PRESSURE RELAXATION STUDIES OF ISOMERISATIONS OF HORSE LIVER ALCOHOL DEHYDROGENASE LINKED TO NAD^+ BINDING

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1. Introduction

In previous experiments we showed that proton liberation during the oxidation of ethanol by horse liver alcohol dehydrogenase (LADH) occurred during formation of the binary and ternary enzyme–substrate complexes rather than during the hydride transfer step [1]. Furthermore this ionisation change is in some way linked to quenching of protein fluorescence, suggesting a change in the environment of one of the two tryptophans of the enzyme subunits [2,3]. From these experiments and further studies of the pH dependence of protein fluorescence a model was proposed for the conformational states of this enzyme [3,4].

In order to account for the different pK_a values for ADP–ribose equilibrium binding (8.9) and the NAD^+ binding rate (9.8), Shore et al. [4] proposed that two conformations of the enzyme exist at neutral pH, E^H and $\overset{*}{E}{}^H$. The latter form showed quenched protein fluorescence, and was linked to the ionization of an enzyme functional group with a pK_a less than 9. The scheme proposed to account for this is similar to our current mechanism. By the use of relaxation techniques in the present study, it has been possible to demonstrate directly the existence of these conformational states and the pH linkage. Coenzyme binding markedly slows down the relaxation of the conformational equilibria.

2. Experimental

LADH was prepared by the method of Theorell et al. [5] and the concentrations in terms of one active site per 40 000 daltons were calculated from extinction at 280 nm using $\epsilon^{280}_{1\ mg/ml}$ 0.455.

The pressure relaxation technique was used as described by Davis and Gutfreund [6]. Protein fluorescence was recorded using a mercury lamp and a monochromator set at 300 nm for excitation. Emission was observed through UG11 and WG335 filters. The photomultiplier output was digitised after amplification and recorded on tape for subsequent processing in a PDP 8/e computer. Averaging of a group of transients was carried out as described elsewhere [7].

In order to avoid possible interference by NADH in experiments involving the enzyme and NAD^+, a regenerating system for NAD^+ was employed in which all solutions contained 7.5×10^{-9} M lactate dehydrogenase and 3.7×10^{-4} M pyruvate.

3. Results and discussion

Fluorescence pH titration measurements [3] show that the enzyme LADH undergoes a conformational change, in the absence of substrates in the pH range 7–10, which is associated with a marked decrease in protein fluorescence. We now report that pressure relaxation measurements of protein fluorescence show a single relaxation process for LADH in the absence of substrates over a similar pH range. Reciprocal relaxation times of approx. 2500 s^{-1} were observed at pH 7.5, increasing to approx. 7000 s^{-1} at pH 10.0. The amplitudes of those relaxations observed using constant pH buffer (Tris-HCl) were very similar to those observed in pressure-dependent buffer (phos-

phate) when measurements were made in the pH range 7.0–9.0. However, at pH values above 9.0 larger relaxation amplitudes were observed in carbonate buffer (in which pH increases with pressure fall) than in glycine buffer (in which pH is essentially pressure invariant). Measurements in carbonate buffer at pH 9.1 did not show any systematic dependence of relaxation time with enzyme concentration over an 8-fold concentration range. These observations are consistent with the titration curves of Wolfe et al. [3] and the postulated hydrogen ion-linked isomerisation of the enzyme of the form

$$EH \underset{H^+}{\rightleftharpoons} E^- \rightleftharpoons \overset{*}{E}{}^-$$

where $\overset{*}{E}{}^-$ represents the alkaline form of the enzyme in a conformational state in which the tryptophan fluorescence is quenched.

Pressure relaxation measurements of the protein fluorescence of LADH in the presence of NAD^+ made over a range of NAD^+ concentrations, show a single relaxation process at high NAD^+ concentrations (fig.1). The relaxation times observed are considerably slower than those observed in the case of the enzyme alone, ranging from 60 s^{-1} to almost 300 s^{-1}. At low degrees of saturation of the NAD^+-binding site a faster relaxation, probably corresponding to that of the free enzyme is also observed. Figure 2 shows the observed reciprocal relaxation time ($1/\tau$) as a function of total NAD^+ concentration measured at pH 7.7 in Tris buffer.

