J. Biochem. 124, 598-601 (1998)

EPR Spectroscopic Evidence for the Mechanism-Based Inactivation of Adenosylcobalamin-Dependent Diol Dehydratase by Coenzyme Analogs[1]

Mamoru Yamanishi,* Seiki Yamada,* Atsuhiko Ishida,* Jun Yamauchi,† and Tetsuo Toraya*,[2]

*Department of Bioscience and Biotechnology, Faculty of Engineering, Okayama University, Tsushima-Naka, Okayama, 700-8530; and †Graduate School of Human and Environmental Studies, Kyoto University, Sakyo-ku, Kyoto 606-8302

Received for publication, April 13, 1998

EPR spectra were measured upon incubation of the complex of diol dehydratase with coenzyme analogs in the presence of 1,2-propanediol, a physiological substrate. When the analog in which the D-ribose moiety of the nucleotide loop was replaced by a trimethylene group was used as coenzyme, essentially the same EPR spectrum as that with adenosylcobalamin was obtained. The higher-field doublet and the lower-field broad signals derived from an organic radical and low-spin Co(II) of cob(II)alamin, respectively, were observed. With the imidazolyl counterpart, base-on cob(II)alamin-like species accumulated, but signals due to an organic radical quickly disappeared. When a coenzyme analog lacking the nucleotide moiety was incubated with apoenzyme in the presence of substrate, the EPR spectrum resembling cob(II)inamide was obtained, but no signals due to an organic radical were observed. From these results, it was concluded that the extinction of organic radical intermediates results in inactivation of the enzyme by these coenzyme analogs. Upon suicide inactivation with a [$^{15}N_2$]imidazolyl analog, the octet signals due to Co(II) showed superhyperfine splitting into doublets, indicating axial coordination of 5,6-dimethylbenzimidazole to the cobalamin bound to diol dehydratase.

Key words: adenosylcobalamin, coenzyme B_{12}, diol dehydratase, electron paramagnetic resonance, mechanism-based inactivation.

AdoCbl (coenzyme B_{12}) is a naturally occurring organometallic compound and undergoes activation of its Co-C bond when bound to apoenzyme. It is widely accepted that the radical species formed by homolysis of the Co-C bond participate in enzymatic catalysis. Diol dehydratase (DL-1,2-propanediol hydro-lyase, EC 4.2.1.28) catalyzes AdoCbl-dependent conversion of 1,2-propanediol, 1,2-ethanediol, and glycerol to propionaldehyde, acetaldehyde, and 3-hydroxypropionaldehyde, respectively (*1-3*). Evidence for the formation of radical intermediate(s) in the diol dehydratase (*4-6*) and glycerol dehydratase (*7*) reactions has been obtained by EPR spectroscopy.

During the course of our studies on the structure-function relationship of AdoCbl, we have synthesized a series of coenzyme analogs in which the D-ribose moiety of the nucleotide loop was replaced by a trimethylene group (*8, 9*). Although the time course of diol dehydratase reaction with the 5,6-dimethylbenzimidazolyl trimethylene analog (AdoCbiP-DBIPr) as coenzyme was essentially linear for at least 20 min, the enzymatic reaction with the imidazolyl counterpart (AdoCbiP-ImPr) was accompanied by inactivation during catalysis. AdoCbiP-Me, an analog lacking the nucleotide moiety, was totally inactive as coenzyme and underwent rapid, irreversible cleavage of the Co-C bond in the presence of substrate, resulting in inactivation of the enzyme (*10*). Thus, it was suggested that a bulky base like 5,6-dimethylbenzimidazole of the coenzyme is important for catalytic turnovers of the diol dehydratase reaction.

In the present paper, we report EPR spectroscopic evidence for the mechanism-based inactivation of diol dehydratase by these nucleotide moiety-modified analogs of the coenzyme.

MATERIALS AND METHODS

Materials—Crystalline AdoCbl was a gift from Eisai, Tokyo. AdoCbiP-DBIPr, AdoCbiP-ImPr, and AdoCbiP-Me were synthesized as described before (*8, 10*). AdoCbiP-[$^{15}N_2$]ImPr was synthesized by the same method as

[1] This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas (Molecular Biometallics, No. 07229234 and 08249226) from the Ministry of Education, Science, Sports and Culture of Japan, and research grants from Okayama Foundation for Science and Technology and from Japan Society for the Promotion of Science (Research for the Future, RFTF96L00506).

