The Coenzymic and Chemical Properties of a Carbocyclic Analogue of Vitamin B$_{12}$ Coenzyme*

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SUMMARY

An analogue of vitamin B$_{12}$ coenzyme in which the ribosyl oxygen has been replaced by —CH$_2$— has been synthesized. This compound has spectral properties very similar to vitamin B$_{12}$ coenzyme. The chemical properties of the analogue differ from those of vitamin B$_{12}$ coenzyme in that reaction with CN$^-$ and BH$_4^-$ does not result in cleavage of the carbon-cobalt bond. The analogue functions as coenzyme in the reaction catalyzed by dioldehydrase. The reaction in the presence of analogue is one-third as fast as with the coenzyme. Other properties of the enzyme reaction in the presence of the analogue are similar to those observed with the coenzyme: spectral changes in the presence of substrate and glycolaldehyde, hydrogen exchange between substrate and coenzyme, and over-all deuterium isotope effect. It was concluded that the ribosyl oxygen of the coenzyme cannot play an essential role in the catalytic process.

Investigations of the mechanism of action of B$_{12}$ coenzyme have led to the hypothesis that the carbon-cobalt bond of the coenzyme undergoes chemical modification, most likely dissociation, during the course of the reaction. Several proposals have been made concerning the mechanism and nature of this modification (1-5). One possibility, which we have considered, is suggested by nonenzymic reaction of cobalamin and cobaloximes containing oxygen $\beta$ to the carbon-cobalt bond. In these compounds, cleavage of the carbon-cobalt bond can occur under relatively mild acid conditions (6, 7). For instance, hydroxethyl cobalamin is converted to ethylene at 25$^\circ$ at pH 3 with $t_1$ of 2 hours. These reactions have led us to consider the possibility that interaction of the enzyme and coenzyme leads to cleavage of the carbon-cobalt bond through protonation of the ribosyl oxygen of the coenzyme by an acidic group on the enzyme. This results in the formation of a complex, consisting of an electron deficient cobalt, which reacts, possibly through a $\pi$ complex, with the double bond of the deoxyribosyl moiety. This complex could function as an intermediate hydride acceptor in the conversion of propanediol to propionaldehyde. The formation of the activated complex is shown in Fig. 1. A similar proposal has been made for the reaction catalyzed by methylmalonyl coenzyme A isomerase (1). The validity of this type of mechanism was tested by preparing a coenzyme analogue in which the oxygen atom of the ribosyl moiety of 5,6-dimethylbenzimidazo-

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2 The abbreviations used are: carbocyclic DBCC, analogue of DBCC in which the ribosyl oxygen of the adenosyl group is replaced by —CH$_2$—; DBCC, 5,6-dimethylbenzimidazolylcobamide 5'-deoxyadenosine; B$_{12}$, vitamin B$_{12}$ containing cobalt in the +1 oxidation state.
with the same mobility as DBCC. Carbocyclic DBCC was eluted from the paper and subjected to descending paper chromatography on Whatman No. 3 paper with water-saturated 2-butanol. Two major pink bands were detected: Fraction A, RF 0.11; Fraction B, RF 0.15; DBCC, RF 0.12. Total yield (Fractions A and B), 14 mg. The homogeneity of these fractions was examined by ascending paper chromatography on Whatman No. 1 paper with the following solvents: isopropanol-HAc-H2O-butanol-1 (70:1:99:100) and butanol-2-NH4OH-H2O (100:36:14). The following RF values were obtained: Fraction A, 0.24; Fraction B, 0.32; DBCC, 0.30; and Fraction A, 0.16; Fraction B, 0.22; DBCC, 0.17. In each case a single spot was observed.

