THE BINDING OF NADH TO LIVER ALCOHOL DEHYDROGENASE: A TWO STEP REACTION.

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Received March 23, 1967

The reaction between Horse Liver Alcohol Dehydrogenase (LADH = 2 E) and NADH (R) has hitherto been regarded as a simple bimolecular addition between R and each of the two independent binding sites of the LADH molecule

$$E + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ER \qquad (I)$$

Since the dissociation constant k_2/k_1, at 23°C, pH 7, now with our chromatographically and electrophoretically homogeneous LADH comes out = 0.20 μM (1) and k_2, the maximum velocity of the catalytic reaction with ethanol and NAD^+, is 3.12 sec^{-1} (2), k_1 is calculated to be = 16 $\mu M^{-1} \times sec^{-1}$. The direct determination of k_1 was tried already in 1951 (3) by a spectrophotometric "rapid flow" method giving a value of $k_1 = 4 \times 10^6$ $\mu M^{-1} \times sec^{-1}$. Since at that time the preparations were less pure than now, and the method not very accurate, we built a new apparatus for "stopped flow", to be described elsewhere, in order to reexamine the combination reaction in some detail. The apparatus registered the increase in fluorescence occuring upon the formation of the binary complex (4). The two syringes of the flow apparatus were of equal size, giving a twofold dilution of each solution after mixing. The time between mixing and observation was 4 msec at a flow rate of 2 m x sec^{-1}. Four sets of experiments were made at 23°C, phosphate buffer μ = 0.1, pH 7.0; 10 μN E + 10 μM R (= 5 μM after mixing), 3 E + 3 R (= 1.5 μM), 1 E + 1 R (= 0.5 μM) and 0.3 E + 0.3 R (= 0.15 μM).

The fluorescence increase after stop of the flow, especially from the two intermediate concentrations, was found to follow S-shaped curves (see Fig. 1). This is an indication that the highly fluorescent complex (ER^*) is preceded by a complex ER, in which the fluorescence of free R is not yet changed:

$$E + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ER \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} ER^* \qquad (II)$$

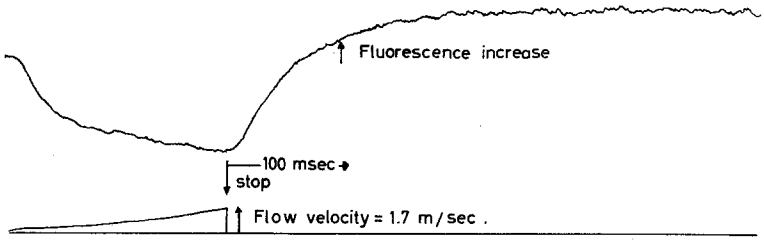

Fig. 1. One of the experimental recorder traces (3 μN E + 3 μM R giving concentrations of 1.5 μM at flow stop).

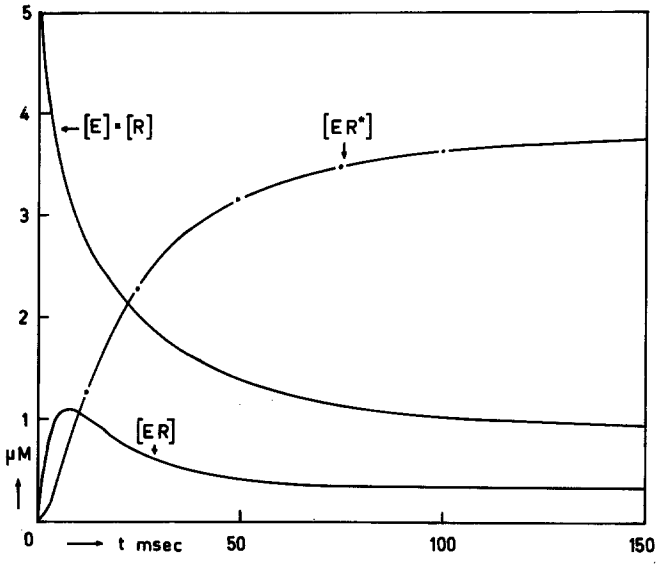

Fig. 2. 10 μN E mixed with 10 μM R to give 5 μM concentration at t = 0. Full drawn curves calculated. Points average of 2 experiments, $\sigma < 0.3$ %. 23°C, pH = 7.0.