[2] To whom correspondence should be addressed. Fax: +81-86-251-8264, E-mail: toraya@biotech.okayama-u.ac.jp

Abbreviations: AdoCbiP-DBIPr, adenosylcobinamide 3-(5,6-dimethylbenzimidazolyl)propyl phosphate; AdoCbiP-ImPr, adenosylcobinamide 3-imidazolylpropyl phosphate; AdoCbiP-Me, adenosylcobinamide methyl phosphate; AdoCbl, adenosylcobalamin; Cbi', cob(I)inamide; Cbl', cob(I)alamin; Cbi'', cob(II)inamide; Cbl'', cob(II)alamin; EtCbi, ethylcobinamide; EtCbl, ethylcobalamin.

AdoCbiP-ImPr, except that [$^{15}N_2$]imidazole (ICON, New York, USA) was used instead of unlabeled imidazole. EtCbl and EtCbi were prepared by reaction of Cbl^{I} and Cbi^{I}, respectively, with ethyl bromide (*11*). Apoenzyme of *Klebsiella oxytoca* diol dehydratase was purified to homogeneity from *Escherichia coli* JM109 harboring expression plasmid pUSI2E(DD) (*12*), as described previously (*13*).

Enzyme Assay—Diol dehydratase activity was determined by the 3-methyl-2-benzothiazolinone hydrazone method (*14*). One unit is defined as the amount of enzyme activity that catalyzes the formation of 1 μmol of propionaldehyde/1 min.

EPR Measurements—Substrate-free apoenzyme was obtained by dialysis against 300-500 volumes of 0.05 M potassium phosphate buffer (pH 8) with two buffer changes. Complexes of the enzyme with coenzyme analogs were formed by incubating the substrate-free apoenzyme (100-500 units, 4.6-23 nmol) at 25°C for 5 min with 50 nmol of corresponding analogs in 0.65 ml of 0.05 M potassium phosphate buffer (pH 8) and 13-18 mM octyl-β-D-thioglucoside or 5-10 mM sucrose monocaprate under a nitrogen atmosphere (*15*). 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 N_2 gas flow controlled by a JEOL JES-VT3A temperature controller. EPR spectra were taken at −130°C on JEOL JES-RE3X spectrometer modified with a Gunn diode X-band microwave unit. EPR microwave frequency, 9.174-9.176 GHz. Modulation amplitude, 1 mT; modulation frequency, 100 kHz. EPR spectra were measured at a microwave power of 20 mW to obtain better signal-to-noise ratio of the signals due to a Co(II) species, because they saturate at a higher power than the doublet signals due to an organic radical. The mixture was then incubated at 25°C for 3 min and frozen again as described above for the second measurement. The mixture was incubated at 25°C for additional 30 min for the third EPR measurement.

RESULTS AND DISCUSSION

EPR Spectrum with the 5,6-Dimethylbenzimidazolyl Trimethylene Analog—EPR spectra were measured upon incubation of the diol dehydratase-AdoCbiP-DBIPr complex with 1,2-propanediol. As shown in Fig. 1A, the reacting complex gave essentially the same spectrum as that with the reacting holoenzyme (enzyme-AdoCbl complex in the presence of substrate) (Fig. 1C). The high-field doublet ($g=2.04$ and 1.95) and the low-field broad signals are assigned to an organic radical and low-spin Co(II), respectively (*16*). The doublet peaks ($J=14.4$ mT) due to the organic radical are suggested to arise from a weak exchange interaction with an unpaired electron of Co(II). The relative spin concentration for the doublet signals was 0.1-0.3 spin/mol of enzyme, which seems to reflect a steady-state concentration of the organic radical intermediate. Upon consumption of the substrate within 3 min of incubation at 25°C, the signals due to an organic radical and low-spin Co(II) disappeared (Fig. 1B). This is probably because of the recombination of adenosyl radical and Co(II), regenerating the coenzyme analog. These results are consistent with the fact that the diol dehydratase reaction with this analog as coenzyme is not accompanied by inactivation at a significant rate ($k_{cat}/k_{inact}=7.0\times10^5$) (*8*).