Treatment of Carbocyclic DBCC with Cyanide—To remove possibly contaminating DBCC the analogue was treated with cyanide under conditions which would convert DBCC to dicyanocobalamin. Carbocyclic DBCC (44 µg) (Fraction B) was dissolved in 1 ml of 0.1 M NaNH2OH-H2O (pH 9.9), containing 0.16 M KCN. The solution was incubated at 37°. After 30 min, 2 ml of 2 N HCl were added and the solution passed through a Dowex 50-H+ column (0.5 X 4 cm). The column was washed with 10 ml of water and carbocyclic DBCC eluted with 10 ml of 1 N NH4OH. The spectrum of the recovered product was identical with that prior to cyanide treatment. Of the material, 82% was recovered.

Analytical Procedures—The following millimolar extinction coefficients were used: DBCC or carbocyclic DBCC, 340 µM at pH 7.0, 12.0; B12a, 352 µM at pH 7.0, 22, and dicyano B12, 367 µM at pH 10.0, 30.4. Spectral measurements were conducted using a Unicam SP-800 recording spectrophotometer.

Diodehydrase was prepared and assayed as described (12). Protein determinations were by the method of Lowry et al. (13). Radiochemical assays were performed by liquid scintillation counting in an Anstis Sp-800 recording spectrophotometer.

RESULTS

Chemical Properties of Carbocyclic DBCC—The spectra of Fractions A and B of carbocyclic DBCC are identical with that of DBCC (Fig. 3) (15). As in the case of DBCC, addition of acid causes a spectral shift and the appearance of a peak at 460

with the same mobility as DBCC. Carbocyclic DBCC was eluted from the paper and subjected to descending paper chromatography on Whatman No. 3 paper with water-saturated 2-butanol. Two major pink bands were detected: Fraction A, RF 0.11; Fraction B, RF 0.15; DBCC, RF 0.12. Total yield (Fractions A and B), 14 mg. The homogeneity of these fractions was examined by ascending paper chromatography on Whatman No. 1 paper with the following solvents: isopropanol-HAc-H2O-butanol-1 (70:1:99:100) and butanol-2-NH4OH-H2O (100:36:14). The following RF values were obtained: Fraction A, 0.24; Fraction B, 0.32; DBCC, 0.30; and Fraction A, 0.16; Fraction B, 0.22; DBCC, 0.17. In each case a single spot was observed.

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The reaction was allowed to proceed for 10 min. Propionaldehyde cases, it was shown by enzymic analysis that at the end of the reaction, no coenzymic activity remained. As expected, after 50 min, the spectrum of DBCC was that of Blzca) and no further spectral change occurred upon exposure to light. The spectrum in Fig. 4 also shows that the compound was not completely converted to B 12~~) since after light exposure, spectral change was complete after 15 min. At that point not all coenzyme activity is lost. It was concluded that carbo cyclic DBCC undergoes a chemical modification in acid, which causes loss of coenzyme activity. Unlike DBCC, the product of the acid reaction may not be B12(a) and certainly is not exclusively B12(a). The significance of the spectral change observed upon heating carbo cyclic DBCC in acid is not clear. Insufficient material was available to further explore this reaction.

In the reactions with CN-, NaBH4, and possibly with acid, the carbon-cobalt bond of DBCC is more susceptible to cleavage than that of carbo cyclic DBCC. This difference in reactivity provides evidence for the contribution of the ribosyl oxygen in reactions in which the carbon-cobalt bond dissociates so that the electrons of that bond remain with the adenosyl moiety.

**Coenzyme Activity of Carboxyclic DBCC**—The coenzyme activity of the two fractions of carbox cyclic DBCC was tested with dioldehydrase and compared to that of DBCC. Saturating amounts of both coenzymes were used. The results are summarized in Table I. Carboxyclic DBCC can function as a coenzyme and therefore the ribosyl oxygen of the adenosyl moiety is not essential for coenzymic activity. Exposure of carboxyclic DBCC to cyanide and subsequent purification did not alter its coenzyme activity when determined under saturating and non-saturating conditions. This eliminates the unlikely possibility that the coenzyme activity observed with carboxyclic DBCC was caused by contamination by DBCC. Carboxyclic DBCC (Fraction B) shows appreciably more coenzyme activity than Fraction A. It is possible that the small amount of activity of Fraction A is caused by contamination by Fraction B, and that Fraction B may also contain some Fraction A so that the activity reported here is a minimal activity. Insufficient carboxyclic DBCC is available at this time to further define the conditions for separation of Fractions A and B.