This assumption leads to the following relations: The overall equilibrum constant K = 0.20 μM becomes, if $k_{-1}/k_{+1} = K_1$ and $k_{-2}/k_{+2} = K_2$

$$K = \frac{K_1}{1+1/K_2} = 0.20\ \mu M \qquad \text{(III)}$$

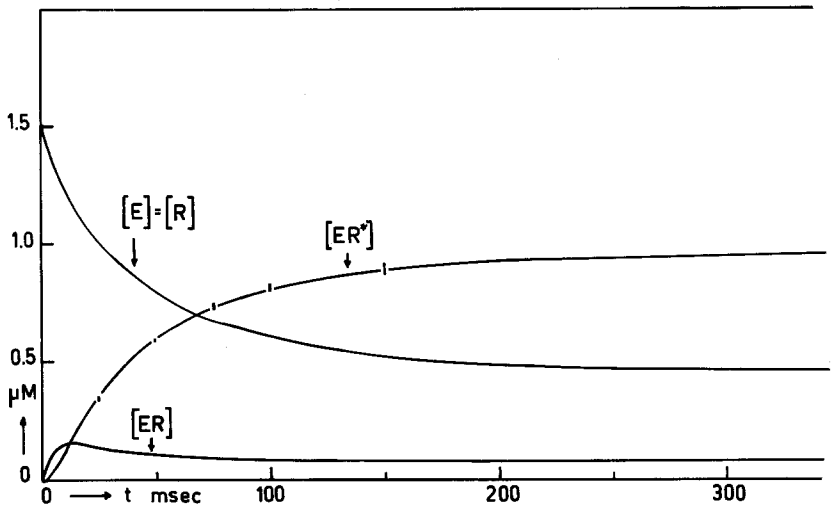

Fig. 3. 3 μN E mixed with 3 μM R to give 1.5 μM concentration at t = 0. Full drawn curves calculated. Points are the average of 6 experiments, height = 2 σ. 23°C, pH = 7.0.

