BBA 63382

The mechanism of action of ethanolamine deaminase, II. The spectrum of the ethanolamine deaminase-coenzyme B_{12} complex during the act of catalysis

The mechanism of action of a number of coenzyme B_{12}-dependent enzymes, including diol dehydrase[1], ethanolamine deaminase[2], glutamate mutase[3] and methylmalonyl-CoA isomerase[4], has been shown to involve the transfer of hydrogen from the substrate to the cobalt-linked carbon atom of the coenzyme. To permit such a transfer to take place, a position must be made available on the coenzyme to accept the hydrogen atom. It has been proposed that this position is created by the splitting of the carbon–cobalt bond during the course of the reaction[5]. To obtain direct evidence concerning this proposed reaction, and to obtain information concerning the nature of the species arising from such a reaction if it does take place, experiments were undertaken to record the spectrum of ethanolamine deaminase while it was in the act of catalyzing the conversion of ethanolamine to acetaldehyde and ammonia.

The enzyme was prepared and resolved of bound cobamides by the method of KAPLAN AND STADTMAN[6]. 5′-Deoxyadenosylcobalamin and 5′-deoxyinosylcobalamin were generously provided by Professor H. Hogenkamp. Spectra were recorded on a Perkin–Elmer Model 350 recording spectrophotometer. The scanning time from 540 to 400 mμ was 12 sec.

The results of such an experiment are illustrated in Fig. 1, which shows spectra

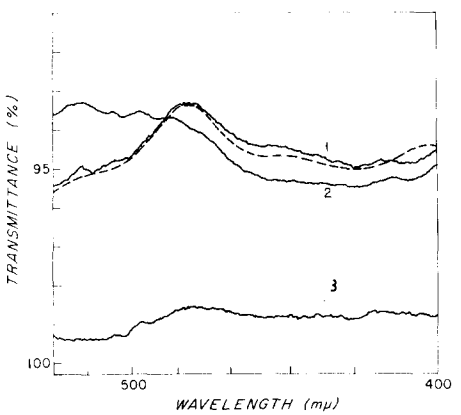

Fig. 1. The spectrum of the ethanolamine deaminase–coenzyme B_{12} complex during catalysis and after completion of catalysis. The reaction mixture contained 20 μmoles ethanolamine·HCl (pH 7.4), 1.5 nmoles (0.79 mg) ethanolamine deaminase, 3.0 nmoles 5′-deoxyadenosylcobalamin and 20 μmoles potassium phosphate buffer (pH 7.4) in a total volume of 1.0 ml. To conduct the experiment, all constituents except 5′-deoxyadenosylcobalamin were added to the cuvette, and an initial spectrum was obtained. 5′-Deoxyadenosylcobalamin was then added in dim light, and the spectrum was recorded at various times after the addition of cobamide as described in the text. The incubation was carried out at 30°. Spectrum 1 was taken 20 sec after the addition of 5′-deoxyadenosylcobalamin; Spectrum 2 was taken 120 sec after the addition of 5′-deoxyadenosylcobalamin; Spectrum 3 is the spectrum taken before the addition of 5′-deoxyadenosylcobalamin. — — — —, the spectrum of a 1:1 mixture of 5′-deoxyadenosylcobalamin and cob(II)-alamin.

of a mixture of enzyme, 5′-deoxyadenosylcobalamin and ethanolamine taken at two time intervals. It can be estimated from the specific activity of the enzyme (35 μmoles/min per mg) and the K_m for 5′-deoxyadenosylcobalamin ($1.5 \cdot 10^{-6}$ M) that in this experiment the substrate would be completely consumed in 80–90 sec. There are obvious differences between the spectrum taken at 20 sec (Spectrum 1), while substrate was still present in the reaction mixture, and the spectrum taken at 120 sec (Spectrum 2), after the substrate was exhausted. Spectrum 1 displays a prominent peak in the vicinity of 475 mμ, reminiscent of the peak observed in the cob(II)alamin spectrum, while Spectrum 2 is indistinguishable from the spectrum of 5′-deoxyadenosylcobalamin. Spectra taken at 160 and 200 sec were superimposible on the spectrum at 120 sec.

