A Nuclear Magnetic Resonance Study of Substrate Binding by Yeast Alcohol Dehydrogenase

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The potential use of high resolution nuclear magnetic resonance (NMR) as a method for the study of the binding of small molecules by large ones is well recognized, and a review of the subject has recently appeared (Jardetzky, 1964). The basis of the method is that line widths in NMR spectra are sensitive to the degree and kinds of molecular motion present in the sample. Increased line widths are generally associated with diminished rates of molecular motion, particularly rotational motion; this decrease accounts for the increased widths observed when a small molecule binds to a large one, since the bound molecule undergoes motions characteristic of the complex. NMR line widths are interpreted in terms of the relaxation times, T_1 and T_2, whose theory is well understood. Zimmerman and Brittin (1964) have developed a theory dealing with the case in which a small molecule exists in equilibrium between two or more phases (i.e. bound and free) having different characteristic relaxation times and hence different line widths.

We have studied NMR spectra of ethanol and acetaldehyde in the presence of the enzyme yeast alcohol dehydrogenase (YADH) and its coenzymes NAD and NADH. From these studies we have concluded that neither ethanol nor acetaldehyde is bound to the enzyme in the absence of coenzyme. However, in the presence of NAD, ethanol does appear to be bound. Due to the large equilibrium constant favoring ethanol, we were unable to observe the acetaldehyde spectrum in the presence of NADH and enzyme. However, we have examined ethanol in the presence of NADH and

enzyme and found no evidence of binding.

Materials and Methods

NAD (Grade III), NADH disodium salt (Grade III) and bovine serum albumin (BSA) were obtained from the Sigma Chemical Company and used as received except that the albumin was lyophylized in D_2O to eliminate the large NMR signal due to exchangeable protons.

The YADH was obtained from G. F. Boehringer. The enzyme was desalted either by passing through a Sephadex G-25 column or by dialysis against .01 M phosphate buffer, pH 7.2. The resulting enzyme solutions were carefully shell frozen and lyophylized , D_2O added, and the lyophylization repeated to remove exchangeable protons. The resulting solid preparations contained 65-70% enzyme as determined spectrophotometrically according to Hayes and Velick (1954). The specific activity varied from 95 to 200 μmoles/min. for different preparations as assayed by the usual method (Kagi and Vallee, 1960).

Nuclear magnetic resonance spectra were obtained using the Varian HA 100 or Varian A-60 NMR spectrometers with the Varian C-1024 Time Averaging Computer. A capillary tube containing tetramethylsilane served as an external reference in all cases. A two cycles per second (cps) per second sweep rate was used throughout. The effective resolution was ascertained to be less than two cps during all runs by observing the residual H_2O peak from the solvent.

Results

Results of a typical experiment are shown in Figure 1. Figure 1A shows the NMR spectrum of a .4 ml sample solution in D_2O containing ethanol (10^{-3}M) and YADH (1.7×10^{-4}M) at 25°C. The solution was buffered at pH 7.2 by .1 M phosphate. Figure 1 B shows the spectrum of the same solution after addition of .002 meq. of NAD. The change in the spectrum appears to result from a decrease in the integrated intensity of the spectrum although some line broadening has also resulted.

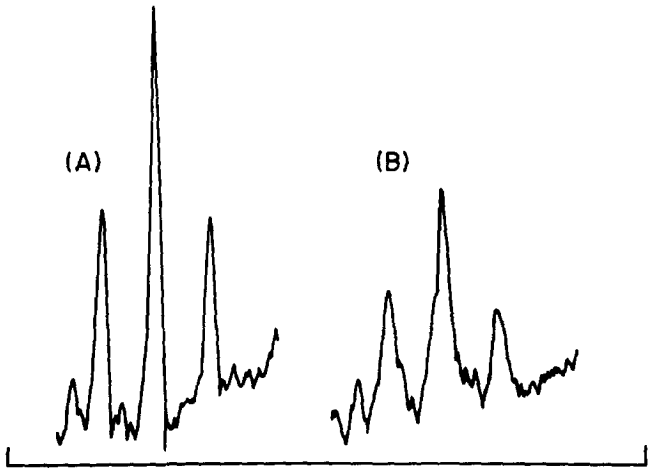

Fig. 1. Comparison of CH_3-spectrum of ETOH-YADH (A) without NAD and (B) with NAD added.