EPR Spectra with the Imidazolyl Trimethylene Analog—When AdoCbiP-ImPr was used as coenzyme, the high-field

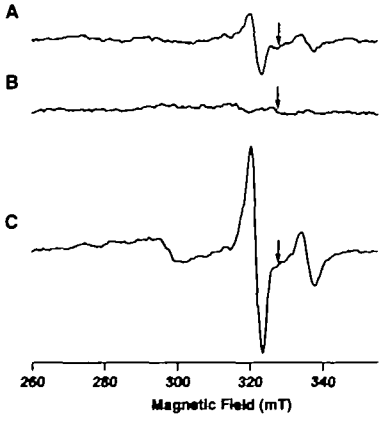


Fig. 1. **EPR spectra of the reacting 5,6-dimethylbenzimidazolyl trimethylene analog-diol dehydratase complex with substrate.** The complex of enzyme (100 units) with AdoCbiP-DBIPr was formed as described in the text and further incubated at 4°C for 1 min (A) and at 25°C for 3 min (B) with 1,2-propanediol. The spectrum of reacting holoenzyme (enzyme-AdoCbl complex) (100 units) with 1,2-propanediol at 4°C for 1 min (C) was measured as control. 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, 20 mW. Modulation amplitude, 1 mT; modulation frequency, 100 kHz. Sweep width, 150 mT; center field, 325 mT; sweep time, 6 min; time constant, 0.3 s.

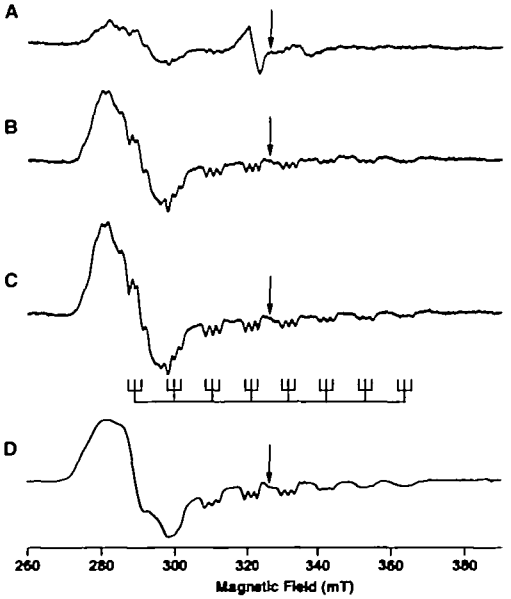


Fig. 2. **EPR spectra obtained upon incubation of the imidazolyl trimethylene analog-diol dehydratase complex with substrate.** The complex of enzyme (480 units) with AdoCbiP-ImPr was formed as described in the text and further incubated at 4°C for 1 min (A), and at 25°C for 3 min (B) and 33 min (C) with 1,2-propanediol. The spectrum of Cbl^{II} (70 μM) (D) was measured as control. Cbl^{II} was formed by photolysis of EtCbl. EPR spectra were taken at −130°C, as described in the legend to Fig. 1. The arrows correspond to $g=2.0$.

doublet signal due to an organic radical was faint even at the initial stage of reaction (Fig. 2A) and completely disappeared within 3 min of incubation at 25°C (Fig. 2B). The spectrum obtained finally after 33 min of incubation at 25 °C (Fig. 2C) resembled that of free, base-on Cbl^{II} (Fig. 2D). A prominent resonance with $g_{\perp}$ of 2.2-2.3 and eight broad signals centered at $g_{\parallel}$ of 2.0 can be assigned to an unpaired electron of Co(II) of Cbi^{II}P-ImPr. Hyperfine splitting into an octet (coupling constant, 10.7 mT) is due to the interaction with the Co nucleus ($I=7/2$), and superhyperfine splitting into triplets (coupling constant, 2.0 mT) is due to the interaction with the ^{14}N nucleus ($I=1$). The relative spin concentration of 0.5-0.8 spin/mol of enzyme seems to represent the amount of Cbi^{II}P-ImPr species accumulated during the inactivation. These results are consistent with the fact that this analog serves as an efficient suicide coenzyme ($k_{cat}/k_{inact}=3.3\times10^3$) for diol dehydratase (*8*) and indicate strongly that the extinction of organic radical intermediate(s) causes rapid inactivation of the enzyme by this coenzyme analog.