The similarity between Spectrum 1 and the cob(II)alamin spectrum becomes more striking when a correction is made for the amount of unbound 5′-deoxyadenosylcobalamin present in the reaction mixture under the conditions of the experiment. From the K_m for 5′-deoxyadenosylcobalamin, the concentrations for enzyme and 5′-deoxyadenosylcobalamin given in Fig. 1 and the fact that the enzyme possesses two equivalent binding sites for 5′-deoxyadenosylcobalamin[7], it can be calculated that only half the coenzyme is bound to enzyme. The broken line in Fig. 1 represents the spectrum calculated for a mixture of equal parts of cob(II)alamin and 5′-deoxyadenosylcobalamin, plotted with Spectrum 3 as the baseline *(vide infra)*. (For purposes of comparison, the calculated spectrum was drawn so that its amplitude at 475 mμ was equal to that of Spectrum 1. The total cobamide concentration of a 1:1 mixture of cob(II)alamin and 5′-deoxyadenosylcobalamin giving a spectrum of this amplitude is $3.1 \cdot 10^{-6}$ M, which agrees closely with the experimental cobamide concentration of $3.0 \cdot 10^{-6}$ M.) It can be seen that except for the absence of the small peak at 405 mμ. Spectrum 1 is almost identical to this calculated spectrum. It thus appears that during the act of catalysis, the enzyme-bound cobalamin develops a spectrum very similar to that of cob(II)alamin.

Spectrum 1 can clearly be distinguished from the spectrum of cob(I)alamin. This latter displays a peak at 460 mμ but none at 475 mμ. Moreover, it shows a very sharp increase in absorption below 450 mμ leading to an intense peak at 385 mμ (ref. 8).

In another experiment carried out under similar conditions, except using only 10 μmoles of ethanolamine, the spectrum taken 5 sec after the start of the reaction resembled Spectrum 1, while those taken at 40 and 80 sec were similar to Spectrum 2. To ascertain whether the spectral changes observed in these experiments could represent the appearance of a new enzyme–cobalamin species arising during the process of catalysis or merely reflected some initial event which occurred on binding of 5′-deoxyadenosylcobalamin to enzyme in the presence of substrate, an additional 10 μmoles of substrate were added at 115 sec and the sequence of spectra was repeated. The spectrum taken at 120 sec was found to have reverted to the form of Spectrum 1, while spectra at 160 and 200 sec again resembled Spectrum 2. These results are consistent with the interpretation that the spectral change represents a species which appears during the catalytic process.

Control experiments showed that Spectrum 1 did not appear if either enzyme or substrate was omitted from the reaction mixture. It also failed to appear if 5′-deoxyadenosylcobalamin was replaced by 5′-deoxyinosylcobalamin, an inhibitor competitive with 5′-deoxyadenosylcobalamin (K_i $2.3 \cdot 10^{-6}$ M) whose spectrum closely

resembles that of the coenzyme. Omission of 5'-deoxyadenosylcobalamin resulted in a spectrum resembling Spectrum 3; this spectrum did not change in the first two minutes.

The occurrence of a spectral change on incubation of a coenzyme B_{12}-dependent enzyme with substrate has been demonstrated previously in the case of diol dehydrase[5]. However, the spectrum of actively functioning diol dehydrase could not be interpreted because of the large excess of unbound coenzyme present in the reaction mixture. In the present experiments, in which enzyme and coenzyme were present in equivalent amounts, it was possible not only to demonstrate the occurrence of a spectral change but also to establish that this change represented the appearance of a species of cobalamin which closely resembled cob(II)alamin in its spectral characteristics. It is reasonable to postulate that this species is involved in the catalytic process. Although it is not possible to interpret such a spectral change in terms of a unique mechanism, the characteristics of the new species raise the possibility that it is generated by a homolytic cleavage of the carbon–cobalt bond of the coenzyme. If so, this result would imply that ethanolamine deaminase operates by a free radical mechanism, as was suggested by EGGERER *et al.*[9] in connection with the mechanism of action of methylmalonyl-CoA isomerase.

Supported in part by U.S. Public Health Service grants AM-09115, AM-5413 and FR-0076 from the National Institutes of Health, Bethesda, Md.

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Received December 13th, 1968

Biochim. Biophys. Acta, 171 (1969) 406–408

BBA 63384

Intramitochondrial localization of 5-aminolaevulinate synthase induced in rat liver with allylisopropylacetamide

The synthesis of haem in higher organisms involves a close cooperation of mitochondria and cell sap. Synthesis of 5-aminolaevulinate is a mitochondrial process, its