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COENZYME ACTIVITY OF 5'-DEOXYADENOSYL-10-CHLOROCOBALAMIN IN PROPANEDIOL DEHYDRATASE SYSTEM

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SUMMARY

The coenzyme action of α-(5,6-dimethylbenzimidazolyl)-*Co*-5'-deoxyadenosyl-10-Cl-cobamide (10-Cl-DBCC) was studied in a cobamide-dependent propanediol dehydratase (EC 4.2.1.28). The K_m value of the coenzyme analogue was almost identical with that of DBCC, but the $v_{\max}$ value was significantly smaller. These facts would indicate an analogous affinity for the apoenzyme of the coenzyme analogue and its lower catalytic activity in comparison with those of DBCC.

The decomposition rate of the cobalt–carbon bond of 10-Cl-DBCC–apoprotein complex caused by oxygen in the absence of the substrate was markedly lower than that of DBCC–apoenzyme complex. This fact seems to correspond with the lower coenzyme activity of 10-Cl-DBCC.

On the other hand, the cobalt–carbon bond of 10-Cl-DBCC was more resistant to cyanide than that of DBCC. This fact would indicate that the bond is polarized toward the cobalt atom which is unfavorable for the dissociation of the bond necessary for the exhibition of the coenzyme activity.

Based on these findings, the possible role of 5'-deoxyadenosyl carbanion and Co^{3+} were proposed for the catalytic action of DBCC.

INTRODUCTION

Recent work in several laboratories strongly suggests that the coenzyme action of α-(5,6-dimethylbenzimidazolyl)-*Co*-5'-deoxyadenosylcobamide (DBCC) is initiated by a heterolytic fission of the linkage between the cobalt atom and 5'-deoxyadenosyl moiety. Furthermore the participation of the 5'-C position of the coenzyme has been observed in the coenzyme-dependent hydrogen transfer reaction, such as in propanediol dehydratase (EC 4.2.1.28)[1], methylmalonyl-CoA mutase (EC 5.4.99.2)[2,3], methyl-

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Abbreviations: DBCC, α-(5,6-dimethylbenzimidazolyl)-*Co*-5'-deoxyadenosylcobamide or 5'-deoxyadenosylcobalamin or coenzyme B_{12}; 10-Cl-DBCC, DBCC chlorinated at C-10 of its corrin nucleus.

aspartate mutase (EC 5.4.99.1) (H. A. BARKER, personal communication to R. H. ABELES) and ribonucleoside triphosphate reductase (R. H. ABELES AND W. S. BECK, personal communication) reactions. On the other hand, it has been shown that the electronegativity of the group attached to the cobalt atom influences the reactivity of the hydrogen on C-10 or C-8 of the corrin ring and, *vice versa*, the substituent at C-10 exerts a profound effect on the cobalt atom[4]. Although the 10-chloro derivative of DBCC was inactive in the methylaspartate mutase reaction (ref. 5; H. A. BARKER, personal communication), it seems of interest to study the effect of the substituent at C-10 on the coenzyme activity in the other reaction systems.

This paper deals with the comparison of the coenyzme activity between DBCC chlorinated at C-10 of its corrin nucleus (10-Cl-DBCC) and DBCC in the propanediol dehydratase system of LEE AND ABELES[6] and discussions concerning the mode of the cleavage of the cobalt–carbon bond of the coenzyme–apoenzyme complex in the reaction. A preliminary note has appeared[7].

MATERIALS AND METHODS

Materials

Cyanocobalamin and DBCC were purchased from Roussel-Uclaf Co., France. All other chemicals were obtained from commercial sources. Methylcobalamin was prepared by the ordinary method[8]. Methyl-10-Cl-cobalamin was obtained from methylcobalamin according to the procedure of DOLPHIN, JOHNSON AND RODRIGO[9], then converted to aquo-10-Cl-cobalamin by irradiation. 10-Cl-DBCC was prepared from aquo-10-Cl-cobalamin enzymatically using a partially purified enzyme from *Propionibacterium shermanii*[12] or by reacting its 2-electron reduced state with 5'-iodoadenosine according to MURAKAMI *et al.*[11,12], followed by the purification by chromatography on TEAE-cellulose and P-cellulose.

