Coenzyme Action of Adenosyl-13-epicobalamin in the Diol Dehydrase System†

Tetsuo Toraya, Tadahiro Shirakashi, Saburo Fukui,* and Harry P. C. Hogenkamp

ABSTRACT: Adenosyl-13-epicobalamin is able to function as a coenzyme in the adenosylcobalamin-dependent diol dehydrase system (EC 4.2.1.28) from *Aerobacter aerogenes*. This coenzyme analog shows approximately 14% of the coenzyme activity of adenosylcobalamin, while its apparent K_m value (12.7 μM) is 13 times higher than that of adenosylcobalamin (0.99 μM). The kinetics of the reaction and the apoenzyme-adenosyl-13-epicobalamin interaction suggest a lower affinity of the enzyme for the coenzyme analog. The coenzyme analog is readily dissociated even from the reacting apoenzyme-adenosyl-13-epicobalamin complex upon gel filtration. Like the native holoenzyme, the apoenzyme-coenzyme analog complex undergoes inactivation by oxygen in the absence of substrate; however, the rate of inactivation is much slower than that of the apoenzyme-adenosylcobalamin complex.

The treatment of cyanocobalamin with trifluoroacetic acid or other highly acidic reagents yields, in addition to cyanocobinamide, two darker red compounds (Bonnett et al., 1971a,b). X-Ray analysis established that in these new corrinoids the propionamide side chain e attached to C-13 is projected up instead of down relative to the plane of the corrin ring (Stoeckli-Evans et al., 1972). This epimerization at C-13 causes a change in the conformation of ring C, but does not significantly alter the rest of the corrin ring. Hodgkin and coworkers (Stoeckli-Evans et al., 1972) demonstrated that in adenosylcobalamin the 5′-deoxyadenosyl moiety is located directly above C-13 and thus in adenosyl-13-epicobalamin the propionamide side chain e should hinder the proper positioning of the adenosyl moiety. Recently Tkachuck et al. (1974) prepared both adenosyl- and methyl-13-epicobalamin and showed that the first epicorrinoid was unable to function as a coenzyme in the ribonucleotide reductase system of *Lactobacillus leichmanni*, while the latter did not yield an active holoenzyme when incubated with N^5-methyltetrahydrofolate-homocysteine cobalamin methyltransferase apoenzyme from *Escherichia coli* B. The ribonucleotide reductase system appears to be particularly sensitive to rather minor changes in its coenzyme, for instance, replacement of the ring oxygen of the adenosyl moiety by a carbon atom, as in aristeromycylcobalamin, results in an inactive coenzyme analog (Sando, 1974). In contrast, in the diol dehydrase system from *Aerobacter aerogenes* the coenzyme requirements appear to be less stringent; indeed, the aristeromycin containing analog is able to function as a coenzyme (Kerwar et al., 1970). In the present communication we demonstrate that adenosyl-13-epicobalamin, which is inactive as a coenzyme in the ribonucleotide reductase system, does serve as a coenzyme in the diol dehydrase system from *A. aerogenes*.

Materials and Methods

Materials. Crystalline adenosylcobalamin was obtained from Glaxo Research Ltd., Greenford, U.K. Adenosyl-13-epicobalamin was prepared as described by Tkachuck et al. (1974). The purity of this analog was established by paper chromatography using water-saturated *sec*-butyl alcohol as the solvent system. R_f values for adenosylcobalamin and adenosyl-13-epicobalamin were 0.28 and 0.23, respectively (R_{AdoCbl} for adenosyl-13-epicobalamin 0.82).[1] Concentrations of both corrinoids were determined spectrophotometrically after converting them into the corresponding dicyano derivatives by photolysis in the presence of 0.1 M KCN. The molar extinction coefficients at 367 nm for dicyanocobalamin and dicyano-13-epicobalamin are 30.4×10^3 and 20.6×10^3 M^{-1} cm^{-1}, respectively (Barker et al., 1960; Bonnett et al., 1971a). All other materials were obtained from commercial sources. The apoenzyme of diol dehydrase (D,L-1,2-propanediol hydro-lyase, EC 4.2.1.28) was prepared from *Aerobacter aerogenes* (ATCC 8724) by a procedure similar to that of Lee and Abeles (1963).