EPR Spectrum with the Nucleotide Moiety-Lacking Analog—Although AdoCbiP-Me is totally inactive as coenzyme, it undergoes rapid Co-C bond homolysis upon binding to diol dehydratase in the presence of substrate and brings about irreversible inactivation of the enzyme (*10*). Upon incubation of the enzyme-AdoCbiP-Me complex with 1,2-propanediol, essentially no signals due to organic radicals were observed even at the initial stage of reaction (Fig. 3A). The EPR spectrum of the resulting enzyme-Cbi^{II}P-Me complex (Fig. 3B) obtained after 3 min of incubation resembled that of free Cbi^{II} (Fig. 3C). No superhyperfine splitting was observed in the hyperfine lines (coupling constant, 14.0 mT), suggesting that a nitrogenous base in the apoprotein is not coordinating to the cobalt atom of Cbi^{II}P-Me. These results are consistent with the fact that this analog behaves as a potent suicide coenzyme ($k_{cat}/k_{inact}\sim0$) (*10*). The relative spin concentration was low (0.1-0.3 spin/mol of enzyme) and was not increased by 33 min of incubation at 25°C (data not shown).

EPR Spectrum with the [^{15}N]Imidazolyl Trimethylene Analog—The EPR spectrum shown in Fig. 2C indicates that a nitrogenous base is coordinating to Co(II) in the lower axial position. To identify the coordinating base, the same experiment was performed using unlabeled AdoCbiP-ImPr and AdoCbiP-[$^{15}N_2$]ImPr as coenzymes. With the unlabeled imidazolyl analog, each line of the hyperfine octet (coupling constant, 10.7 mT) showed superhyperfine splitting into triplets (coupling constant, 2.0 mT) (Fig. 4A). With the [$^{15}N_2$]imidazolyl analog, however, the hyperfine lines (coupling constant, 10.7 mT) showed superhyperfine splitting into doublets (coupling constant, 2.7 mT) (Fig. 4B). The ratio of the coupling constant with ^{14}N (A^{14}_N) to that with ^{15}N (A^{15}_N) can be expressed theoretically as follows:

$$A^{14}_N/A^{15}_N=\gamma^{14}_N/\gamma^{15}_N=0.713 \quad \text{(theoretical)}$$

where γ is a gyromagnetic ratio. The A^{14}_N/A^{15}_N value observed here was 0.741, which is in good agreement with the theoretical one. Both of these observations indicate clearly that the axial base coordinating to Co(II) of the enzyme-bound [$^{15}N_2$]imidazolyl analog is not ^{14}N ($I=1$) of the apoenzyme but ^{15}N ($I=1/2$) of the coenzyme's imidazole. The fact that the EPR spectrum shown in Fig. 3B resembling that of Cbi^{II} did not show superhyperfine splitting also indicates that no nitrogenous bases in the apoenzyme are coordinating to the cobalt atom. These results offer clear evidence for axial coordination of 5,6-dimethylbenzimidazole to the diol dehydratase-bound AdoCbl.

Mechanism of Inactivation of Diol Dehydratase by Coenzyme Analogs—With AdoCbiP-ImPr, it was demonstrated by EPR spectroscopy that the extinction of organic radical intermediate(s) results in inactivation of diol dehydratase by this coenzyme analog during catalysis. With AdoCbiP-Me, almost no signals due to organic

