AN EPR SIGNAL GENERATED BY THE ETHANOLAMINE DEAMINASE-COENZYME B_{12} COMPLEX IN THE PRESENCE OF SUBSTRATE.*

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Coenzyme B12- dependent rearrangements characteristically involve the transfer of an alkyl group or an electronegative atom from one carbon atom to an adjacent one, accompanied by the migration of a hydrogen atom in the opposite direction (1). In the coenzyme B12-dependent ethanolamine deaminase reaction, in which ethanolamine is converted to acetaldehyde and ammonia, analogy with the diol dehydrase reaction (2) suggests that the group transferred is the amino group, forming 1-aminoethanol as the initial product, since hydrogen has been shown to migrate from the carbinol carbon to the amino carbon of the substrate (3). It has been demonstrated with ethanolamine deaminase as well as with a number of other coenzyme B12-dependent enzymes that in these reactions, the coenzyme serves as an intermediate hydrogen carrier, with the migrating hydrogen atom becoming transiently bound to the 5'-carbon of the cobalt-linked adenosyl group (4,5).

An acceptor site for the migrating hydrogen atom could be created on the coenzyme by the temporary abstraction of a hydrogen from the 5'-carbon of the adenosine, or, as usually proposed (6, 7) by the enzymatic cleavage of the coenzyme at the carbon-cobalt bond. In either case the bond could be ruptured homolytically or heterolytically. To examine the possibility of a

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homolytic cleavage, EPR spectroscopy was used to obtain evidence for the appearance of unpaired electrons during the course of the reaction.

Ethanolamine deaminase was prepared and resolved of bound cobamide by the method of Kaplan and Stadtman (8). 5'-Deoxyadenosylcobalamin (DBC) was the generous gift of Professor H. Hogenkamp. 2-Amino-1,1-dideuterioethanolamine was prepared by the method previously described (3). Electron paramagnetic resonance spectroscopy (EPR) was performed with a Varian V-4500 x-band spectrometer with 100 kHz field modulation; sample temperature was maintained at -196° with a Varian V-4557 variable temperature device. The constituents of the reaction mixtures were introduced into the EPR sample tubes by means of syringes to which suitable lengths of polyethylene tubing were attached. Mixing was accomplished by agitating the solutions with the polyethylene tubing. The incubations were conducted at 23°. At selected times, the reactions were arrested by quickly freezing the samples in heptane cooled in a dry ice/trichloroethylene mixture (-78°). After freezing, the samples were transferred to liquid nitrogen and then to the spectrometer. All experiments were conducted in dim light, to avoid photolysis of the coenzyme (9).

These experiments were performed by freezing a mixture of enzyme, coenzyme and substrate before the substrate was completely consumed in the reaction, in the hope that by this method it might be possible to trap a paramagnetic intermediate arising while the enzyme was engaged in catalysis. To be able to see a signal from such a species, large amounts of ethanolamine deaminase were used. With these amounts of enzyme, very large quantities of substrate had to be available at the beginning of the reaction, to insure that some unreacted substrate was still present when the reaction mixture was frozen. When unlabeled ethanolamine was used as substrate in the required amount, the enzyme was found to precipitate during the course of the reaction. However, precipitation of the enzyme was not observed when deuterated ethanolamine was used, presumably because this compound, which reacts at only one-

sixth the rate of the undeuterated material (3), was used in substantially smaller quantities.

It can be calculated, assuming a constant reaction rate, that under the conditions of the incubation the deuterated ethanolamine in the reaction mixture would be completely consumed in 20 seconds (3). This period of time is a minimum value, since the reaction displays an initial lag (8) which seems to be greater with deuterated than protonated substrate.*

In the first experiment (fig. 1), the reaction was begun by adding enzyme to a mixture of DBC and ethanolamine. The spectrum of a sample frozen 8 seconds