Enzyme Assay. The activity of diol dehydrase was assayed as described before (Toraya et al., 1971). In this assay with 25 μM adenosyl-13-epicobalamin constant velocity was attained only after a lag period of approximately 20 min. After this 20-min lag period the reaction rate was linear for at least 30 min.

Protein Determination. Protein concentration was determined by the procedure of Lowry et al. (1951). Crystalline bovine serum albumin was used as the standard.

Results

Coenzyme Activity of Adenosyl-13-epicobalamin. As shown in Figure 1 adenosyl-13-epicobalamin is able to function as a coenzyme in the adenosylcobalamin-dependent diol dehydrase system from *A. aerogenes*. From the double reciprocal plot of reaction velocity against coenzyme analog concentration an apparent K_m of 12.7 μM for adenosyl-13-epicobalamin was calculated. This value is about 13 times

† From the Laboratory of Industrial Biochemistry, Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Yoshida, Sakyo-Ku, Kyoto, Japan, and from the Department of Biochemistry, College of Medicine, The University of Iowa, Iowa City, Iowa 52242. *Received May 22, 1975.* Supported in part by the U.S. Public Health Grant GM-20307 from the National Institutes of Health. Paper VI in this series. The preceding paper in the series is Toraya et al. (in press). This paper is dedicated to the memory of Professor K. Bernhauer.

[1] Abbreviations used are: AdoCbl, adenosylcobalamin or *Coα*-[α-(5,6-dimethylbenzimidazolyl)]-*Coβ*-adenosylcobamide; AdoCbl(13-epi), adenoxyl-13-epicobalamin.

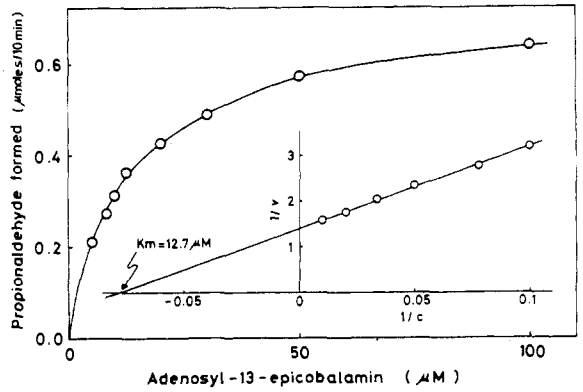

FIGURE 1: Effect of adenosyl-13-epicobalamin concentration on diol dehydrase activity. The reaction mixture contained 1 unit of apoenzyme, 0.2 *M* 1,2-propanediol, 0.1 *M* KCl, 0.025 *M* potassium phosphate buffer (pH 8.0), and the indicated amount of adenosyl-13-epicobalamin, in a total volume of 1.0 ml. The reaction was carried out at 37° for 10 min.

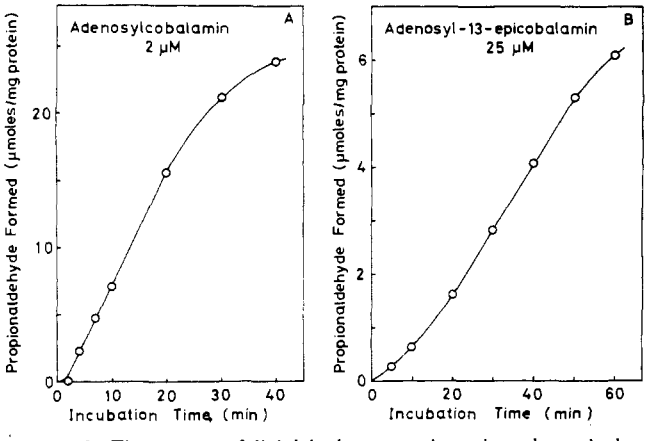

FIGURE 2: Time course of diol dehydrase reaction using adenosylcobalamin (A) and adenosyl-13-epicobalamin (B) as a coenzyme. (A) The reaction mixture contained 0.03 unit of apoenzyme, 0.2 *M* 1,2-propanediol, 0.1 *M* KCl, 0.025 *M* potassium phosphate buffer (pH 8.0), and 2 μM adenosylcobalamin, in a total volume of 1.0 ml. (B) The mixture was identical with that described in Figure 1 except that the total volume was 2.0 ml. At the indicated time, a 0.2-ml aliquot was sampled from the mixture and the propionaldehyde formed was measured.

higher than the K_m value of 0.99 μM for adenosylcobalamin, indicating that the epimerization at C-13 affects the enzyme–coenzyme interaction.

