PN 1289

Effect of oxidized nicotinamide-adenine dinucleotide on hydrogen-deuterium exchange of yeast alcohol dehydrogenase as measured by infrared spectrophotometry

Previous work from this laboratory has established hydrogen–deuterium exchange as a sensitive index of protein structure. The solvent analysis technique of LINDERSTRØM-LANG[1] has yielded reproducible results, but its applicability is seriously hampered by the inherent necessity of drying the protein completely. The infrared technique recently developed by NIELSEN[2], by BLOUT *et al.*[3], and by HVIDT[4] avoids this difficulty. It utilizes the disappearance of the amide II absorption band (1550 cm^{-1}) on deuteration of the peptide group. The present report demonstrates the usefulness of this method in detection of changes in hydrogen–deuterium exchange accompanying the interaction of yeast alcohol dehydrogenase with NAD^+.

Volumes of 200 μl of a 3.15% solution of 4-times recrystallized yeast alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1) were lyophilized[5] and subsequently dissolved in 200 μl deuterium oxide buffer (0.015 M NaH_2PO_4, 0.085 M Na_2HPO_4, $p^2H = 8$) containing NAD^+ if appropriate. The exchanging solution was transferred to a CaF_2 cell of 0.01 cm optical pathlength and infrared spectra in the region from 1760 cm^{-1} to 1350 cm^{-1} were recorded at intervals by means of a Perkin-Elmer Model 13 ratio-recording spectrophotometer. The reference cell contained solvent.

Difference spectra were obtained by subtracting pairs of spectra which had been recorded, (a) on the same sample after different times of exchange (time-difference spectra), or (b) on two different samples (*i.e.* with and without coenzyme present) after the same time of exchange (constituent-difference spectra). The effects of instrumental fluctuations were minimized by superimposition of the spectra to be subtracted in regions which were not affected by deuteration. The precision was $\pm$ 0.002 absorbancy units. Estimates of the numbers of exchangeable peptide hydrogens were obtained from absorption parameters determined for the amide II band of lysozyme[4].

Infrared spectra of yeast alcohol dehydrogenase in ^2H_2O are depicted in Fig. 1.

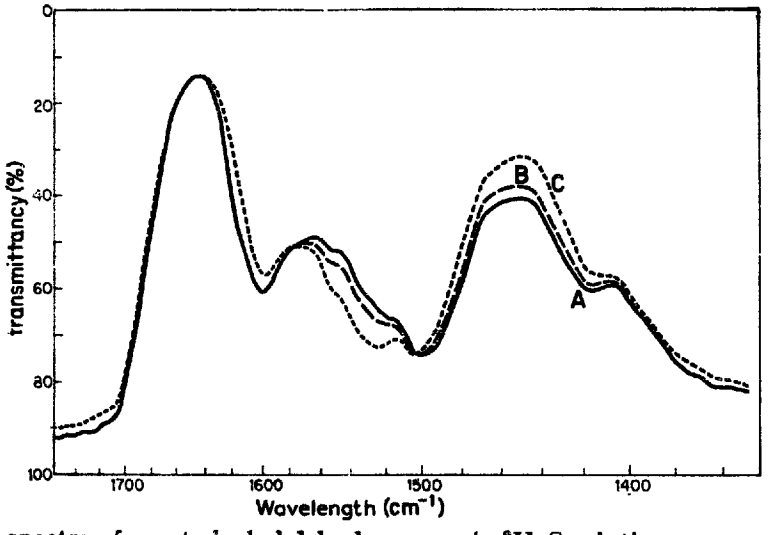

Fig. 1. Infrared spectra of yeast alcohol dehydrogenase in ^2H_2O solution. $2.1 \cdot 10^{-4}$ M yeast alcohol dehydrogenase in ^2H_2O (0.1 M sodium phosphate, p^2H 8, 21°). Curve A (———): after 27 min of exchange; Curve B (– – –): after 152 min of exchange; Curve C (-------): after 450 min of exchange in presence of 0.2% sodium dodecyl sulfate.

The amide I band at 1645 cm^{-1} is due to the carbonyl stretching motion. It is flanked by a carboxylate band at 1573 cm^{-1} (ref. 6), which is overlapping the amide II band at 1544.5 cm^{-1}. Originating from coupled CN-vibration and NH-deformation[7], the amide II band decreases on deuteration. Concomitantly a new absorption band appears at 1450 cm^{-1}. The crossing at about 1500 cm^{-1} represents an isosbestic point of the system. Fig. 2A shows the time-difference spectrum derived from spectra A and B of Fig. 1. The shoulder at 1525 cm^{-1} indicates heterogeneity of the amide II absorption reflecting perhaps different hydrogen-bonded structures within the protein molecule[8]. The disappearance of the amide II absorption is shown in Fig. 3, Curve A. The exchange reaction levels off after about 180 min, but there is still unexchanged peptide hydrogen left at this time (Fig. 1, Curve B). Estimates from the amide II absorbancy indicate that approx. 80% of all peptide hydrogens exchange rapidly,