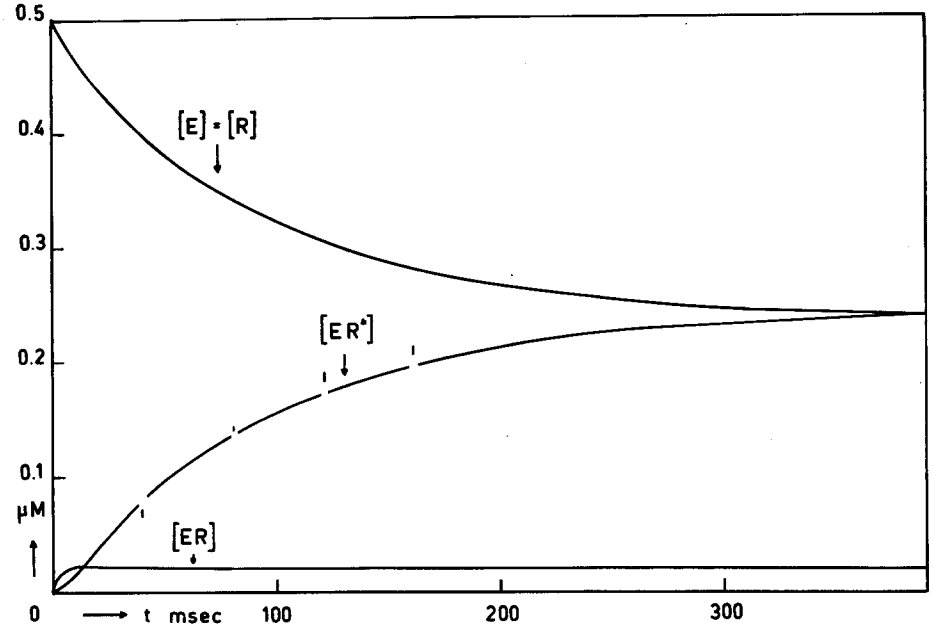

Fig. 4. 1.0 μN E mixed with 1.0 μM R to give 0.5 μM concentration at t = 0. Full drawn curves calculated. Points are the average of 3 experiments, height = 2 σ. 23°C, pH = 7.0.

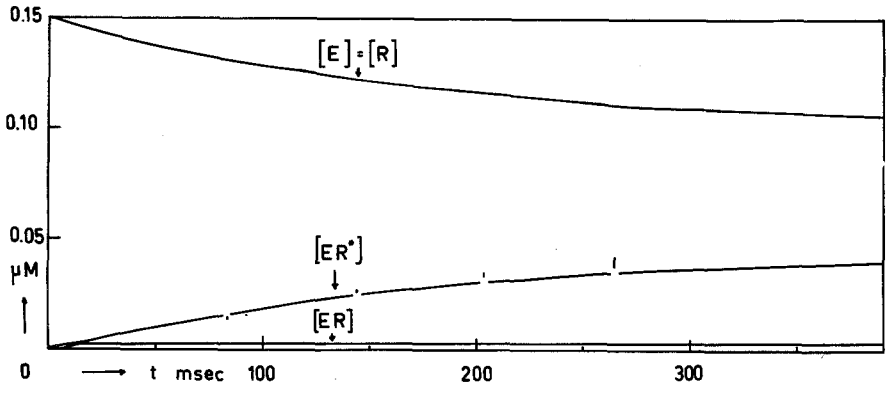

Fig. 5. 0.3 μN E mixed with 0.3 μM R to give 0.15 μM concentration at t = 0. Full drawn curves calculated. Points are the average of 3 experiments, height = 2 σ. 23°C, pH = 7.0.

The maximum velocity of the catalytic reaction between E, NAD^+ and alcohol, assuming ER^* to be the complex remaining after release of the aldehyde, is

$$e/v_{t=0} = \frac{1}{k_{-1}} + \frac{k_{+2}}{k_{-2} \times k_{-1}} = \frac{1}{k_{-1}} (1 + 1/K_2) = 3.12 \text{ sec}^{-1} \qquad \text{(IV)}$$

Combining Eq. (III) and (IV) again gives $k_{+1} = 16\ \mu M^{-1} \times \text{sec}^{-1}$. The maximum velocity of ER^* formation, when E and R $\longrightarrow \infty$ is = $(k_{+2} + k_{-2})\ \text{sec}^{-1}$. This could be extrapolated from the experiments with the highest concentration to be $\approx 100\ \text{sec}^{-1}$.

Digital computing with IBM 1401 was used for finding the combination of the four rate constants that fitted best to the average of the experimental curves. Intervals of 1 msec were found to be sufficiently short for the three higher concentrations, 2 msec for 0.3 μN + 0.3 μM. The number of experiments was 6 for the combination "3 + 3", 2 for "10 + 10" and 3 for each of the others. When selecting the values we kept the dissociation constant K = 0.20 μM, since this value was checked in repeated experiments and found to be reliable. k_{+1} was varied between 15 and 19 $\mu M^{-1} \times \text{sec}^{-1}$.

The following series of constants was found to fit best of those tested:

$k_{+1} = 17\ \mu M^{-1} \times \text{sec}^{-1}$, $k_{+2} = 120\ \text{sec}^{-1}$,

$k_{-1} = 44\ \text{sec}^{-1}$, $k_{-2} = 10\ \text{sec}^{-1}$.

