COENZYME-B_{12}-DEPENDENT ENZYME REACTIONS: A SPECTROPHOTOMETRIC RAPID KINETIC STUDY OF ETHANOLAMINE AMMONIA-LYASE

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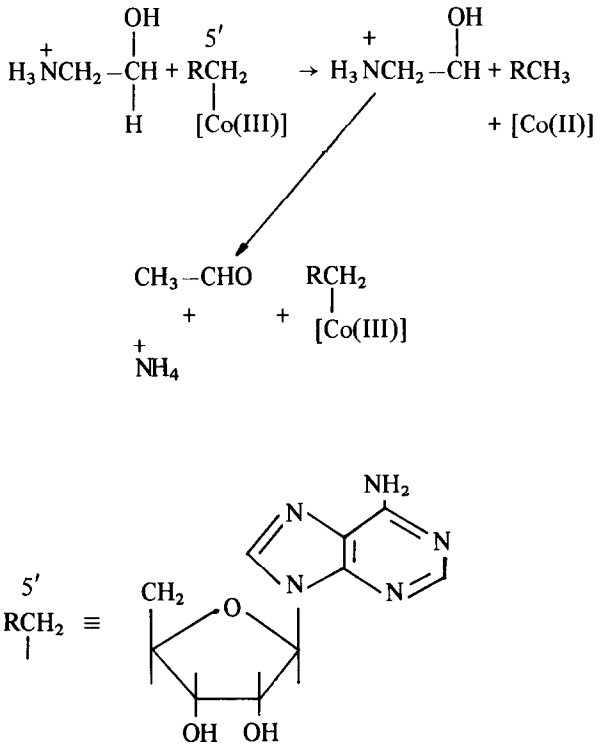
1. Introduction

The mechanisms of enzymic reactions involving coenzyme B_{12} as a cofactor have been studied widely (for references see [1]) and convincing evidence has been presented to show these reactions involve an intermolecular migration of a hydrogen atom between the substrate and the 5′-methylene group of the coenzyme, as exemplified by ethanolamine-ammonia-lyase (Scheme 1). In the present communication we present results from the use of stopped-flow, rapid scanning spectrophotometry which demonstrate the rapid formation of a cobalt (II) species in the pre-steady state phase of ethanolamine–ammonia lyase catalysed deamination of L-2-aminopropanol but we have failed to obtain evidence for the formation of any cobalt (I) intermediate.

In the case of the enzymes diol dehydrase, glycerol dehydrase, and ethanolamine ammonia-lyase, electronic absorption spectra [2,3] and e.p.r. spectroscopy [4,5] have been used to detect a cobalt (II) species during the steady-state phase of the reaction. Although these observations suggested the involvement of such a species in the catalytic process, it was not shown that the kinetics of formation and breakdown of the Co(II) intermediate were consistent with it being on the main reaction pathway. More recently, application of a rapid mixing, freeze-quenching procedure followed by



Scheme 1

e.p.r. spectroscopy on the quenched reaction mixture, enabled the time course of the appearance of the paramagnetic species to be followed during the pre-steady phases of the diol dehydrase [6] and ethanolamine ammonia lyase [7] catalysed reactions. In these cases the values of the rate constants for the appearence of the Co(II) species and of an organic radical were shown to be greater than the turnover number of the enzyme. It has also been shown that with ribonucleotide reductase [8], a coenzyme B_{12}-dependent enzyme which does not catalyse a rearrangement reaction, a Co(II) species appears at a kinetically competent rate during the reduction of the substrate.