Time Course of the Enzyme–Coenzyme Analog Interaction. The higher K_m value for adenosyl-13-epicobalamin could be due to a slower binding rate of the analog to the enzyme. To investigate the binding rates of the analog and of adenosylcobalamin both corrinoids were incubated in a complete reaction mixture at concentrations twice their K_m values, i.e., 25 and 2 μM for adenosyl-13-epicobalamin and adenosylcobalamin, respectively. With the analog an approximate 20-min lag period was required before constant velocity was attained. After this lag period the reaction rate was linear for at least 30 min. With adenosylcobalamin only a 2-min lag period was observed (Figure 2). Increasing the concentration of adenosyl-13-epicobalamin to 50 μM did not shorten the lag period. At constant velocity the coenzyme activity of adenosyl-13-epicobalamin at 25 and 50 μM was about 12 and 14% of that of a saturating concentration of adenosylcobalamin.

Effect of Anaerobic Preincubation of the Coenzyme Activity of Adenosyl-13-epicobalamin. The effect of preincubation under anaerobic conditions of 25 μM adenosyl-13-epicobalamin with diol dehydrase is presented in Figure 3. The results show that the initial reaction rate increases with preincubation up to 45 min. The maximum initial velocity after 45-min preincubation was about 15% of that attained with a saturating concentration of adenosylcobalamin, in good agreement with the maximum velocity observed in the time-course study (vide supra). The observation that in the absence of substrate a longer lag period is required suggests that the substrate promotes the binding of the coenzyme analog to the apoenzyme. Previous studies by Toraya et al. (1971) demonstrated that propanediol facilitates the binding of adenosylcobalamin to diol dehydrase.

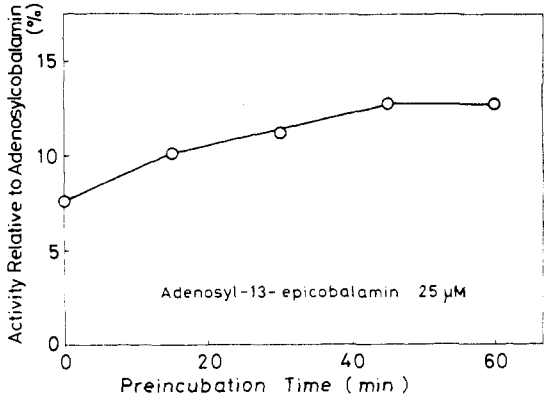

FIGURE 3: Effect of time of preincubation with adenosyl-13-epicobalamin on diol dehydrase activity. The apoenzyme (0.2 unit) was preincubated with 25 nmol of adenosyl-13-epicobalamin in the presence of 0.05 *M* KCl and 0.025 *M* potassium phosphate buffer (pH 8.0) under a helium atmosphere. After the indicated time of preincubation, the reaction was started by addition of 0.2 *M* 1,2-propanediol.

Resolution of the Apoenzyme–Adenosyl-13-epicobalamin Complex by Gel Filtration on Sephadex G-25. Earlier studies indicated that in the presence of a monovalent cation, such as K^+, NH_4^+, or Rb^+, adenosylcobalamin and diol dehydrase apoenzyme form a tight complex (holoenzyme) which cannot be resolved by gel filtration on Sephadex G-25 in the presence of K^+ and substrate (reacting holoenzyme[2]) (Toraya et al., 1971). However, in the absence of a monovalent cation, irrespective of the presence of substrate, the holoenzyme is completely resolved upon gel filtration. As shown in Table I, the apoenzyme–adenosyl-13-epicobalamin complex can be completely resolved even in the presence of K^+. The results demonstrate that the coenzyme analog is loosely bound by the apoenzyme, such that it is readily dissociated even from the reacting apoenzyme–coenzyme analog complex.

