, the holoenzyme incubated with 2-methyl-1,2-propanediol at 25 °C for 33 min showed essentially the same EPR spectrum as that obtained with [^{14}N]apoenzyme (Figure 3A). That is, superhyperfine splitting of the hyperfine lines into triplets was clearly observed, which highly suggests that Co(II) of Cbl^{II} bound to diol dehydratase is coordinated by ^{14}N of the coenzyme's 5,6-dimethylbenzimidazole but not by ^{15}N of the apoenzyme. If ^{15}N were the Coα ligand, the peaks should split into doublets due to the coupling with the nuclear spin of ^{15}N ($I = 1/2$). Essentially the same spectrum was also obtained with the apoenzyme purified from the *E. coli* strain grown in the medium containing both $^{15}NH_4Cl$ and unlabeled histidine (data not shown).

EPR Spectroscopic Evidence for Coordination of the Coenzyme's Base to the Cobalt Atom in the Diol Dehydratase−Imidazolyl Coenzyme Analogue Complex. To demonstrate that superhyperfine splitting of the hyperfine lines reflects a nuclear spin of the nitrogen atom of the Co-coordinating base, similar experiments of suicide inactivation by 2-methyl-1,2-propanediol were carried out using unlabeled apoenzyme (480 units) and AdoCbiP-ImPr or AdoCbiP-[$^{15}N_2$]-

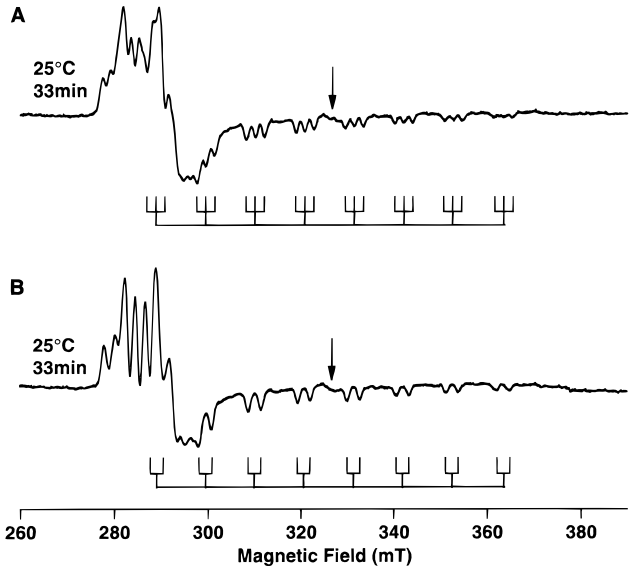

FIGURE 4: EPR spectra of 2-methyl-1,2-propanediol-inactivated complexes of diol dehydratase with [$^{14}N_2$]- and [$^{15}N_2$]imidazolyl trimethylene analogues. The complexes of the enzyme with AdoCbiP−ImPr (A) and AdoCbiP-[$^{15}N_2$]ImPr (B) were formed as described in the text from 480 units of apoenzyme and further incubated at 25 °C for 33 min with 7 mM 2-methyl-1,2-propanediol. EPR spectra were taken at −130 °C as described in the legend to Figure 2, except for microwave power of 20 mW. The arrows correspond to $g = 2.0$.

ImPr. AdoCbiP-ImPr is an analogue of AdoCbl in which the D-ribose moiety and 5,6-dimethylbenzimidazole are replaced by the trimethylene group and imidazole, respectively (*12*). This analogue serves as both a poor coenzyme and a potent suicide coenzyme for diol dehydratase (*12*). The ESI-mass spectra indicated that CN-CbiP-ImPr (MW 1203) and CN-CbiP-[$^{15}N_2$]ImPr (MW 1205) gave quasi-molecular ions (MH^+) at *m*/*e* 1204 and 1206, respectively.