The latter may reflect a short lifetime of the ethanol against conversion to acetaldehyde. At 5°C about 40% of the intensity of the ethanol spectrum is lost on addition of NAD under the conditions described above with only a minor line width change. The spectra of 10^{-3}M ethanol and acetaldehyde were unchanged, except for a possible slight broadening in the presence of 1.7 x 10^{-4}M enzyme. The methyl spectra of both the aldehyde and hydrated forms of acetaldehyde were examined. Each of the experiments described has also been repeated using an equal weight of BSA in place of the YADH. The effect of BSA on both substrates is identical to that of YADH alone; only a slight broadening is effected. Addition of DPN to the ethanol-BSA solution produced no effect. Similarly, the ethanol methyl spectrum of a solution containing 2.5 x 10^{-3}M ethanol, 5 x 10^{-3}M NADH and 1.7 x 10^{-4}M enzyme was the same as that of the solution prior to addition of the enzyme.

Discussion

To explain the intensity decrease observed for ethanol in the presence of enzyme and NAD we have considered the following propositions: (1) Some of the ethanol is converted to acetaldehyde. (2) The protons of the ethanol methyl group are exchanged for solvent deuterium. (3) A ternary enzyme-NAD-ethanol complex is formed in which the bound ethanol has its NMR spectrum either shifted or broadened beyond detection or both.

Proposition (1) is not possible if the published values of the equilibrium constant are correct. The values range from about 10^{-11}M to 10^{-10}M depending on the enzyme concentration (Hayes and Velick, 1954). Only when the enzyme concentration exceeded that of the coenzyme was the value of 10^{-10} reached. In our experiments the coenzyme concentration exceeds that of the enzyme by a factor of about seven so that the equilibrium constant should be nearer 10^{-11}. We searched for the acetaldehyde resonance in the appropriate regions and found no evidence for its presence.

Proposition (2) is excluded by the fact that the intensity decrease is complete within five minutes (the time required to obtain the spectrum in our briefest experiment); no further decrease in intensity occurs even after several hours. Furthermore, with partial deuterium exchange the methyl spectrum should show coupling between deuterons and protons, which is not observed. The necessity that NAD be present for observation of the effect would also be difficult to explain on this basis.

Proposition (3) appears therefore to be the most likely explanation. Whether the effect is due to a chemical shift or a broadening of the resonance of the bound species beyond detection is not determined by these experiments but in either case it is implied that the average lifetime in each state is at least a few hundredths of a second. Otherwise the chemical shifts of the two forms would be averaged (Pople, Schneider

and Berstein, 1959) since methyl group shifts normally extend over only a few hundred cps (Varian Associates, 1963). In the fast exchange limit line widths are also averaged (Zimmerman and Brittin, 1957). Assuming a line width of at least 20 cps for the bound form which is a reasonable value for a methyl proton in a protein, (Bovey, et al., 1959) the width of the methyl resonance should be increased considerably when the lifetime of the unbound ethanol approaches the reciprocal of this value or about .05 seconds.

The lack of broadening of the acetaldehyde and ethanol resonances in the presence of enzyme alone is strong evidence that neither substrate is bound with a dissociation constant of the order of magnitude calculated from kinetic studies (i.e. $\sim 10^{-1}$M for ethanol and 10^{-4}M for acetaldehyde)(Nygaard and Theorell, 1955). Fisher and Jardetzky (1965), for example, have observed considerable line broadening when penicillin is bound to BSA to the extent of only one per cent.

Our results, then, suggest a compulsory binding order in which the coenzyme binds first. Previous studies on this point have not been in agreement (Wratten and Cleland, 1963; Silverstein and Boyer, 1964).

A more detailed account of this and other related work on this system will appear elsewhere.

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