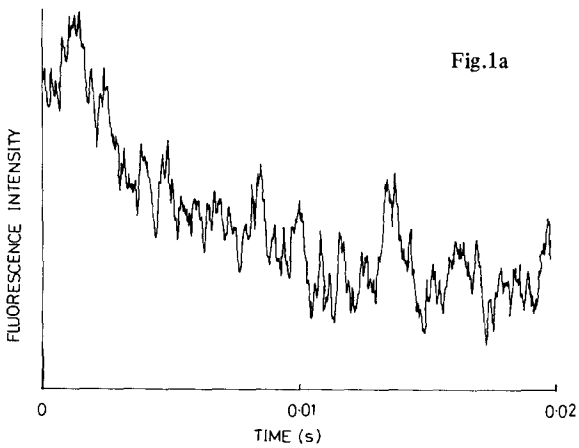
The full line is calculated assuming that the binding of NAD^+ is given by the mechanism:

$$E^H + NAD^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E^H_{NAD^+} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \overset{*}{E}{}^H_{NAD^+}$$

in which the NAD^+-binding step is assumed to equilibrate rapidly compared with the isomerisation step. The slower of the two theoretically possible reciprocal relaxation times for this mechanism is given by the expression:

$$\frac{1}{\tau} = \frac{k_2}{1 + k_{-1}/(k_1\{[E^H] + [NAD^+]\})} + k_{-2}$$

Over the range of enzyme and NAD^+ concentrations used, the sum of the equilibrium concentrations of unbound enzyme and NAD^+ may be replaced by the total NAD^+ concentration without incurring unacceptable error. The value of $1/\tau$ extrapolated to $1/[NAD^+]_{total} = 0$ will be approximately equal to k_{-2}. The above expression may then be written as



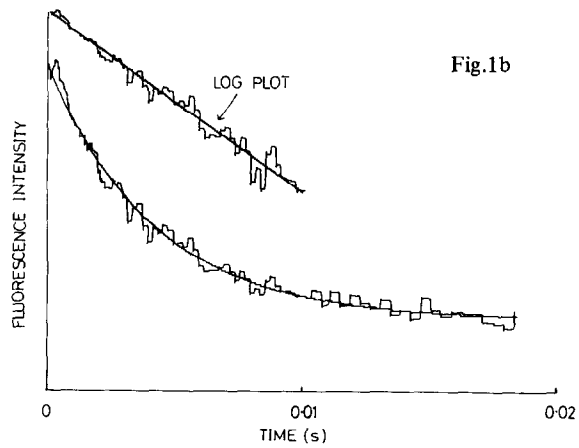


Fig.1a. The relaxation of protein fluorescence following a single pressure relaxation from 180 at. to 1 at. in a solution containing 11 μM LADH and 1.2 mM NAD^+ at pH 7.7. Fig.1b. The computed average transient relaxation (CAT) obtained from eight identical sequential relaxations of the same solution. The averaged data is also shown as a logarithmic plot, linear regression analysis of which gave $1/\tau = 244 \pm 29\ s^{-1}$. The least squared straight line shown was used to compute the smooth curve drawn through the averaged data.

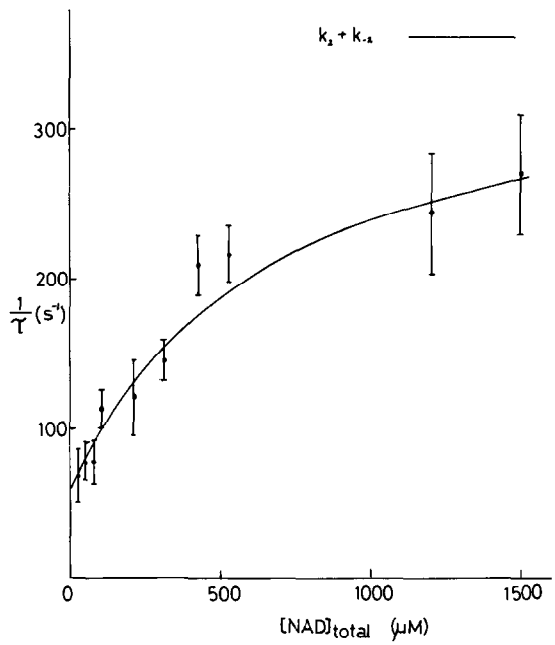


Fig.2. The reciprocal relaxation time for protein fluorescence of a solution (pH 7.7) containing 11 μM LADH and added NAD^+ as a function of total NAD^+ concentration. The smooth curve was obtained as described in the text.

