The Mechanism of Action of Ethanolamine Ammonia Lyase, a B$_{12}$-dependent Enzyme

X. A STUDY OF THE REACTION BY ELECTRON SPIN RESONANCE SPECTROMETRY

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SUMMARY

Ethanolamine ammonia lyase catalyzes the coenzyme B$_{12}$-dependent conversion of ethanolamine to acetaldehyde and NH$_3$. When the enzyme-coenzyme complex is frozen in liquid nitrogen during the act of catalysis, an electron spin resonance signal equivalent to about 0.04 spin per active site can be detected. By saturation experiments, the signal consists of two components: a broad derivative signal composed of two peaks ($g = 2.34$ and $g = 2.08$) assigned to the cobalt of B$_{12}$, and a narrow signal ($g = 2.007$) assigned to a free radical. With [1,1-D$_2$]ethanolamine as substrate, the shape and position of the signal was unchanged, but its size was increased. In the absence of substrate, a signal was still observed, but the concentration of unpaired electrons was reduced. The broad metal signal was unchanged except for a decrease in size. The radical signal was narrower (10 gauss, compared with 18 gauss in the presence of substrate) and was shifted ($g = 2.003$) with respect to the substrate-dependent signal, suggesting that the radical in the absence of substrate may have been different from the substrate-dependent radical. These observations support the proposal that the mechanism of action of this coenzyme B$_{12}$-dependent enzyme involves homolysis of the carbon-cobalt bond of the coenzyme.

Ethanolamine ammonia lyase (EC 4.1.2) catalyzes the coenzyme B$_{12}$-dependent conversion of ethanolamine to acetaldehyde and ammonia. Investigations of the mechanism of action of this enzyme have indicated that the initial step in this reaction involves cleavage of the carbon-cobalt bond of the coenzyme, followed by the transfer of hydrogen from the substrate to the adenosyl group of the coenzyme to form 5'-deoxyadenosine (1). Rearrangement then takes place, followed by the reaction of hydrogen from 5'-deoxyadenosine to acetaldehyde (or more likely, 1-aminoethanol (2)) and release of the products from the enzyme-coenzyme complex.

There is considerable evidence that in this reaction, the carbon cobalt bond is broken homolytically. Experiments involving the trapping of enzyme-bound intermediates (1) as well as experiments using spin-labeled cobamide coenzyme (3) both point to this conclusion. The first evidence, however, was the demonstration that upon addition of substrate to enzyme-coenzyme complex, an electron spin resonance signal appears (4). The instrumentation used for that study was not adequate for a close analysis of the properties of the signal, which was present at very low levels. The availability of better equipment now has permitted the signal to be studied in more detail. This study is the subject of the present report.

MATERIALS AND METHODS

Ethanolamine ammonia lyase from Clostridium sp. was prepared and resolved of bound cobamides by the method of Kaplan and Stadtman (5). Enzyme concentration was calculated on the basis of a molecular weight of 520,000 (6). The enzyme had previously been shown to possess two active sites per molecule (7). The turnover number of the enzyme preparation used in these experiments was 7,600 min$^{-1}$ at 23°C, using 10$^{-4}$m coenzyme.

Coenzyme B$_{12}$ was purchased from Calbiochem. Isotopically labeled ethanolamine was prepared by the reduction of glycine ethyl ester by LiAlD$_4$ (Alfa Inorganics) as previously described (8). Incubations were conducted at 23°C in quartz ESR tubes. The reaction was begun with enzyme which had been allowed to stand at room temperature for 10 min. At the appropriate time, the incubation was terminated by plunging the ESR tube into a container of isopentane immersed in liquid nitrogen ($-196$ K). The frozen sample was then transferred to the cavity of a Varian E-9 electron spin resonance spectrometer for spectroscopy. All operations were conducted in dim light to prevent photolysis of the coenzyme.

RESULTS

When a reaction mixture containing enzyme, coenzyme, and ethanolamine was frozen in liquid nitrogen 45 s after starting...
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The ESR spectrum of the enzyme-DMBC complex in the presence and absence of substrate. The reaction mixtures contained 15.4 nmoles of ethanolamine ammonia lyase, 44.7 nmoles of DMBC, 15.0 nmoles of ethanolamine-HCl (pH 7.4), and 2.0 nmoles of potassium phosphate buffer (pH 7.4), with omissions as noted, in a volume of 0.3 ml. Incubations (45 s) were conducted as described in the text. The ESR spectra were taken at 95° K. The microwave frequency was 9.180 GHz, and the microwave power was 1.6 milliwatts. Modulation amplitude (10 gauss) and gain were the same for both spectra.

Fig. 2. Rate of appearance of the substrate-dependent ESR signal. The reaction mixtures contained 7.0 nmoles of ethanolamine ammonia lyase, 44.7 nmoles of DMBC, 15.0 nmoles of ethanolamine-HCl (pH 7.4), and 2.0 nmoles of potassium phosphate buffer (pH 7.4) in a volume of 0.3 ml. Incubations were conducted for the times noted as described in the text. ESR spectra were taken at 95° K. The conditions were as described under Fig. 1 except that the microwave power was 10 milliwatts. Gain was the same for all spectra. The relative amplitude was estimated by measuring the distance between the peak and the trough of the major signal.