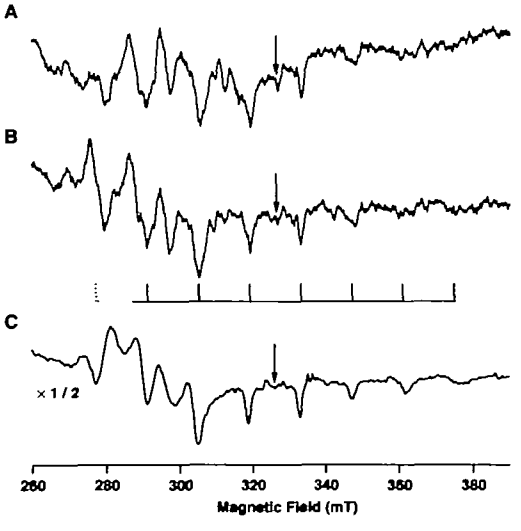


Fig. 3. **EPR spectra obtained upon incubation of the nucleotide moiety-lacking analog-diol dehydratase complex with substrate.** The complex of enzyme (240 units) with AdoCbiP-Me was formed as described in the text and further incubated at 4°C for 1 min (A) and at 25°C for 3 min (B) with 1,2-propanediol. The spectrum of Cbi^{II} (70 μM) (C) was measured as control. Cbi^{II} was formed by photolysis of EtCbi. EPR spectra were taken at −130°C, as described in the legend to Fig. 1. The arrows correspond to $g=2.0$.

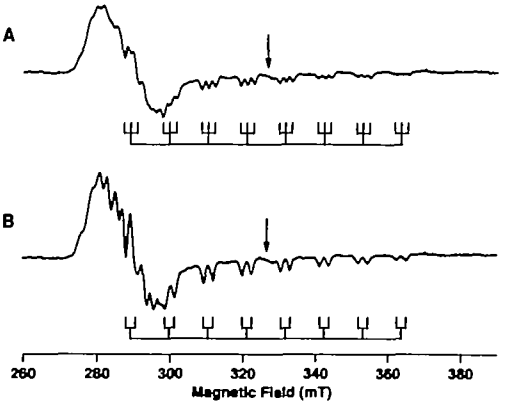
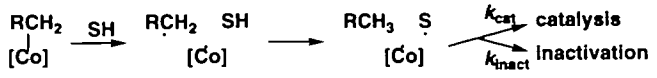


Fig. 4. **EPR spectra of 1,2-propanediol-inactivated complexes of diol dehydratase with [$^{14}N_2$] and [$^{15}N_2$]imidazolyl trimethylene analogs.** The complexes of enzyme (480 units) with unlabeled AdoCbiP-ImPr (A) and AdoCbiP-[$^{15}N_2$]ImPr (B) were formed as described in the text and further incubated at 25°C for 33 min with 1,2-propanediol. EPR spectra were taken at −130°C as described in the legend to Fig. 1. The arrows correspond to $g=2.0$.



Scheme 1. **A possible mechanism of inactivation of diol dehydratase by nucleotide moiety-modified coenzyme analogs.** [Co], CbiP-ImPr or CbiP-Me; RCH_3, 5′-deoxyadenosine; SH, substrate.

radical(s) were detected, although its Co-C bond underwent rapid and irreversible cleavage upon binding to the enzyme in the presence of substrate. In both cases, a Co(II) corrinoid accumulated during the inactivation. The only product derived from the adenosyl group was identified as 5′-deoxyadenosine (*8, 10*). The apoenzyme itself was considered not to be modified in the inactivation process, since the apoenzyme recovered from the inactivated complexes by acid ammonium sulfate treatment was reconstitutable into catalytically active holoenzyme upon incubation with AdoCbl. The inactivation took place only in the presence of substrate. From these lines of evidence, it was concluded that the inactivation by AdoCbiP-ImPr and AdoCbiP-Me is mechanism-based. That is, these analogs undergo activation of their Co-C bond by the enzyme in the presence of substrate, and the initial steps of the catalytic cycle take place (Scheme 1). An adenosyl radical formed by the Co-C bond homolysis abstracts a hydrogen atom from the substrate, forming 5′-deoxyadenosine and a substrate radical. At least one of the radical intermediates is unstable and undergoes undesired side reactions. As a result, the organic radical(s) disappears. The highly reactive radical intermediates must become extinct in the only way destined for the reaction. Once a reactive radical intermediate is quenched by side reactions, regeneration of the coenzyme becomes impossible, resulting in suicide inactivation of the enzyme. From k_{cat}/k_{inact} ratios of <18 and 3.3×10^3 for AdoCbiP-Me and AdoCbiP-ImPr, respectively (*8, 10*), it is evident that AdoCbiP-Me is a much more efficient suicide inactivator than AdoCbiP-ImPr. We therefore propose to call the former a suicide coenzyme and the latter a pseudocoenzyme ($k_{cat}/k_{inact}\sim0$). EPR spectroscopic evidence for the mechanism-based inactivation by AdoCbl analogs is reported here for the first time.