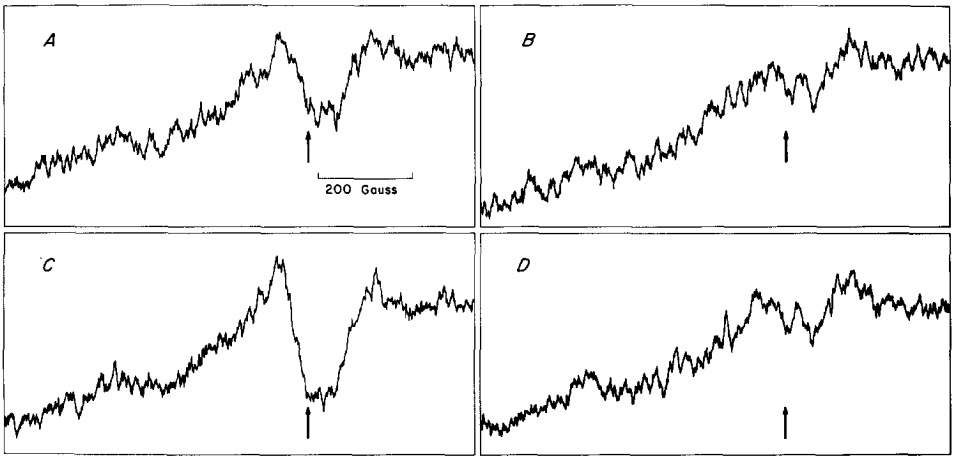

Figure 1. EPR spectra of a mixture of enzyme, DBC and substrate frozen during the act of catalysis. The sample contained 5 nmoles ethanolamine deaminase (10 nmoles in terms of active sites), 10 nmoles DBC, 4 μmoles 2-amino-1,1-dideuterioethanolamine ·HCl and 2 μmoles potassium phosphate buffer (pH 7.4) in a total volume of 0.25 ml. The reaction was begun with enzyme, and the experiment was conducted as described in the text. a. Sample frozen after 8 seconds, then thawed and refrozen after an additional 25 seconds. b. Sample thawed and refrozen after an additional 65 seconds. c. Sample thawed, 4 μmoles more of deuterated ethanolamine added, then sample frozen 15 seconds after the addition of the second aliquot of substrate. d. Sample thawed and refrozen after an additional 45 seconds. In each spectrum, the arrow indicates the position of g = 2.07 as determined from the Cu^{++}/EDTA signal. The magnetic field strength increases from left to right. The microwave frequency was 9.14 GHz, and the microwave power was 5 mW. No saturation was observed at this power level. The gain and modulation amplitude were constant for all spectra.

* B. Babior, unpublished results

after the addition of enzyme showed a signal with a peak to peak width of about 90 gauss in the vicinity of g = 2.09. On thawing the enzyme and permitting the reaction to proceed for 25 seconds more, the signal grew in size (fig. 1a). However, when the reaction was permitted to proceed for an additional 65 seconds, so that the substrate was completely consumed, the signal almost disappeared (fig. 1b). Additional substrate was then added, and the reaction was permitted to continue for 15 seconds more. Figure 1c shows that the signal was restored. After an additional 45 seconds of reaction, however, the signal again disappeared (fig. 1d). Thus, the appearance of the EPR peak appears to correlate with the presence of substrate in the reaction mixture.

In fig. 2 are the spectra of samples in which one component was omitted from the reaction mixture. Each sample was frozen 15 seconds after the two constituents were mixed. No signal was observed when enzyme and substrate were incubated alone, or when coenzyme and substrate were incubated alone. However, in the EPR spectrum of the sample containing enzyme and coenzyme in the absence of substrate, two small signals can be distinguished. These signals resemble the B12r signals observed by Barker et al. upon anerobic photolysis of DBC (10); moreover, the signal at higher field strength bears some similarity to the signals shown in figs. 1a and 1c.

The concentration of unpaired electrons in the sample represented in fig. 1c is estimated to be approximately 0.1 per active site (11). This value was obtained by integrating the signal twice, using the spectrum in fig. 2 (bottom) as the baseline, and using a solution of 0.5 mM $CuCl_2$ in 5 mM Na_2EDTA as standard.

The observation that an EPR signal appears in a mixture of ethanolamine deaminase, DBC and ethanolamine which has been frozen quickly during the act of catalysis, and that this signal disappears when the substrate is exhausted, suggests that the mechanism of action of coenzyme B12-dependent enzymes involves species with unpaired electrons. This was first proposed by Eggerer et al. on the basis of studies on methylmalonyl CoA isomerase (12).