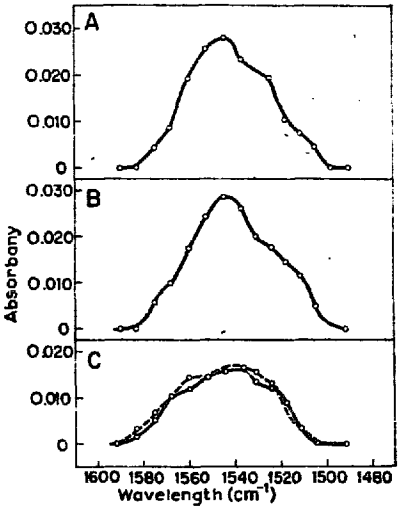

Fig. 2. Effect of deuteration on amide II absorption of yeast alcohol dehydrogenase: Difference spectra. Time-difference spectra corresponding to differences of spectra obtained on the same sample after 27 and 152 min of exchange: without coenzyme present (A); in presence of $5 \cdot 10^{-2}$ M NAD^+ (B). Constituent-difference spectrum (C) obtained from a spectrum of yeast alcohol dehydrogenase in presence of $5 \cdot 10^{-2}$ M NAD^+ by substracting the spectrum of a control sample which was exposed to ^2H_2O for the same length of time but did not contain NAD^+. Full line: after 21 min of exchange; broken line: after 152 min of exchange.

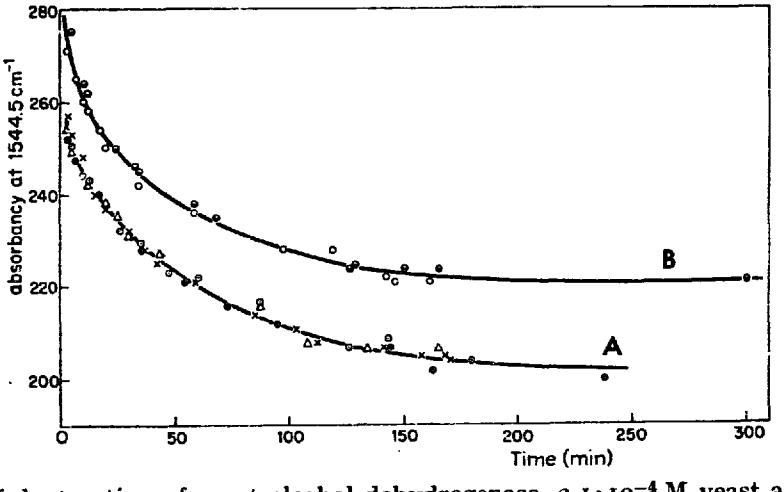

Fig. 3. Rates of deuteration of yeast alcohol dehydrogenase. $2.1 \cdot 10^{-4}$ M yeast alcohol dehydrogenase in ^2H_2O (0.1 M sodium phosphate, p^{2}H 8). Curve A, experiments without coenzyme present (×, ●, ⊙, △); Curve B, experiments in presence of $5 \cdot 10^{-2}$ M NAD^+ (○, ◒).

while 9% exchange slowly between 10 and 180 min. The remaining 11% exchange upon exposure to 0.2% sodium dodecyl sulfate (Fig. 1, Curve C).

The effect of 0.05 M NAD^+ on the hydrogen–deuterium exchange of yeast alcohol dehydrogenase is borne out by Fig. 3. The upward displacement of the exchange curve (Curve B) represents a net increase in the number of non-exchangeable peptide hydrogen atoms. In contrast, the proportion of slowly exchanging peptide hydrogens seems unchanged by the coenzyme as indicated by the similarity of the time-difference spectra in 2A and B. Apparently the hydrogen atoms prevented from exchanging by NAD^+ belong to peptide groups open to instantaneous exchange in the absence of the coenzyme. The spectral characteristics of these groups are shown by the constituent-difference spectrum (Fig. 2C). From its area it was estimated that about 4% of all peptide hydrogens were prevented from exchanging by the coenzyme, *i.e.* about 56 atoms per enzyme molecule (molecular wt. 150000) or 14 atoms per coenzyme binding site. Partial inactivation of yeast alcohol dehydrogenase by prolonged storage at room temperature resulted in a comparable reduction of area of the constituent-difference spectrum.

According to the usual interpretation of hydrogen–deuterium exchange of proteins it appears most likely that the differences observed reflect a change in the 3-dimensional structure of yeast alcohol dehydrogenase induced by the coenzyme.

It has been suggested that the action of yeast alcohol dehydrogenase proceeds in accordance with a compulsory order mechanism allowing a combination with the substrate only after the enzyme–coenzyme complex has been formed[9,10]. It might be speculated that the observed structural change leads to the critical configuration for substrate binding.

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