**Table II**

| Additions                                      | Activity after prior incubation |
|-----------------------------------------------|---------------------------------|
| None                                          | 100                             |
| Hydroxycobalamin                              | 13                              |
| Carboxyclic DBCC (Fraction A)                 | 12                              |

Fraction A inhibits the conversion of propanediol to propionaldehyde as illustrated by the results in Table II. The results show that Fraction A is as inhibitory as hydroxy B12, an irreversible inhibitor of dioldehydrase.

**Fig. 5** shows double reciprocal plots of the initial velocities against DBCC or carboxyclic DBCC concentrations. The apparent K_m obtained for the two coenzymes is quite similar (6.1 X 10^-7 M for DBCC and 8.7 X 10^-7 M for carboxyclic DBCC). It is difficult to assign a mechanistic significance to these apparent K_m values since the combination between enzyme and coenzyme is essentially irreversible. One of the characteristics of the reaction catalyzed by dioldehydrase is that tritium from the C-1 position of the substrate is incorporated, during the course of the catalytic process, into the C-5' position of the coenzyme (4). To establish whether this tritium exchange also occurs with carboxyclic DBCC, 60 μg of carboxyclic DBCC, Fraction B, were incubated at 10° with 242 units of dioldehydrase, 20 μmoles of potassium phosphate buffer,
Stability of dioldehydrase in presence of DBCC and carbocyclic DBCC

Incubation mixture contained: potassium phosphate buffer, pH 8.0, 0.04 M; dioldehydrase, 0.48 unit; serum albumin, 0.4 mg per ml; DBCC or carbocyclic DBCC (Fraction B), or both, as indicated, in a total volume of 5.0 ml at 37°. At times indicated, 0.1-ml aliquot of reaction mixture was removed and assayed for enzymatic activity under standard assay conditions (12).

| Time | Activity in presence of |
|------|-------------------------|
|      | None | DBCC (4.8 x 10^(-7) M) | Carbocyclic DBCC (2.4 x 10^(-7) M) | DBCC (4.8 x 10^(-7) M) + carbocyclic DBCC (2.4 x 10^(-7) M) |
| 0    | 100% | 100% | 100% | 100% |
| 10   | 100% | 35%  | 100% | 46%  |
| 20   | 100% | 13%  | 100% | 37%  |

Fig. 6. Isolation of dioldehydrase-carbocyclic DBCC complex. In a total volume of 0.845 ml 70 units of dioldehydrase, 7.1 x 10^(-8) M carbocyclic DBCC (tritium-labeled, specific activity = 1.16 x 10^6 cpm per pmole), 5.9 x 10^(-8) M K_2HPO_4 were incubated at 37° for 10 min. The reaction mixture was layered onto a Sephadex G-75 column, 30 x 2.5 cm, equilibrated with 1 x 10^(-3) M K_2HPO_4. At a flow rate of 2 ml per min, 3-ml fractions were collected. Each fraction was analyzed for protein concentration (X), radioactivity (Y), and enzyme activity (Z). Tritium-labeled carbocyclic DBCC was prepared with dioldehydrase and DL-1,2-propanediol-1,3H as described in the text.

pH 8.0, and 16 µmoles of DL-1,2-propanediol-1,3H (specific activity 1.5 x 10^6 cpm per µmole) in a total volume of 2.8 ml. The reaction was allowed to proceed for 58 sec and was stopped by the addition of 0.4 ml of 90% trichloroacetic acid. Carbocyclic DBCC was then isolated and purified by procedures previously used with DBCC (4). The specific activity of the re-isolated carbocyclic DBCC was 1.1 x 10^8 cpm per µmole. Qualitatively, carbocyclic DBCC resembles DBCC in that hydrogen exchange occurs during the catalytic process between substrate and carbocyclic DBCC. Insufficient material was available to carry out quantitative comparisons.