DBCC and 10-Cl-DBCC were assayed spectrophotometrically after being converted to their dicyano forms, respectively. The amount of DBCC was calculated from the molar extinction coefficient of dicyanocobalamin, $30.4 \cdot 10^3$ at 368 mμ according to BARKER *et al.*[13], and that of 10-Cl-DBCC was determined by the molar extinction coefficient of its dicyano form at 370 mμ, $26 \cdot 10^3$ which was calculated on the basis of that of methyl-10-Cl-cobalamin at 347 mμ, $12 \cdot 10^3$ (ref. 9).

Assay of coenzyme activity of 10-Cl-DBCC. Propanediol dehydratase (EC 4.2.1.28) was used for the examination of the activity of 10-Cl-DBCC. The apoenzyme was prepared from *Aerobacter aerogenes* (ATCC 8724) according to the procedure of LEE AND ABELES[6]. In this study the E-3 fraction of the original method was used as an apoprotein after dialysis.

The assay mixture contained 0.05 M phosphate buffer (pH 8.0), 0.10 unit of apoprotein, 0.1 mmole of 1,2-propanediol, and graded amounts of DBCC or 10-Cl-DBCC in a total volume of 1.0 ml. The enzyme reaction was allowed to proceed for 10 min at 37° and stopped by the addition of 0.5 ml of 10% trichloroacetic acid. The amount of propionaldehyde formed was colorimetrically determined as the 2,4-dinitrophenylhydrazone according to the method of ABELES[14].

In order to examine the possibility that 10-Cl-DBCC would be converted to DBCC during the enzyme reaction, the reaction mixture was photolyzed by irradiating it with 500-W tungsten lamp at a distance of 15 cm for 10 min. A cobalamin separated from the apopropein was extracted with phenol, transferred back to the aqueous phase by shaking with a small amount of a mixture of ether and water[15], then examined by paper chromatography, paper electrophoresis and spectroscopy. In this case 150 ml of the enzyme reaction mixture containing 2 mg of 10-Cl-DBCC, 15 units of the apoenzyme, 15 mmoles of the substrate and 0.05 M phosphate buffer (pH 8.0) were incubated for 10 min at 37°.

RESULTS

The coenzyme activity of 10-Cl-DBCC in propanediol dehydratase system

It was observed that 10-Cl-DBCC exhibited the coenzyme activity in the propanediol dehydratase system. As shown in Fig. 1, the K_m value for 10-Cl-DBCC was almost identical with that of DBCC, about $6 \cdot 10^{-7}$ M, whereas the maximal velocities (v_{max}) were markedly different. These results seem to indicate that the coenzyme analogue is able to combine with the apoprotein as efficiently as DBCC, but the catalytic activity of the coenzyme analogue–apoenzyme complex is much lower than that of the normal enzyme.

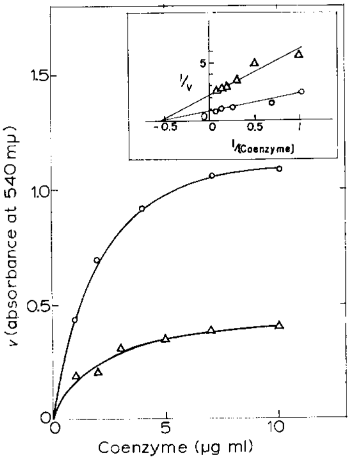


Fig. 1. Comparison of the coenzyme activities of DBCC and 10-Cl-DBCC in a cobamide-dependent propanediol dehydratase reaction. The reaction mixture contained the following in a final volume of 1 ml: apoenzyme, 0.10 unit; 1,2-propanediol, 0.1 mmole; potassium phosphate buffer (pH 8.0), 0.05 M; coenzyme, as indicated in the figure. Incubation was carried out for 10 min at 37°. The propionaldehyde formed was assayed colorimetrically as its 2,4-dinitrophenylhydrazone at 540 mμ. ○—○, DBCC; △—△, 10-Cl-DBCC.