The incubation and then subjected to ESR spectrometry at liquid nitrogen temperatures, a well defined narrow signal was observed. This signal (shown in Fig. 1, top) was located at g = 2.0072 and had a peak to peak width of 18 gauss. These properties suggest that it was generated by a free radical.

A signal was also observed in the absence of substrate (Fig. 1, bottom). This signal was much smaller than the one generated in the presence of substrate. From its width and location, it too appears to represent a free radical. However, when compared with the substrate-dependent signal, it is shifted slightly toward higher field strength (g = 2.003) and is substantially narrower (10 gauss). This result suggests that the signals observed in the presence and absence of substrate represent two different species of radicals, although the possibility cannot be excluded that the differences between these signals are due to differences in the environment of a single radical species in the presence and absence of substrate.

The rate of appearance of the substrate-dependent signal is shown in Fig. 2. The first measurement was made on a sample frozen 15 s after the addition of enzyme, by which time the bulk of the signal had already appeared. Over the following 45 s, there seemed to be a slow increase in the size of the signal. However, the difference between the 15-s value and the 60-s value was close enough to experimental error that a conclusion cannot be drawn about whether this increase in signal size actually represents a change in the concentration of free radical or not.

When the spectrum was scanned over a wider range of field strengths, it became apparent that there were two other signals present in addition to the signal generated by the free radical. These signals are shown in Fig. 3. In order to bring out their features, this particular spectrum was taken under conditions in which the radical signal was extensively saturated. The weak field signal is located at g = 2.34, and the strong field signal at g = 2.08. They are both broad, with peak to peak widths of 90 gauss and about 65 gauss, respectively. With respect to both their position and their width, these signals are reminiscent of those generated by the paramagnetic d7 cobalt atom in CoCp2

In order to obtain evidence as to how many chemical species were responsible for generating these three signals, saturation experiments were performed. In the first of these experiments, a comparison was made of the amplitudes of the "radical" peak and the broad peak at g = 2.08 as a function of microwave power delivered to the cavity of the spectrometer. The results of this experiment, presented in Fig. 4, show that the "radical" peak is severely saturated at levels of power well below those required to saturate the broad peak. This observation confirms that the two signals are generated by different species and supports the idea that the narrow signal represents a radical and the broad signal represents a paramagnetic metal ion. On the other hand, the ratio of amplitudes of the two broad peaks is constant over a wide range of temperatures and power levels (Table I), suggesting that both these peaks are generated by
The amplitude of the signal at 1 milliwatt was arbitrarily set at 1.0. Power was taken at 95° K. Except for the microwave power, which varied as shown in the figure, the conditions were as described under Fig. 1. The relative amplitude was estimated by dividing the peak-to-trough distance of the signal in question by the gain. The amplitude of the signal at 1 milliwatt was arbitrarily set at 1.0.

The reaction mixture was the same one used for the substrate-dependent spectrum in Fig. 1. ESR spectra were taken at various temperatures and power levels as shown in the table. Modulation amplitude was 10 gauss for all spectra. The relative amplitudes of the peaks in question were determined as described in Fig. 4. The amplitude of the g = 2.34 peak at 95° K and 1 milliwatt power was arbitrarily set at 1.0.

Table I
Amplitudes of g = 2.08 and g = 2.34 signals as a function of power and temperature

| Conditions | Peak size | Power | Temperature (°K) | Low field (g = 2.34) | High field (g = 2.08) | Ratio |
|------------|-----------|-------|-----------------|----------------------|----------------------|-------|
|            |           |       |                 |                      |                      |       |
| *K         | milliwatt |       | 95              | 1                    | 1.6                  | 1.6   |
|            |           |       | 55              | 1                    | 2.0                  | 2.9   | 1.4   |
|            |           |       | 26              | 1                    | 2.4                  | 4.3   | 1.6   |
|            |           |       | 26              | 10                   | 3.9                  | 6.3   | 1.6   |

The relative amplitude was estimated by dividing the peak-to-trough distance of the signal in question by the gain. The amplitude of the signal at 1 milliwatt was arbitrarily set at 1.0.

Table II
Effect of deuterium-labeled substrate on amplitude of radical (g = 2.007) signal

The reaction mixtures contained 15.4 nmoles of ethanolamine ammonia lyase, 44.7 nmoles of DMBC, 15 amoles of substrate (pH 7.4), and 2 amoles of potassium phosphate buffer (pH 7.4) in a volume of 0.3 ml. Incubations were conducted as described in the text.

| Substrate | Relative peak size |
|-----------|--------------------|
| Unlabeled ethanolamine | 1.0 |
| [1,1-Dl]ethanolamine (5-min incubation) | 1.3 |
| [1,1-Dl]ethanolamine (45-s incubation) | 1.4 |

additional experiment was performed in which the incubation lasted 5 min.

With deuterated substrate there was a significant increase in the amplitude of both the radical and the metal signals (Table II). This was seen in both the 45-s and 5-min incubations. It indicates that the concentration of unpaired electrons generated in the presence of deuterated ethanolamine is greater than the concentration in the presence of unlabeled substrate.