As shown in Figure 4A, superhyperfine splitting of the hyperfine lines into triplets was observed again with the unlabeled imidazolyl analogue (AdoCbiP-ImPr). However, the octet showed superhyperfine splitting into doublets (coupling constant, 2.7 mT) when the [$^{15}N_2$]imidazolyl analogue (AdoCbiP-[$^{15}N_2$]ImPr) was used as coenzyme (Figure 4B). These results clearly indicate that superhyperfine splitting of the hyperfine lines is due to the coupling with a nuclear spin of the nitrogen atom of the Co-coordinating base and that Co(II) of the enzyme-bound [$^{15}N_2$]-imidazolyl analogue is coordinated by ^{15}N of the coenzyme's imidazole but not by ^{14}N of the apoenzyme. Coupling constants of the hyperfine and superhyperfine splitting summarized in Table 1 are in good agreements with the reported values for free Cbl^{II} and Cbi^{II} (*27*, *28*) and protein-bound Co(II) corrinoids (*1*, *6*−*8*, *29*). From these results, it can be concluded that the cobalamin coenzyme is bound to diol dehydratase with the 5,6-dimethylbenzimidazole ligand coordinating to the cobalt atom in the lower axial position.

Utility of CbiP-Me as a Probe for Exploring the Cobalamin-Binding Sites. To develop a convenient method for determining whether the replacement of 5,6-dimethylbenzimidazole by a histidine residue of proteins takes place upon binding of cobalamin to proteins without X-ray crystallographic analysis or ^{15}N-labeling of proteins, diagnosis using CbiP-Me was attempted. AdoCbiP-Me behaves as a pseudocoenzyme or a very efficient suicide coenzyme for

Table 1: Coupling Constants of the Hyperfine and Superhyperfine Splitting in the $g_{||}$ Region for Free and Diol Dehydratase-Bound Co(II) Corrinoids[a]

Co(II) corrinoid	$A_{||}$ (mT) (multiplicity[b]) Co	^{14}N	^{15}N
Cbl^{II} (free)	10.7 (*o*)	1.9 (*t*)	
Cbi^{II} (free)	14.1 (*o*)		
[^{14}N]E·[$^{14}N_2$-DBI]Cbl^{II}	10.5 (*o*)	2.0 (*t*)	
[^{15}N]E·[$^{14}N_2$-DBI]Cbl^{II}	10.5 (*o*)	2.0 (*t*)	
[^{14}N]E·Cbi^{II}P-[$^{14}N_2$]ImPr	10.7 (*o*)	2.0 (*t*)	
[^{14}N]E·Cbi^{II}P-[$^{15}N_2$]ImPr	10.7 (*o*)		2.7 (*d*)
[^{14}N]E·Cbi^{II}P-Me	14.0 (*o*)		

[a] Relative spin concentrations observed were 0.5−0.8 spin/mol of enzyme for E·Cbl^{II} and E·Cbi^{II}P-ImPr and 0.1−0.3 spin/mol of enzyme for E·Cbi^{II}P-Me. [b] *o*, *t*, and *d* indicate octet, triplet, and doublet, respectively.

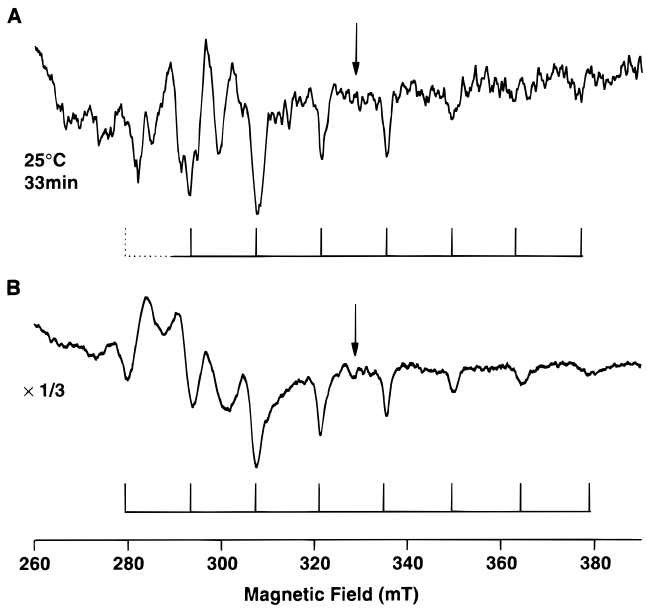

FIGURE 5: The EPR spectrum obtained upon incubation of the enzyme−AdoCbiP-Me complex with 2-methyl-1,2-propanediol. The enzyme−AdoCbiP-Me complex formed as described in the text from 240 units of apoenzyme was incubated with 7 mM 2-methyl-1,2-propanediol at 25 °C for 3 min (A). The spectrum of Cbi^{II} (70 μM) was measured as a control (B). Cbi^{II} was obtained by photolysis of EtCbi. EPR spectra were taken at −130 °C as described in the legend to Figure 2, except for microwave power of 20 mW. The arrows correspond to $g = 2.0$.