The full drawn curves are calculated, the points are averages from the

experimental recordings. The height of the points reflects the simple standard deviation $\pm \sigma$. The agreement is perfect using the two higher concentrations; the moderate discrepancies occuring with the lowest concentrations are ascribed to the lower experimental accuracy under these conditions. The uncertainty of the velocity constants may be estimatedto around $\pm$ 10 %.

DISCUSSION

1. Previous X-ray crystallographical data (5) indicated that the crystals of ER had the same orthorhombic unit cells as the free E. This was difficult to understand in the light of the new evidence indicating a conformational change of 92 % of ER to ER^{*}. There was an additional reason to suspect that this result was unreliable, since our enzyme preparations three years ago seem to have had some impurity that destroyed NADH, so the crystals, supposed to be ER at that time might have been mainly E. Our present LADH preparations do not contain this destructive impurity, so we made new ER crystals from 0.05 μ phosphate buffer, pH 7.0, and Dr. Brändén reexamined them by X-ray crystallography. They were now found to be monoclinic just as the ternary complexes E-pyrazole-NAD^{+} and E-isobutyramide-NADH.

It is most interesting to remember, that the complexes E-adenosine diphosphate ribose (EA), E-phenanthroline (EPh) and the "mosaic" EPhA all give orthorhombic crystals (6). In EPhA, Ph and A are independent of one another, with the same dissociation constants as in EPh and EA (7). Ph is coupled to Zn^{++} in the active sites, A to the A-site. This indicates that active Zn^{++} and the A-site are fairly independent of, perhaps remote from one another in the orthorhombic configuration. In the coupling reaction $E + R \xrightarrow{k_{+1}} ER$ only the ADPR moiety of R is presumably bound to the A-site, because the fluorescence emanating from the nicotinic acid amide (N) moiety is not changed. The E - A coupling by an "allosteric effect" induces the conformational change in E which brings N into the correct position for coupling at the active site, as revealed by the increased fluorescence in ER^{*}. This process is accompanied by the change in crystal shape from orthorhombic to monoclinic.

2. These observations seem to give a very plausible explanation of the recent finding of Theorell et al. (8) that the appearance of the increased fluorescence following the combination of E and R is retarded by a factor of 1000 in a crystal suspension of E, compared with the reaction in solution. The first reaction $E + R \longrightarrow ER$ may very well occur rapidly in the crystals, though we cannot observe it; the conformational change in the second step, $ER \longrightarrow ER^{*}$ is presumably hampered by the close packing of the molecules in the crystals and therefore the fluorescence increase is slow.

3. The "compulsory order" in the "Theorell-Chance mechanism" which requires that the coenzyme should be bound to the enzyme before the substrate, can now be easily understood. The active substrate site is formed through the conformational change induced by the coenzyme, whereby the nicotinic acid amide is brought into the proper distance from the Zn^{++} to let the reacting substrate group be adjusted between them in optimal position for the hydrogen transfer. It should be noted that the lipophilic binding site for the substrate may function independently of this mechanism.

4. The conformational change gives a very plausible explanation to the remarkable stabilizing influence of coenzyme and ternary ligands on LADH against denaturation by p-chloro-mercuribenzoate, heat, acids or alkali (9).

5. To our knowledge this is the first direct determination of the velocity constants for a reversible, allosteric change.

The reaction scheme presented here seems to be the simplest one compatible with the experimental results. It presumes mutual functional independence of the two coenzyme binding sites in the ADH molecule; this is supported by the fact that the equilibrium between LADH and NADH follows a monovalent dissociation curve. However, it is possible that more sophisticated models involving some interdependence of the two subunits of LADH might also conform to the experimental results. This is presently being investigated.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. Å. Åkeson for providing the enzyme preparations. This investigation was supported in part by grants from Institutet för Maltdrycksforskning, The Swedish Medical Research Council and U.S. Public Health Service (AM-05895).

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