It has been known that aquocobalamin acts as a competitive inhibitor against DBCC in the diol dehydratase system[6]. We observed that aquo-10-Cl-cobalamin also exerted a competitive inhibitory action with a 50% inhibition index of about 1.7 which was almost identical with that of aquocobalamin. The fact would suggest a good affinity of 10-Cl-DBCC for the apoprotein.

On the other hand, both aquo-10-Cl-cobalamin and 10-Cl-DBCC are known to be inactive in the methylaspartate mutase system (ref. 5; H. A. BARKER, personal communication). The discrepancy would be attributed to the difference in the affinities between the corrinoids and the apoproteins in these two systems.

Even though the coenzyme activity of 10-Cl-DBCC was observed, there remains some doubt whether the coenzyme analogue was transformed into DBCC during the enzyme reaction. In order to study this possibility, after the enzyme reaction was finished we recovered a cobalamin from the reaction mixture and studied its properties. The behaviors of the cobalamin in paper chromatography and paper electrophoresis using several kinds of solvent systems were coincident with those of aquo-10-Cl-cobalamin. The presence of aquocobalamin could not be detected. Fig. 2 shows the results of the paper electrophoresis carried out in 0.5 M acetic acid (pH 2.7).

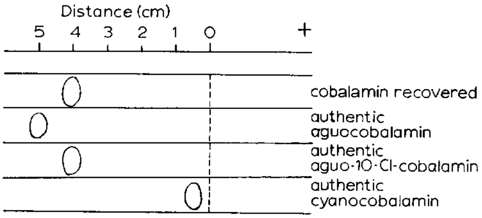


Fig. 2. Paper electrophoretic behavior of a cobalamin recovered from the reaction mixture of the propanediol dehydratase system. The paper electrophoresis was carried out in 0.5 M acetic acid (pH 2.7) at 13 V/cm for 2.5 h.

The absorption spectrum of the cobalamin recovered was identical with that of authentic aquo-10-Cl-cobalamin having absorption peaks at 355, 525 and 555 mμ. These results would confirm the identity of the cobalamin with aquo-10-Cl-cobalamin and the maintenance of the chlorine in 10-Cl-DBCC during the course of the enzyme reaction.

The time-course study of the propionaldehyde formation catalyzed by 10-Cl-DBCC showed that the reaction proceeded with a definitively lower rate than that catalyzed by DBCC. The result suggested that the lower catalytic activity of 10-Cl-DBCC would be due to the resistance of its cobalt–carbon bond to cleavage in the enzyme reaction caused by the change of the polarization of the bond.

Cleavage of the cobalt–carbon bond of 10-Cl-DBCC–apoenzyme complex

The DBCC–apoenzyme complex has been known to be inactivated by oxygen when incubated without adding the substrate[6,16]. The inactivation velocity of the 10-Cl-DBCC–apoprotein complex was markedly smaller than that of DBCC–apoprotein complex as illustrated in Fig. 3. WAGNER *et al.*[16] have observed that the inactivation resulted from the stoichiometric reaction of the coenzyme–apoenzyme complex with oxygen and postulated that the nature of the cobalt–carbon bond of the coenzyme is modified by the apoprotein to react readily with oxygen. Hence the result mentioned above suggests that the cobalt–carbon bond of 10-Cl-DBCC is more resistant to oxidative cleavage than that of DBCC.

Moreover the decomposition rate of the coenzyme analogue by cyanide was significantly lower than that of DBCC (Fig. 4). According to MÜLLER AND MÜLLER[17] the susceptibility to cyanide has been considered to reflect the polarization of the cobalt–carbon bond.

These facts appear to indicate that the Cl at C-10 of the corrin nucleus enhances

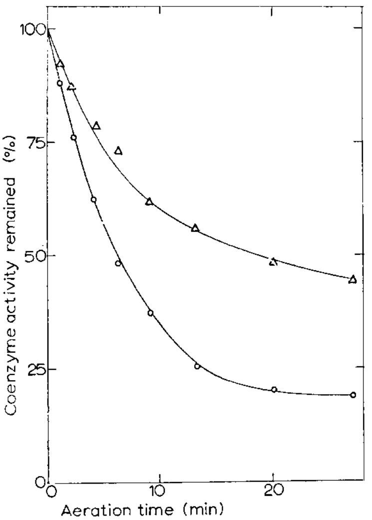


Fig. 3. Comparison of inactivation rates of DBCC–apoprotein complex and 10-Cl-DBCC–apoprotein complex incubated without adding propanediol under aerobic conditions. The reaction mixture contained the following in a final volume of 1 ml: DBCC or 10-Cl-DBCC, 1 μg; other components were the same as in Fig. 1 except propanediol omitted. Aeration was carried out at 37°. At the indicated time of aeration, 100 μmoles of 1,2-propanediol was added and the coenzyme activity remaining was assayed.