Identification of intermediates by e.p.r. spectroscopy is of course limited to paramagnetic species, so that applications of this method would not have detected the presence of a diamagnetic species such as a Co(I) complex, which has been suggested as an intermediate in coenzyme B_{12} reactions [9]. As Co(I), Co(II), and Co(III) have readily identifiable visible electronic absorption spectra it should be possible to detect the presence of any these species in the pre-steady state phase of the reaction by using the technique of stopped-flow, rapid scanning spectrophotometry. This technique, of scanning the spectrum at a frequency of about 1000 per sec from 3 msec after mixing enzyme and substrates, enables direct observation of the spectra of reaction intermediates in the approach to the steady state, and so is not subject to the problems associated with the qualitative and quantitative assessment of frozen intermediates. As pointed out by Ballou and Palmer [10] 'the interpretation of results obtained by the rapid freezing method must be made with care and, whenever possible, correlated with parallel fast kinetic methods by optical or other means'. In studies on the steady state of the diol dehydrase catalysed reaction it has been noted [3] that the amount of Co(II) as measured by e.p.r. on the frozen sample (−150°C) is less than the amount as measured by absorption spectroscopy on the same sample (25°C). From more recent observations [7] on ethanolamine ammonia lyase, it appears that the interpretation of the e.p.r. Co(II) signal is complicated by the presence of a nearby radical. While the spectrophotometric observation of Co(II) does not suffer from this limitation it should be emphasised that e.p.r. spectroscopy and the spectrophotometric technique used in this paper are complementary.

2. Materials and methods

Ethanolamine ammonia lyase was purified from *Clostridium* sp. (EC 4.1.3.7.) and resolved of bound cobalamins according to the procedure of Kaplan and Stadtman [11]. Active site concentrations were calculated on the basis of an equiv. wt of 260 000 [12,13] and on a reported maximum spec. act. of 45 μmol/min/mg [14]. Substrate-free enzyme was prepared by dialysis against 0.01 M potassium phosphate, pH 7.4 for 24 hr. Spectrophotometric enzyme assays and thiol coenzyme reactions were carried out using a Cary 14 spectrophotometer. Coenzyme B_{12} (Glaxo) was recrystallised twice from aqueous acetone. Immediately before use an aqueous solution of the coenzyme was filtered through Whatman CM cellulose to remove any hydroxocobalamin. Thin layer chromatography of the aqueous eluate on cellulose (butan-2-ol/88% ammonia/water, 19/1.35/8) indicated that the coenzyme was homogeneous. An extinction coefficient at 342 nm of 1.11×10^4 M^{-1} cm^{-1} was used to calculate coenzyme B_{12} concentrations. All work involving the coenzyme was carried out in very subdued red light to avoid photolysis, and enzyme experiments were carried out in an atmosphere of oxygen-free argon. L-2-aminopropanol was purchased from Aldrich and redistilled before use. 2-mercaptoethanol, cysteine (free base), and reduced glutathione were purchased from Sigma.

Rapid-scanning, stopped-flow spectrophotometric measurements were carried out using the apparatus developed by Hollaway and White [15]. This apparatus comprises a Norcon model 501 rapid-scanning spectrometer (Norcon Ltd., 132 Water Street, South Norwalk, Connecticut, USA) equipped with a light source and suitable optics so as to produce two light beams each of which vary in wavelength over a span of about 200 nm in 1 msec at a repeat rate of 800 cycles per sec. One light beam passes through the observation cuvette of a stopped-flow apparatus to a photomultiplier tube and the other through a reference cuvette to the reference photomultiplier. Suitable electronic circuitry measures the difference in absorbance between the two cuvettes and, after analogue to digital conversion, the resulting spectra are stored in an ESL data-capture system (ESL Ltd., High Wycombe, Bucks, UK). Up to 32 spectra each comprising 256 × 8 bit words can be recorded at any preselected time during the reaction

after the 3 msec dead-time of the stopped-flow apparatus. The spectra are displayed on an oscilloscope screen, photographed and drawings made from the negatives. The drawings were processed manually by substracting appropriate base-lines to generate the spectra shown in the figures.

3. Results and discussion

Fig.1(a) shows spectra recorded during the first second of the ethanolamine ammonia lyase-catalysed deamination of L-2-aminopropanol, which is a poor substrate for this enzyme (k_{cat} 1–2 sec^{-1}) [14]. By comparing the spectrum obtained 0.94 sec after initiation of the reaction (fig.1(a) with published spectra of Co(I) and Co(II) cobalamins and of the Co(III) coenzyme (fig.1(b)) it can be seen that the intermediate has a typical Co II spectrum with the characteristic absorption maximum near 475 nm. The pronounced shoulder in the region of 500 to 525 nm and the position of the absorption maximum make it unlikely that the spectrum is that of a 'base-off' form of the Co(III) coenzyme (cf [3]). It is also similar to published spectra [2,3] observed during the steady state of other coenzyme B_{12}-dependent reactions.