Discussion

The first suggestion that coenzyme B12-dependent reactions might involve intermediates with unpaired electrons was made by Eggerer et al. (12) on the basis of studies on the coenzyme B12-dependent rearrangement of methylmalonyl-CoA. Direct evidence supporting this hypothesis only became available with the observation that ESR signals appear in reaction mixtures containing ethanolamine ammonia lyase, coenzyme B12, and substrate (4). Since this observation was made, much evidence has accumulated indicating that homolysis of the carbon-cobalt bond takes place during the ethanolamine ammonia lyase reaction (see above). In addition, recent evidence has indicated that other coenzyme B12-dependent enzymes may rupture the carbon-cobalt bond of the coenzyme homolytically. There are several papers concerning ESR signals generated by coenzyme B12-dependent ribonucleotide reductase under various circumstances (13, 14). Most recently, reports have appeared documenting the appearance of ESR signals during catalysis by diol dehydrase and glycerol dehydrase, both coenzyme B12-dependent enzymes which catalyze the conversion of glycols to aldehydes (15, 16).

If catalysis by ethanolamine ammonia lyase involves homolysis of the carbon-cobalt bond of the coenzyme, it would be expected that an ESR signal generated by this process should show a component due to the paramagnetic cobalt atom and one due to an unpaired electron on a free radical. Although previously obtained ESR spectra showed only a metal signal (4), both of these components were observed in the present study. Failure to observe the free radical component in the previous ESR study was probably due to a combination of instrumental inadequacy and saturation of the radical signal at the power levels used.

An additional cobalamin species has been described which can be observed by ESR spectroscopy, viz. peroxocobalamin (17). It is unlikely that either of the signals reported here represents this species. The g values for the metal signal seen in the present...
experiments agree closely with the $g$ values of $B_{12}$ and are in substantial disagreement with the $g$ values reported for peroxocobalamin ($g = 2.079$ and 2.003). With regard to the radical signal, not only is its $g$ value in rather poor agreement with either of the peroxocobalamin $g$ values, but its width is only about one-fifth that of the signal produced by peroxocobalamin.

From the properties of the signal, it appears that a steady state situation exists involving enzyme-coenzyme complex with an intact carbon-cobalt bond (diamagnetic) and complex with a dissociated bond (paramagnetic). The fact that an ESR signal is present in the absence of substrate indicates that under these conditions the carbon-cobalt bond is dissociated to a small extent. In the presence of substrate the signal undergoes alterations indicating a shift in the steady state in favor of the species in which the bond is dissociated as well as a probable change in the identity of the radical. That the situation in the presence of substrate is a steady state (or quasi-steady state), involving complexes with intact and broken carbon-cobalt bonds, is indicated by the time course (Fig. 2), which shows that the concentration of unpaired electrons rises rapidly within the first 15 s or less to a steady state level which then either remains constant or rises slowly over the next 45 s. The steady state level of carbon-cobalt bond cleavage of 45%, calculated from the concentration of unpaired electrons on the cobalt atom, agrees with previous experiments in which the steady state level of carbon-cobalt bond cleavage was measured directly with $^{13}$C-labeled coenzyme $B_{12}$ (1).

The ethanolamine ammonia lyase reaction displays a significant lag during its early stages, the maximum reaction rate not being achieved until 30 sec to 1 min after the reaction is begun (5). The present experiments show that the rate at which the concentration of unpaired electrons approaches the steady state is at least as rapid as the rate at which the deamination of ethanolamine is achieved until 30 sec to 1 min after the reaction is begun. This result supports the concept that the paramagnetic species generated in the ammonia-dependent exchange of hydrogen between acetaldehyde and coenzyme $B_{12}$ is at least as rapid as the rate at which the deamination of ethanolamine approaches its final velocity. This result suggests that the cleavage of the carbon-cobalt bond in the presence of substrate is directly connected to the hydrogen transfer step and is not merely related to the binding of substrate to the enzyme.

In most of the experiments, the conditions were such that the substrate had been completely consumed by the time the reaction was terminated by freezing in liquid nitrogen. The ESR signals in these experiments must therefore be due to paramagnetic species involved in the ammonia-dependent exchange of hydrogen between acetaldehyde and coenzyme $B_{12}$, a process which constitutes a partial reversal of the ethanolamine ammonia lyase reaction (2). The single experiment in which the deamination of ethanolamine was still taking place when the reaction mixture was frozen was the 45-s incubation with deuterium-labeled substrate. The ESR signal from this incubation was virtually identical in configuration and amplitude with the signal from the 5-min incubation with deuterated substrate, indicating that the same paramagnetic species are produced during the deamination of ethanolamine as during the acetaldehyde-coenzyme $B_{12}$ hydrogen exchange. This result provides further evidence supporting the formulation shown in Scheme 1.

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\[ \text{Scheme 1} \]

\[ \text{CH}_3 \xrightarrow{\text{Co}} \text{X} \xrightarrow{\text{slow}} \text{CH}_3 + \text{products} \]
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