As shown here with AdoCbl-dependent diol dehydratase, it is very likely that radical enzyme catalysis generally tends to be accompanied by mechanism-based inactivation with high probability.

We thank Ms. Yukiko Kurimoto for her assistance in manuscript preparation.

REFERENCES

1. Lee, H.A., Jr. and Abeles, R.H. (1963) Purification and properties of dioldehydrase, an enzyme requiring a cobamide coenzyme. *J. Biol. Chem.* **238**, 2367-2373
2. Toraya, T., Shirakashi, T., Kosuga, T., and Fukui, S. (1976) B_{12}-dependent diol dehydrase: glycerol as both a good substrate and a potent inactivator. *Biochem. Biophys. Res. Commun.* **69**, 475-480
3. Toraya, T. (1994) Diol dehydrase and glycerol dehydrase, coenzyme B_{12}-dependent isozymes. *Metal Ions Biol. Systems* **30**, 217-254
4. Finlay, T.H., Valinsky, J. Mildvan, A.S., and Abeles, R.H. (1973) Electron spin resonance studies with dioldehydrase. Evidence for radical intermediates in reactions catalyzed by coenzyme B_{12}. *J. Biol. Chem.* **248**, 1285-1290
5. Valinsky, J.E., Abeles, R.H., and Mildvan, A.S. (1974) Electron spin resonance studies with dioldehydrase. II. The hyperfine structure of proposed radical intermediates derived from isotopic substitution in 2-chloroacetaldehyde. *J. Biol. Chem.* **249**, 2751-2755
6. Valinsky, J.E., Abeles, R.H., and Fee, J.A. (1974) Electron spin resonance studies on diol dehydrase. 3. Rapid kinetic studies on the rate of formation of radicals in the reaction with propanediol. *J. Am. Chem. Soc.* **96**, 4709-4710
7. Cockle, S.A., Hill, H.A.O., Williams, R.J.P., Davies, S.P., and Foster, M.A. (1972) The detection of intermediates during the conversion of propane-1,2-diol to propionaldehyde by glycerol dehydrase, a coenzyme B_{12} dependent reaction. *J. Am. Chem. Soc.* **94**, 275-277
8. 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
9. Toraya, T., Miyoshi, S., Mori, M., and Wada, K. (1994) The synthesis of a pyridyl analog of adenosylcobalamin and its coenzymic function in the diol dehydratase reaction. *Biochim. Biophys. Acta* **1204**, 169-174
10. Ishida, A. and Toraya, T. (1993) Adenosylcobinamide methyl phosphate as a pseudocoenzyme for diol dehydrase. *Biochemistry* **32**, 1535-1540
11. Smith, E.L. and Mervyn, L. (1963) Coenzyme B_{12} and analogs synthesized from hydridocobalamin. *Biochem. J.* **86**, 2p-3p
12. Tobimatsu, T., Hara, T., Sakaguchi, M., Kishimoto, Y., Wada, Y., Isoda, M., Sakai, T., and Toraya, T. (1995) Molecular cloning, sequencing, and expression of the genes encoding adenosylcobalamin-dependent diol dehydrase of *Klebsiella oxytoca*. *J. Biol. Chem.* **270**, 7142-7148
13. Tobimatsu, T., Sakai, T., Hashida, Y., Mizoguchi, N., Miyoshi, S., and Toraya, T. (1997) Heterologous expression, purification, and properties of diol dehydratase, an adenosylcobalamin-dependent enzyme of *Klebsiella oxytoca*. *Arch. Biochem. Biophys.* **347**, 132-140
14. 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
15. Toraya, T., Yamanishi, M., Muguruma, H., Ushio, K., Yamauchi, J., and Kawamura, T. (1997) An electron paramagnetic resonance study on the mechanism-based inactivation of adenosylcobalamin-dependent diol dehydrase by glycerol and other substrates. *Biochim. Biophys. Acta* **1337**, 11-16
16. Schepler, K.L., Dunham, W.R., Sands, R.H., Fee, J.A., and Abeles, R.H. (1975) A physical explanation of the EPR spectrum observed during catalysis by enzymes utilizing coenzyme B_{12}. *Biochim. Biophys. Acta* **397**, 510-518