Thus the intermediate can be identified as a Co(II) cobalamin species although it is not possible to decide unambiguously whether the benzimidazole ligand on the cobalt atom *trans* to the 5'deoxyadenosyl moiety is co-ordinated or not, i.e. whether it is in the 'base-on' or 'base-off' form (cf. figs.1(a) and 1(b).

Spectra recorded up to 1 sec showed an isosbestic point at 478 nm which indicates that the Co(II) species is formed directly from enzyme-bound coenzyme, or, if there is another intermediate between the coenzyme and the Co(II) species, then the rate of its breakdown is much faster than that of its formation. Within experimental error, the half-lives of time-courses at different wave-lengths were the same. A first-order rate constant of about 3 sec^{-1} was calculated from time-courses at both 460 nm and 525 nm (see fig.2). This is compatible with the Co(II) species being a true intermediate on the catalytic pathway since the turnover number per active site under the conditions of these experiments is approx. 1–2 sec^{-1} [13]. Earlier studies [7], involving rapid freeze-quenching studies with ethanolamine ammonia lyase and the same substrate, showed that a paramagnetic Co(II) species formed with a rate constant of 7 sec^{-1} which is in fair agreement with the present findings.

It is noteworthy that time-courses for the formation

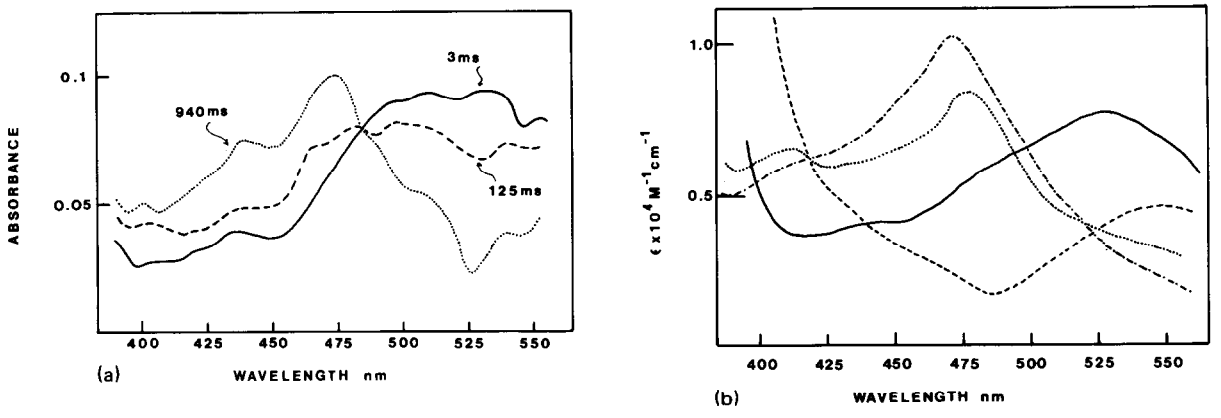


Fig.1(a). Spectral changes during the reaction of ethanolamine ammonia lyase with coenzyme B_{12} and propanolamine. The enzyme solution (10.5 μM) was placed in one syringe of the stopped flow apparatus and mixed with coenzyme B_{12} (10.5 μM) and propanolamine (1.75 mM) in the other syringe. The concentrations of the components in the reaction mixture are given in parenthesis. The reaction medium comprised carefully degassed 0.01 M phosphate buffer, pH 7.4 at 23°C. Spectra at the times shown were each recorded in 1 msec during which time they were not significantly distorted by the progress of the reaction.

Fig.1(b). Spectra of Coenzyme B_{12} derivatives. These spectra were drawn using data from [3 and 16]. They are for 'base-on' coenzyme B_{12}, Co(III) (——); 'base-on' $B_{12\,r}$, Co(II) (.....); 'base-off' $B_{12\,r}$, Co(II) (–·–·–·) and $B_{12\,s}$, Co(I) (– – –) respectively and are drawn on the same wavelength scale as the spectra in fig.1(a) for comparison.