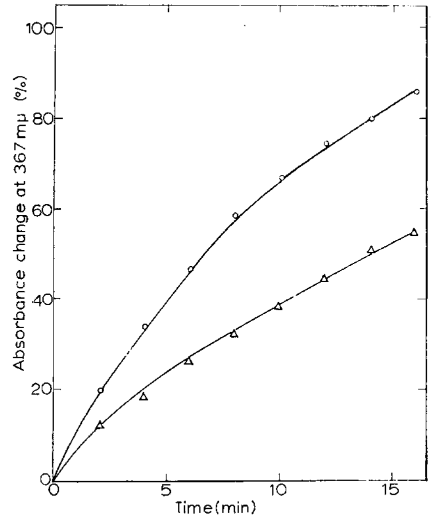


Fig. 4. Comparison of decomposition rates of DBCC and 10-Cl-DBCC by cyanide. The reaction mixture contained 0.2 μmole of DBCC or 10-Cl-DBCC, 0.5 mmole of KOH and 50 μmoles of KCN. The reaction was started by the addition of KCN. The absorbance of the cyano form produced was determined at 367 mμ. ○—○, DBCC; △—△, 10-Cl-DBCC.

the polarization of the cobalt–carbon bond in which the electrons are attracted towards the cobalt atom.

Analogous effects of the substituents at C-10 have been observed on the reactivity of the Co–S bond of 10-halogenocobalamin sulfonate[19].

DISCUSSION

It has been postulated that the enzyme reaction involving DBCC would be initiated by a reversible dissociation of the cobalt–carbon bond resulting from the interaction of the coenzyme and the apoprotein[16]. In this case the two possible ways in which the dissociation could occur can be supposed as follows.

First is the case in which the electron pair of this bond remains with the cobalt (Reaction 1) or, in the second case, the pair remains with the 5′-deoxyadenosyl moiety (Reaction 2). In either case the dissociation gives rise to an electron-rich species and an electron-deficient species which could function as electron donor and acceptor, respectively. Despite the fact that polarization of the bond caused by the chlorine at C-10 is considered to be favorable to yield an electron-rich cobalt, and finally B_{12s}, the coenzyme activity as well as the inactivation rate of 10-Cl-DBCC–apoprotein complex in the absence of the substrate was lower than that of DBCC. This discrepancy would be explained by the supposition that the polarization of the cobalt–carbon bond in the coenzyme analogue is inadequate and the dissociation necessary for the exhibition of the coenzyme action of the coenzyme produces a protein-bound B_{12b} and 5'-deoxyadenosyl carbanion (Reaction 2).

Based on this supposition, the mechanism of the function of the protein-bound 5'-deoxyadenosyl carbanion may be postulated as illustrated in Fig. 5. The reaction sequence is as follows: The transformation of State I to State II, that is, a heterolytic cleavage yielding 5'-deoxyadenosyl carbanion and Co^{3+}, is presumed to be a rate-limiting step. Abstraction of a hydrogen (expressed as H^* in Fig. 5) occurs from C-1 of the substrate to Co^{3+} of the coenzyme–apoenzyme complex as a hydride ion, then the OH^- at C-2 transfers to C-1. From the 5'-C of State III, representing a reduced state of the coenzyme–apoenzyme complex interacting with the substrate (Substrate–Enzyme–DBCC–H), a hydride ion transfers to C-2 of the substrate, followed by the dehydration to propionaldehyde. The coenzyme–apoprotein complex goes back to State I through State IV consisting of the protein-bound Co^+ and 5'-deoxyadenosyl carbonium ion. It appears likely that this postulated mechanism can explain the findings of FREY AND ABELES[1] and ABELES AND ZAGALAK[19] that a hydrogen abstracted from C-1 of the substrate was shifted to C-2 of the other substrate molecule and a hydrogen attached to 5'-C of the deoxyadenosyl moiety of the coenzyme was incorporated into the substrate.