Inactivation of the Apoenzyme–Adenosyl-13-epicobalamin Complex by Oxygen in the Absence of Substrate. Abeles and coworkers (Lee and Abeles, 1963; Wagner et al., 1966) reported that incubation of diol dehydrase apoenzyme with adenosylcobalamin in the absence of substrate but in the presence of oxygen leads to rapid inactivation of the holoenzyme. Evidently the enzyme-bound coenzyme reacts with oxygen causing irreversible cleavage of the carbon–cobalt bond. Inactivation of the apoenzyme–adenosyl-13-epicobalamin complex also occurs when the complex is incubated in the presence of oxygen. However, as shown in

[2] Reacting holoenzyme: holodiol dehydrase in the presence of 1,2-propanediol.

Table 1: Resolution of Apoenzyme–Adenosyl-13-epicobalamin Complex by Gel Filtration on Sephadex G-25.[a]

Enzyme[b]	Buffer Used[c]	Specific Activity (units/mg)			Resolution (%)
		− AdoCbl (13-epi)	+ AdoCbl (13-epi)	+ AdoCbl[d]	
E·AdoCbl (13-epi)	0.05 *M* Tris-HCl–0.1 *M* KCl–0.1 *M* 1,2-propanediol	0.00	0.15	1.14	100
	0.05 *M* Tris-HCl–0.1 *M* 1,2-propanediol	0.00	0.13	1.19	100
E·AdoCbl[e]	0.05 *M* Tris-HCl–0.1 *M* KCl–0.1 *M* 1,2-propanediol				2
	0.05 *M* Tris-HCl–0.1 *M* 1,2-propanediol				100

[a] The mixture containing 10 units of apoenzyme, 0.2 *M* 1,2-propanediol, 0.1 *M*-KCl, 0.025 *M* potassium phosphate buffer (pH 8.0), and 25 μM adenosyl-13-epicobalamin, in a total volume of 1.0 ml, was incubated at 37° for 30 min and then submitted to gel filtration through Sephadex G-25 (fine) column (1.6 × 20 cm) using an appropriate buffer. Specific activity of the protein-containing fractions was assayed with or without adenosyl-13-epicobalamin or adenosylcobalamin, and the extent of resolution was estimated as described previously (Toraya et al., 1971). [b] E = apoenzyme. [c] All buffers, pH 8.0. [d] Specific activity of the original apoenzyme was 1.05 unit/mg. [e] The data from Toraya et al. (1971).

Figure 4 the rate of inactivation of the analog–apoenzyme complex is much slower than that of the adenosylcobalamin–apoenzyme complex. This slower rate of inactivation suggests not only a slower rate of binding for the analog but also a difference in the susceptibility of the carbon–cobalt bond of the two adenosylcorrinoids to homolytic cleavage.

Possible Enzymatic Conversion of Adenosyl-13-epicobalamin to Adenosylcobalamin. The low activity of adenosyl-13-epicobalamin as a coenzyme as well as the long lag period required to attain maximum activity suggested the possibility that the enzyme preparation is able to catalyze the epimerization of the analog to the coenzyme. To test this possibility adenosyl-13-epicobalamin was incubated in a complete reaction mixture (Table I) for 30 min, the corrinoid was then extracted with 80% aqueous ethanol as described by Ertel et al. (1968). Paper chromatography of the recovered corrinoid showed only one spot with a mobility identical with that of adenosyl-13-epicobalamin. This observation indicates that the diol dehydrase preparation does not possess any epimerase activity and that adenosyl-13-epicobalamin is not degraded during the enzymatic reaction. Furthermore, the data presented in Table I show that the apoenzyme–adenosyl-13-epicobalamin complex is completely resolved upon gel filtration even in the presence of both K^+ and substrate and that the specific activity of the recovered apoenzyme is virtually identical with that of the original apoprotein. If the enzyme-bound adenosyl-13-epicobalamin was converted into adenosylcobalamin or hydroxocobalamin, the resulting apoenzyme–adenosylcobalamin or apoenzyme–hydroxocobalamin complex would not be resolved upon gel filtration in the presence of both K^+ and substrate (Toraya et al., 1971, 1972). Thus, it can be concluded that neither free nor bound adenosyl-13-epicobalamin changes during the enzymatic reaction.