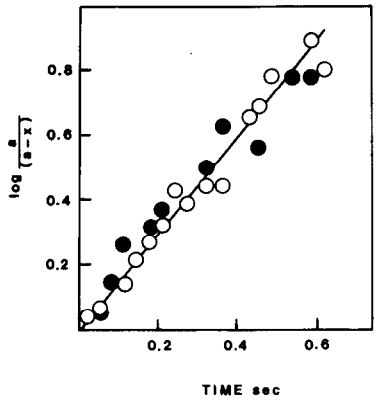


Fig.2. Logarithmic first-order plot of the time-course of the reaction of ethanolamine ammonia lyase with coenzyme B_{12} and propanolamine. Logarithmic plots were derived from the stored data from the reaction in fig.1(a). Data are presented for the time-courses at 525 nm (–○–) and 450 nm (–●–). The symbol 'a' represents the absorbance change for the complete reaction at the given wavelength and 'x' represents the absorbance change at time t after the start of the reaction.

of the Co(II) species (fig.2) did not show any observable lag phase which could have resulted from a slow combination of coenzyme and apoenzyme to form enzymically active holoenzyme. Spectra recorded during the first 40 msec of the reactions (32 spectra each of 1.25 msec duration) were all closely similar to the spectrum of enzyme mixed with coenzyme in the absence of substrate.

By assuming that the value of the molar extinction coefficient at 473 nm of the Co(II) enzyme intermediate approximates to that of B_{12r} (a Co(II) cobalamin) in aqueous solution, i.e. 0.92×10^4 M^{-1} cm^{-1}, [15] it can be calculated that at least 90% of the bound coenzyme was converted to the Co(II) species within 1 sec (figs.1(a) and 2). Thus, within experimental error there was a complete cleavage of the Co–C bond on reaching the steady-state phase of the reaction. It should be noted that an intermediate which is formed with a rate constant of 3 sec^{-1} and breaks down with a rate constant of 1 sec^{-1} (i.e. k_{cat}) should reach a steady-state concentration of 75% which is appreciably lower than the value of 90% estimated above. An explanation for this apparent inconsistency may be that the first turnover of enzyme which is necessary for cleavage of the Co–C bond of the coenzyme is slow, but once this has occurred, subsequent turnovers take place at a faster rate. Upon exhaustion of substrate the Co–C bond of the coenzyme reforms.

During this study a careful search was made for the presence of other transient spectral changes in the 400–600 nm range which would have indicated the presence of other intermediates. In particular, no evidence was obtained for the presence of a Co(I) species which would have displayed a weak maximum at 455 nm and not at 473 nm. Thus if a Co(I) species is an intermediate on the main reaction pathway in the ethanolamine ammonia lyase catalysed reaction, it must be present in a very small amount.

It seems likely that the first step in catalysis involves activation of the B_{12} coenzyme towards Co–C bond cleavage. This process may be facilitated by a substrate-induced conformation change in the holoenzyme, since mixing enzyme and coenzyme in the absence of substrate does not result in any significant formation of the Co(II) species. Binding of coenzyme to the enzyme leading to changes in the type of ligands bound to the cobalt atom would be expected to result in spectral changes, but minor alterations, such as hydrogen-bonding of the amide side-chains of the macrocyclic ligand would not be expected to alter the spectra significantly. In the present experiments the spectra recorded in the first few milliseconds of the reaction differed only slightly from the spectrum of free coenzyme, (a slight broadening at lower wavelengths) so that it seems unlikely that there had been any change of ligands at the Co atom. In particular the spectra are not shifted in the way expected for a 'base-off' coenzyme spectrum.

It has been suggested from the results of model experiments with the coenzyme [16] that the initial activation of the Co–C bond could involve displacement of the dimethylbenzimidazole base from the Co atom by a thiol group in the active site of the enzyme, as addition of a large molar excess of reduced glutathione in a buffered solution of coenzyme at pH 7.4 was shown to result in a shift in the visible absorbance spectrum towards lower wavelengths. Using our present technique we attempted to follow the time-course of the reported displacement reactions using 2-mercaptoethanol and cysteine as well as reduced glutathione. Even concentrations of thiols up to 0.1 M failed to alter the visible spectrum of the buffered coenzyme as long as the pH value was maintained at pH 7.4. Using higher concentrations of reduced glutathione it was

possible to reproduce the published results but tests on the resulting solutions showed that the pH value had been lowered to 3.5 after addition of the glutathione. We found that this was a result of the manufacturer's unmarked reduced glutathione being in the hydrochloride form. Repeating the experiment using a stock reduced glutathione solution pre-adjusted to pH 7.4 gave no change in the coenzyme spectrum and we conclude that there is no spectral evidence for a coenzyme B_{12}–thiol adduct and that the published spectral changes resulted from the formation of 'base-off' cobalamin as a result of lowering the pH value.