To establish whether a different rate-limiting step occurs with carbocyclic DBCC than with DBCC, the isotope effect obtained with C-1-deutero-DL-propanediol in the presence of carbocyclic DBCC was determined. Under standard assay conditions the nondeuterated substrate reacted 8- to 10-fold faster than the deuterated substrate. This was previously shown (18) that with DBCC an isotope effect of 10 to 12 is obtained under these conditions. It is therefore highly probable that the same step, the breaking of the substrate C-H bond, is rate-limiting with both coenzymes.

The dioldehydrase-DBCC complex reacts with oxygen in the absence of substrate to give a catalytically inactive complex (19). This oxygen inactivation involves breaking of the carbon-cobalt bond. The stability of the dioldehydrase-carbocyclic DBCC complex was examined. The results are summarized in Table III. The enzyme-carbocyclic DBCC complex is completely stable under conditions where the corresponding DBCC complex is largely inactivated. Furthermore, carbocyclic DBCC retards inactivation by DBCC. The failure to observe loss of catalytic activity in the presence of carbocyclic DBCC might be attributed to the stability of the carbocyclic DBCC-enzyme complex or to the inability of the analogue to form a stable complex with the apoenzyme in the absence of substrate. This latter interpretation is made unlikely by the demonstration that the carbocyclic analogue protects against inactivation by DBCC. To test more directly whether a stable complex is formed, dioldehydrase was allowed to react with tritiated carbocyclic DBCC in the absence of substrate and then passed through a Sephadex column. The experimental conditions and the elution pattern from the Sephadex column are shown in Fig. 6. Fractions 14, 15, and 16 contained, respectively, 6.6, 9.0, and 6.7 units of enzyme per µg of carbocyclic DBCC. It is in this respect similar to DBCC. It
Table IV
Effect of glycolaldehyde on activity of enzyme carbocyclic DBCC complex

| Time (min) | No addition | Glycolaldehyde |
|-----------|-------------|----------------|
| 0         | 100         | 98             |
| 2         | 104         | 11             |
| 5         | 100         | 1              |

has been shown that 7 to 10 units of enzyme bind 1 µg of DBCC (11). The enzyme in the column effluent was completely saturated with coenzyme, since further addition of carbocyclic DBCC or DBCC did not increase the catalytic activity. This experiment establishes that the apoenzyme has high affinity for carbocyclic DBCC in the absence of substrate and further illustrates the stability of the carbocyclic DBCC-enzyme complex. It is not possible to isolate an enzymatically active enzyme-DBCC complex under the conditions employed for the isolation of the enzyme-carbocyclic DBCC complex.

There are two additional processes which lead to the inactivation of the enzyme-DBCC complex. (a) When ethylene glycol is converted to acetaldehyde, the enzyme-coenzyme complex becomes catalytically inactive (12). This inactivation does not occur when D,L-1,2-propanediol is the substrate. (b) Addition of glycolaldehyde to diodehydrase and DBCC leads to the formation of a catalytically inactive enzyme-DBCC-glycolaldehyde complex. Formation of this complex is accompanied by a spectral change identical with that observed in the presence of substrate (12). Carboxylic DBCC was examined with respect to both these points. The time course of the reaction in the presence of ethylene glycol and D,L-1,2-propanediol is shown in Fig. 7. When ethylene glycol is the substrate, inactivation occurs with both coenzymes. With D,L-1,2-propanediol inactivation is observed only with carbocyclic DBCC. Inhibition of diodehydrase by glycolaldehyde in the presence of carbocyclic DBCC is summarized in Table IV. These results are similar to those obtained with DBCC. The spectral changes observed when glycolaldehyde or D,L-1,2-propanediol are added to carbocyclic DBCC-diodehydrase complex are identical with that previously observed in the presence of DBCC (19).