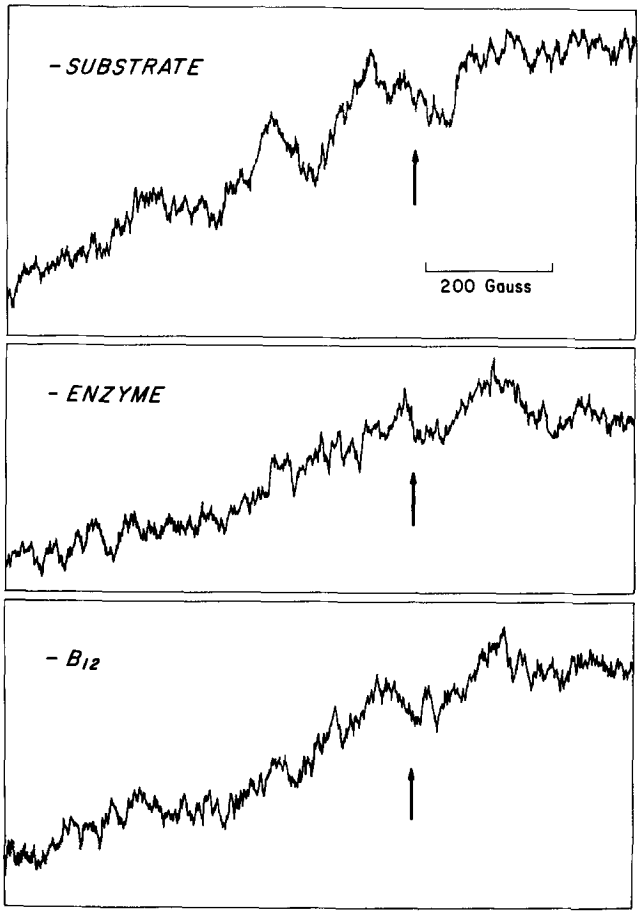

Figure 2. EPR spectra of reaction mixtures from which one component was omitted. The complete mixture was as described in fig. 1. The omitted component is noted in the figure. The experiments were conducted as described in the text. The arrow indicates the position of g = 2.07. The microwave frequency and microwave power are given in fig. 1. The gain and modulation amplitude were the same as for the spectra in fig. 1.

The fact that this signal is not associated with a process resulting in inactivation of the enzyme was shown by the observation that a solution of enzyme, coenzyme and substrate identical in composition to that used for EPR spectroscopy could be carried through two cycles of freezing and thawing without loss of activity of the enzyme.* Even though the species responsible for the signal may be on a branch rather than the main reaction path, this

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result can still be construed as evidence for such a mechanism, since it indicates that the enzyme is able to generate a paramagnetic species during the course of its activity.

Because of the lag in the reaction, it is difficult to predict exactly when the substrate will be entirely consumed. This is illustrated by the fact that in one case a signal was observed in the reaction mixture even after the reaction had gone for a total of 33 seconds, although according to calculation the substrate should have been exhausted by then (fig. 1a.). We attribute the difference in the size of the signals in fig. 1a and 1c to the possibility that at the time the sample represented in fig. 1a was frozen the ethanolamine concentration had fallen to the point where the enzyme was no longer saturated with substrate. In a similar experiment (not shown) in which the sample was frozen 15 seconds after each addition of ethanolamine, the signals were much more nearly the same size.

Although the location and breadth of the signal suggests that it is generated by a transition metal ion, presumably the cobalt of the coenzyme, we feel that it is premature to speculate at length on the identity of the paramagnetic species. Experiments are currently in progress to characterize the signal further.

SUMMARY

EPR spectroscopy of a mixture of ethanolamine deaminase, 5'-deoxyadenosylcobalamin and deuterated ethanolamine frozen during the act of catalysis revealed the appearance of a signal at g = 2.09. This signal disappeared when the reaction was permitted to continue until the substrate was exhausted, but reappeared upon subsequent addition of substrate. No signal was observed when enzyme or coenzyme was omitted from the reaction mixture, but small signals did appear when substrate was omitted. There were significant differences between these latter signals and the signal obtained from a sample containing all three components. This evidence is consistent with the hypothesis that species with unpaired electrons are involved in the mechanism of action of ethanolamine deaminase.

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