$$\frac{1}{\frac{1}{\tau} - k_{-2}} = \frac{1 + \frac{k_{-1}}{k_1}\frac{1}{[NAD^+]_{total}}}{k_2}$$

In order to calculate the full line shown in fig.2 the value of $k_{-2} = 60\ s^{-1}$ obtained as described above was used to obtain a plot of $1/[(1/\tau - k_{-2})]$ against $1/[NAD^+]_{total}$. For the above mechanism this plot should be linear with the negative intercept at $1/[(1/\tau) - k_{-2}] = 0$ equal to k_1/k_{-1} and the intercept at $(1/[NAD^+]) = 0$ equal to $1/k_2$. The data from fig.2 were plotted as described and found by least squares linear regression to lie on a straight line given by $1/[(1/\tau) - 60] = 2.6/[NAD^+] + 3.82 \times 10^{-3}$ with a correlation coefficient $r^2 = 0.94$. These regression parameters gave values of 680 μM for k_{-1}/k_1 and 262 s^{-1} for k_2. Use of these values to calculate $1/\tau$ as a function of $[NAD^+]$ did not give good agreement with the experimental data at high NAD^+ concentration. However, with k_2 adjusted to 302 s^{-1} the other constants being unaltered, the full line shown in fig.2 was obtained giving reasonable agreement with the experimental data. The latter procedure is justifiable in that the reciprocal of the assumed k_2 lies within the uncertainty of the intercept of the line with the axis at $1/[NAD^+] = 0$. Furthermore, the values for $K_1 = 680\ \mu M$, $K_2 = 0.2\ K_1$, $K_2 = 136\ \mu M$ are not unreasonable when compared with the dissociation constant for NAD^+ binding under these conditions.

It is well established that on the binding of NAD^+ to LADH the pK_A of a group adjacent to the binding site shifts from about 9.8 to about 7.6 [3,8]. Accordingly, since it is also known (loc. cit.) that protein fluorescence is quenched on NAD^+ binding, it is to be expected that in the NAD^+-binding relaxation experiments described here at pH 7.7, the solution must contain the quenched enzyme in both the protonated and unprotonated form. As a consequence k_{-2} should properly be regarded as an apparent first order rate constant; since the mechanism written to take account of the quenched species present at pH 7.7 would be as follows:

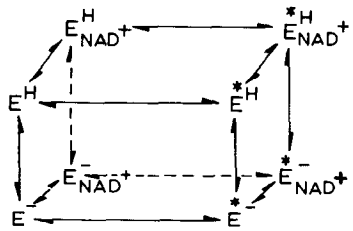
$$E^H + NAD^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E^H_{NAD^+} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \overset{*}{E}{}^H_{NAD^+} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \overset{*}{E}{}^-_{NAD^+} + H^+$$

The reciprocal relaxation time for this mechanism, assuming rapid equilibration of steps 1 and 3 compared with step 2 and with the hydrogen ion concentration buffered is given by

$$\frac{1}{\tau} = \frac{k_2}{1 + k_{-1}/(k_1\{[E^H] + [NAD^+]\})} + \frac{k_{-2}}{1 + (K_A/[H^+])}$$

Where $K_A = k_3/k_{-3}$ the acid dissociation constant of the species $\overset{*}{E}{}^H_{NAD^+}$; K_A is believed to have a value of $10^{-7.6}$. Thus at pH 7.6 the apparent value of k_{-2} would be half the true k_{-2} value. Increasing the pH from 7.7 through 8.1 to 8.7 leads to approximate apparent k_{-2} values of 60 s^{-1}, 20 s^{-1} and 5 s^{-1}, respectively, in qualitative accordance with the postulated mechanism.

In order to accommodate the relaxation behaviour observed for NAD^+ binding and for proton release together with the equilibrium data available on this system the following mechanism may be postulated:



This system presents a nice example of a substrate induced conformation change linked to a proton equilibrium. The latter is an essential feature of the uptake and release of one of the reactants (H^+). The conformation change, characterised by k_2 and k_{-2} appears to play a significant role in the rate of the reaction in both directions.

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