Discussion

DBCC and carbocyclic DBCC have similar spectra, light sensitivity, and chromatographic properties. An important chemical difference between DBCC and carbocyclic DBCC becomes apparent in the reactions with CN− and NaBH4. Carbocyclic DBCC, unlike DBCC, is unreactive toward these reagents. Both CN− and NaBH4 are known to bring about heterolytic cleavage of the carbon—cobalt bond of cobalamin in which the electrons of the bond remain with the leaving group. Presumably, this reaction is facilitated by the electron-releasing oxygen of DBCC which can stabilize the incipient negative charge at C-5'. No corresponding stabilization can occur with carbocyclic DBCC, where the oxygen is replaced by —CH2—. The acid-catalyzed cleavage of the carbon—cobalt bond of cobalamin resembles the reaction with CN− and BH4− in that it also is a heterolytic cleavage in which the electrons remain with the leaving group. It differs from the reaction with CN− and BH4− in that it probably does not involve attack of nucleophile on the carbon—cobalt bond. It was therefore of interest to compare the susceptibility of the carbon—cobalt bond of carbocyclic DBCC and DBCC to acid. Our results, pertaining to this point, are ambiguous, since we have not identified the products of the acid reaction. Although, upon exposure of carbocyclic DBCC to acid, coenzymatic activity was lost, a light sensitive compound was still present. This suggests that loss of coenzyme activity may not be caused by cleavage of the carbon—cobalt bond, but by modification of another part of the molecule, and it is possible that carbon—cobalt bond of DBCC is less susceptible to acid cleavage than that of DBCC.

The diodehydrase-DBCC complex and diodehydrase-carbocyclic DBCC complex have similar properties in many respects. Vmax with the carbocyclic coenzyme for the conversion of D,L-1,2-propanediol to propionaldehyde is one-third that with DBCC. With both coenzymes, tritium exchange between substrate and coenzyme is observed during the course of the catalytic process. Identical spectral changes are observed when either substrate or glycolaldehyde are added to the complex. Glycolaldehyde inactivates both complexes. In addition, the Km and over-all kinetic isotope effects are quite similar. The mechanisms of the reactions with the carbocyclic coenzyme and with DBCC are probably identical and it can be concluded that the ribosyl oxygen does not play an important part in the catalytic process. Therefore, any mechanism involving carbon—cobalt bond cleavage in which the stabilization of the incipient negative charge at C-5' is dependent on oxygen must be excluded for the reactions in which B12 enzymes participate. A specific example of this type of mechanism was discussed in the introduction of this paper. This conclusion does not imply that carbon—cobalt bond cleavage does not occur by other mechanisms. We believe that it is very likely that such a process occurs during the course of the reaction.

An important difference between the two coenzymes is the greater stability of the carbocyclic DBCC-diodehydrase complex than the corresponding DBCC-enzyme complex, in the absence of substrate. Although it is known that oxygen is involved in the inactivation of DBCC-enzyme complex, the mechanism of the inactivation process is unknown. Therefore, it is difficult to propose a reason for the stability of the carbocyclic DBCC-enzyme complex.

The catalytic conversion of D,L-1,2-propanediol to propionaldehyde in the presence of carbocyclic DBCC ceases after about 30 min, whereas in the presence of DBCC, it is linear for more than an hour. With both coenzymes, the conversion of ethylene

3The products of the reductive cleavage of the carbon-cobalt bond are B12+, adenade, and a mixture of L-erythro-2,3-dihydroxy-Δ4-pentenal and its reduction product, D-erythro-2,3-dihydroxy-Δ4-pentanol. C. P. Dumme and R. H. Abeles, unpublished results.
glycol to acetaldehyde stops after 30 min. We have shown that in the presence of ethylene glycol, inactivation of DBCC occurs. Presumably, a similar phenomenon occurs with propanediol and carbocyclic DBCC. Why this occurs with carbocyclic DBCC and not DBCC is not known. It could be, as many phenomena in enzymology, blamed on conformational effects.

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