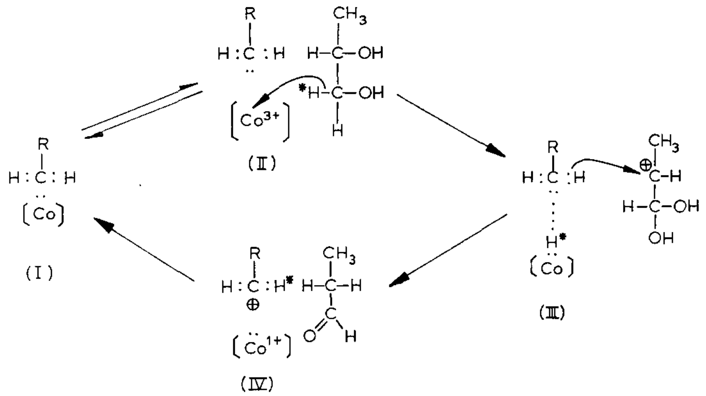
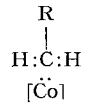


Fig. 5. Postulated mechanism of a propanediol dehydratase reaction catalyzed by DBCC represented as:


$$\begin{array}{c}\mathrm{R}\\ |\\ \mathrm{H{:}\underset{..}{C}{:}H}\\ [\mathrm{Co}]\end{array}$$

REFERENCES

1 P. A. FREY AND R. H. ABELES, *J. Biol. Chem.*, 241 (1966) 2732.
2 J. RETEY AND D. ARIGONI, *Experientia*, 22 (1966) 783.
3 J. G. CARDINALE AND R. H. ABELES, *Biochim. Biophys. Acta*, 132 (1967) 517.
4 H. P. C. HOGENKAMP, *Federation Proc.*, 25 (1966) 1623.
5 F. WAGNER, *Ann. Rev. Biochem.*, 35 (1966) 405.
6 H. A. LEE, JR. AND R. H. ABELES, *J. Biol. Chem.*, 238 (1963) 2368.
7 Y. TAMAO, Y. MORIKAWA, S. SHIMIZU AND S. FUKUI, *Biochem. Biophys. Res. Commun.*, 28 (1967) 692.

8 E. L. SMITH, L. MERVYN, A. W. JOHNSON AND N. SHAW, *Nature*, 194 (1962) 1175.
9 D. DOLPHIN, A. W. JOHNSON AND R. RODRIGO, *J. Chem. Soc.*, (1964) 3186.
10 R. O. BRADY, E. C. CASTERNA AND H. A. BARKER, *J. Biol. Chem.*, 237 (1962) 2325.
11 M. MURAKAMI, K. TAKAHASHI, H. IWAMOTO AND A. MATSUMOTO, *Bitamin*, 34 (1966) 598.
12 M. MURAKAMI, K. TAKAHASHI, H. IWAMOTO AND A. MATSUMOTO, *Bitamin*, 35 (1967) 68.
13 H. A. BARKER, R. D. SMYTHE, H. WEISSBACH, J. L. TOOHEY, J. N. LADD AND B. E. VOLCANI, *J. Biol. Chem.*, 235 (1960) 480.
14 R. H. ABELES, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. IX, Academic Press, New York, 1966, p. 686.
15 W. FRIEDRICH AND K. BERNHAUER, *Z. Naturforschung*, 12 (1954) 757.
16 O. WAGNER, H. A. LEE, JR., P. A. FREY AND R. H. ABELES, *J. Biol. Chem.*, 241 (1966) 1751.
17 O. MÜLLER AND G. MÜLLER, *Biochem. Z.*, 336 (1962) 299.
18 F. WAGNER, *Proc. Roy. Soc. London, Ser. A*, 288 (1965) 344.
19 R. H. ABELES AND B. ZAGALAK, *J. Biol. Chem.*, 241 (1966) 1245.