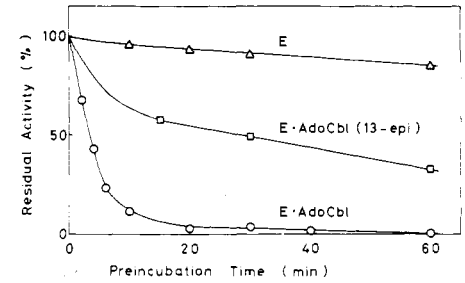

FIGURE 4: Inactivation of apoenzyme–adenosylcobalamin and apoenzyme–adenosyl-13-epicobalamin complexes by oxygen in the absence of substrate. The apoenzyme (0.1 unit) was aerobically incubated with and without 2 nmol of adenosylcobalamin in the presence of 0.05 *M* KCl and 0.025 *M* potassium phosphate buffer (pH 8.0), in a total volume of 0.9 ml. After the indicated time of incubation, the remaining reactants of the usual assay mixture were added and the remaining activity was measured. In the case of apoenzyme–epimer complex, apoenzyme (1.5 units) was incubated with 37.5 nmol of adenosyl-13-epicobalamin in the presence of 0.05 *M* KCl and 0.025 *M* potassium phosphate buffer (pH 8.0), in a total volume of 1.35 ml. After the indicated time of incubation, 0.15 ml of 2 *M* 1,2-propanediol was added and the remaining activity was evaluated from a value of constant velocity as described in the text.

Discussion

Adenosyl-13-epicobalamin is an analog of adenosylcobalamin in which the adenosyl moiety in the upper coordination position is positioned differently above the corrin ring because the inverted e-propionamide side chain blocks its normal location above C-13. Furthermore, the lower pK_a of the "base on" ⇄ "base off" conversion of adenosyl-13-epicobalamin (2.8) compared to that of adenosylcobalamin (3.5) suggests that the inversion at C-13 also affects the electronic character of the cobalt atom. Tkachuck et al. (1974) reported that adenosyl-13-epicobalamin was unable to serve as a coenzyme in the ribonucleotide reductase system of *L. leichmannii,* while methyl-13-epicobalamin did not yield an active holoenzyme when incubated with N^5-methyltetrahydrofolate–homocysteine cobalamin methyltransferase apoenzyme from *E. coli.* However, both coenzyme analogs did inhibit their respective enzymes. In contrast to these observations, the present work shows that adenosyl-13-epicobalamin is able to function as a coenzyme in the diol dehydrase system of *A. aerogenes,* although both the coenzyme activity of the epimer and its affinity for the apoenzyme are lower than those of adenosylcobalamin. The lower affinity of the apoenzyme for adenosyl-13-epicobalamin is reflected in several observations: (a) a longer lag period is required before constant velocity is reached; (b) a longer preincubation time is necessary for maximum initial

velocity; (c) the reacting apoenzyme–coenzyme analog complex is readily resolved by gel filtration; (d) the rate of inactivation of the reacting complex by oxygen is much lower than that of the reacting diol dehydrase–adenosylcobalamin complex. On the other hand, the interaction of the analog with the enzyme is very similar to that of adenosylcobalamin. As is the case with the adenosylcobalamin–apoenzyme complex (Toraya et al., 1971), the presence of the substrate, propanediol, also accelerates the binding of the epimer analog to the apoenzyme. It is conceivable that the substrate promotes the binding of the corrinoid to the apoenzyme facilitating the association of the enzyme subunits F and S, since earlier studies have demonstrated that both subunits are necessary for coenzyme binding and that the substrate promotes their interaction (Toraya et al., 1974, 1975). The inversion of configuration at C-13 and the resulting change in the conformation of ring C are probably the major factors which lower the affinity of the analog for the enzyme, because the major enzyme binding sites involve the corrin ring and the lower 5,6-dimethylbenzimidazole ligand.

The lower coenzyme activity of adenosyl-13-epicobalamin as well as the slower rate of inactivation of the apoenzyme–coenzyme analog complex by oxygen suggests that the carbon–cobalt bond of adenosyl-13-epicobalamin is more resistant to homolytic cleavage than that of adenosylcobalamin.

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