It has been shown [2] that with L-2-aminopropanol as a substrate, the ethanolamine ammonia lyase holoenzyme is irreversibly inactivated by a slow side-reaction. This process prevented us from observing the disappearance of the Co(II) spectrum and the reappearance of the coenzyme B_{12} Co(III) spectrum after all the substrate had been exhausted (within 2 min under the condition of figs.1(a) and 2 and 3. Spectra recorded in the later stages of the reaction are shown in fig.3, and these agree with the spectra obtained by Babior et al. [2] for this irreversible inactivation phase. The half-life of the process is about 4 min ($k_1{}^{obs} = 3 \times 10^{-3}$ sec^{-1}) and thus these spectral changes are too slow to have caused any perturbation of pre-steady state spectra shown in fig.1. The isosbestic point at

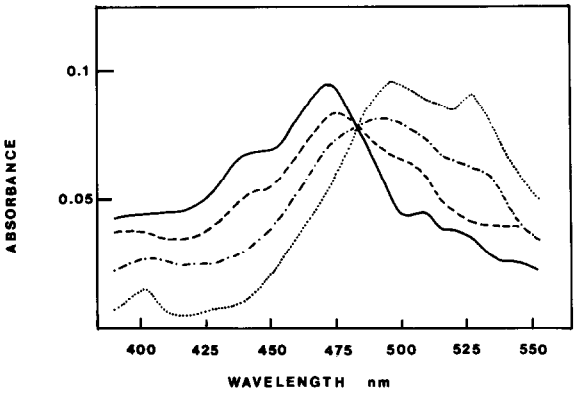


Fig.3. Time-course of the 'slow' spectral changes observed during the reaction of ethanolamine ammonia lyase with coenzyme B_{12} and propanolamine. Conditions and reactant concentrations were as for fig.1(a) and 'averaged-mode' (2^6) spectra [15] were collected at the following times: 30 sec (——); 135 sec (– – –); 240 sec (–·–·–) and 660 sec (.....).

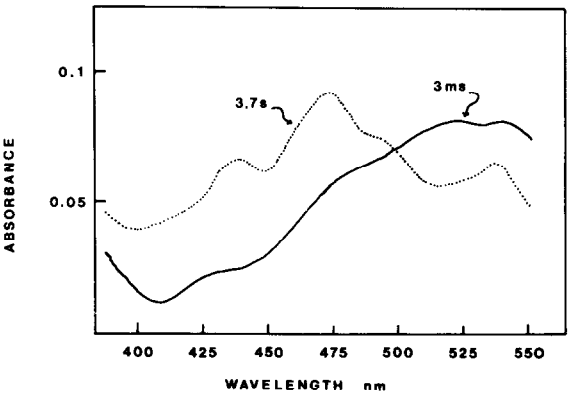


Fig.4. Spectra recorded during the reaction of ethanolamine ammonia lyase with coenzyme B_{12} and ethanolamine. Enzyme (10.5 μM) in one syringe was reacted with coenzyme B_{12} (10.5 μM) and ethanolamine (18 mM) in the other syringe. The concentrations shown in parentheses are those in the reaction cuvette. Other conditions are as for fig.1(a).

485 nm suggests that the slow reaction may involve a single process although of course it does not exclude the presence of much faster steps.

The k_{cat} value for ethanolamine ammonia lyase acting on propanolamine is between 1 and 2 sec^{-1} whereas it is about 140 sec^{-1} with ethanolamine as substrate, so that it is possible that there could be either a different rate-limiting step in this case or that there could be a different reaction pathway. In an experiment carried out with ethanolamine as substrate, a spectrum recorded 3.7 sec after mixing (fig.4) agrees with a published [17] spectrum for this reaction and is clearly similar to the Co(II) spectrum observed with propanolamine as substrate. It is concluded that the reaction with a 'good' substrate also proceeds through a rapidly formed Co(II) intermediate.

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