diol dehydratase (*13*). This coenzyme analogue was rapidly converted to Cbi^{II}P-Me upon incubation with apodiol dehydratase in the presence of 2-methyl-1,2-propanediol. The EPR spectrum of the enzyme−Cbi^{II}P-Me complex obtained by 3-min incubation at 25 °C is shown in Figure 5A. The spectrum resembled that of Cbi^{II} (Figure 5B), and the hyperfine lines with a coupling constant of 14.0 mT did not show superhyperfine splitting into triplets. Essentially the same EPR spectrum was obtained by 33-min incubation as well (data not shown). Therefore, it is evident that a nitrogenous base of the apoenzyme does not coordinate to the cobalt atom of Cbi^{II}P-Me.

This conclusion is consistent with that obtained by optical spectroscopy. Absorption maximum in the spectrum of the enzyme−Cbi^{II}P-Me complex (λ_{max} = 474 nm) (*13*), which was obtained by incubation of apoenzyme with AdoCbiP-Me in the presence of 1,2-propanediol, was compared with

those of various Co(II) corrinoids bound to diol dehydratase. 5,6-Dimethylbenzimidazole-coordinated and 5,6-dimethylbenzimidazole-uncoordinated forms of free Cbl^{II} possess λ_{max} at 473 and 470 nm, respectively (*30*, *31*). On the other hand, diol dehydratase-bound Cbl^{II} in reacting holoenzyme or in the reconstituted complex shows λ_{max} at 478 nm (*18*, *32*). The complexes of the enzyme with imidazolyl and pyridyl trimethylene analogues of AdoCbl undergo suicide inactivation during catalysis, forming Co(II) corrinoid species whose absorption spectra with λ_{max} at 477−478 nm are very similar to Cbl^{II} (*12*, *14*). From the comparison of these λ_{max} values, it was strongly suggested that the absorption spectrum of Cbl^{II} exhibits red shift by 4−5 nm upon binding to the enzyme and that diol dehydratase binds CbiP-Me without coordination to the cobalt atom in the lower axial position. Thus, CbiP-Me seems to be a useful probe for exploring the Cbl-binding sites by EPR and optical spectroscopy.

Padmakumar et al. (*6*) have recently reported that AdoCbi undergoes spectral changes to a base-on form upon binding to methylmalonyl-CoA mutase. However, AdoCbi is not a useful probe for diol dehydratase because this analogue shows a very low affinity for diol dehydratase as compared with AdoCbiP-Me (*33*).

Recently, Poppe and Rétey (*34*) have found that *p*-cresolylcobamide coenzyme serves as a highly active coenzyme for methylmalonyl-CoA mutase but as an inhibitor for diol dehydratase and glycerol dehydratase. Since the *p*-cresolyl analogue exists exclusively in the base-off form, this observation supports our conclusion described above.

All the data presented in this paper offer conclusive evidence for coordination of 5,6-dimethylbenzimidazole to the cobalt atom of AdoCbl bound to diol dehydratase. We have demonstrated with diol dehydratase that the presence of a bulky base like 5,6-dimethylbenzimidazole in the loop is essential for protecting reactive radical intermediates from undesired side reactions (*12*−*14*), but not absolutely required for activation of the Co−C bond by apoenzyme, since AdoCbiP-Me undergoes homolysis of the Co−C bond upon binding to the enzyme (*13*). It is now evident in diol dehydratase that the bulky base exerts such effect through coordination to the cobalt atom. However, the mechanism of stabilization of the radical intermediates during catalysis by coordination of the bulky base remains at present to be elucidated.

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