THE PUTATIVE ISOMERIZATION OF THE LACTATE DEHYDROGENASE – NADH COMPLEX

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1. Introduction

Transient kinetic studies of the mechanism of lactate dehydrogenase were helped considerably by the variety of optical signals available. This permitted the direct observation of the formation and interconversion of intermediates [1]. The formation of the binary complex, *E*.NADH, from free enzyme and reduced nucleotide can be observed both through the enhancement of nucleotide fluorescence and the quenching of protein fluorescence. Quantitative analysis of equilibrium [2] and kinetic [3] experiments indicated no distinction between binding of NADH to pig heart lactate dehydrogenase as monitored by the two signals, provided the correction for the non-linearity of protein fluorescence quenching [2] is applied.

Everse, Berger and Kaplan reported [4–6] that the reaction of reduced acetylpyridine adenine nucleotide with chicken-heart lactate dehydrogenase proceeds in two steps distinguishable through a second-order protein fluorescence signal and a first-order nucleotide fluorescence signal. The process monitored by nucleotide fluorescence was reported to have a rate constant of about 95 s^{-1} and this was interpreted in terms of a first-order isomerisation, following a second-order binding process. We therefore re-examined the kinetics of the reaction of the pig heart enzyme with NADH to see whether we could detect a rearrangement with this species.

2. Results and discussion

The binding of NADH to the pig heart LDH was studied in a stopped-flow apparatus under pseudo first-order conditions and was monitored by nucleotide fluorescence. Equal volumes of purified [2] pig heart lactate dehydrogenase and NADH in 20 mM phosphate buffer pH 6.5 were rapidly mixed at 22°C. The concentration of NADH binding sites after mixing was 1 μM (for [NADH] = 10 μM, 7.5 μM and 5 μM) or 0.5 μM [NADH] = 3.75 μM and 2.5 μM). The course of the reaction was monitored by the NADH fluorescence increase (transmitted by Wratten 95 and Shott KV 408 filter at about 440 nm) after excitation at 335 nm (nucleotide fluorescence) or excitation at 290 nM ('Double transfer'). Figure 1A shows results with [NADH] = 5 μM and [sites] = 1 μM. Each of the points in fig.1B represents the mean and standard deviation of the linear regression of the log plot of 12 consecutive experiments summed by a transient averaging programme in a PDP8/E digital computer. One is left in no doubt that these data correspond to a second-order association process. Any limiting rearrangements must be faster than 1000 s^{-1}. The pseudo first-order rates exceed by nearly an order of magnitude the value of 95 s^{-1} which is assumed to be the limiting rate of the rearrangement by Everse et al. It is also shown in fig.1B that there is no significant difference in the result obtained with different optical signals. The observed amplitudes varied with reactant concentrations as predicted from the measured dissociation constant (0.6 μM) and the dead time of our stopped-flow machine (1 ms).

Heck [7] and Südi [8] studied the same reaction by temperature-jump relaxation and stopped-flow respectively at 4°C and also observed a bimolecular process. Any isomerisation is likely to be more temperature-dependent than the association process. The above conclusions are the same as those reached by

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Figure 1A

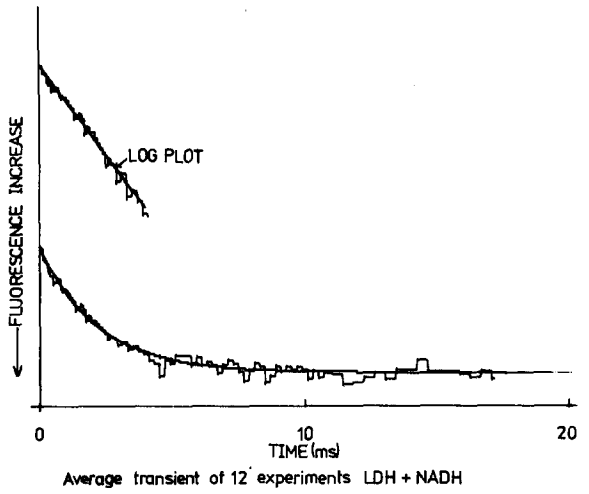

Figure 1B

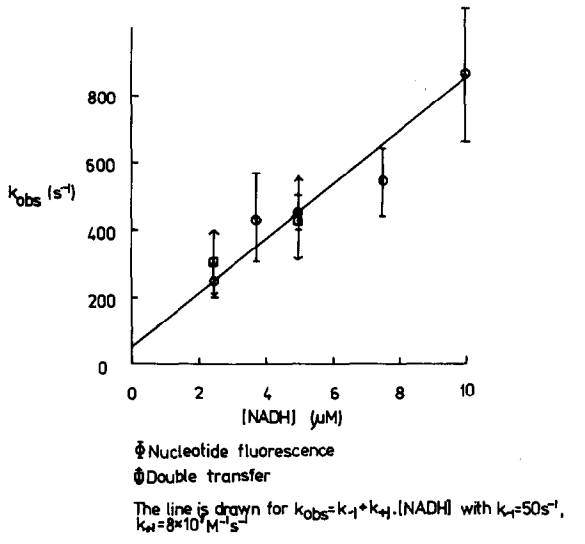

Fig.1A. The progress of NADH-binding to lactate dehydrogenase under pseudo first-order conditions, monitored by the increase in nucleotide fluorescence (ΔF). [Sites] = 1 μM, [NADH] = 5 μM. The straight line is the plot of log $[(\Delta F_\infty - \Delta F)/\Delta F_\infty]$ against time and gave an observed rate of 450 ± 50 s^{-1}. The solid line is the exponential curve corresponding to the derived rate constant. The result shown is the average of 12 consecutive transients. Fig.1B. The observed rate at which NADH (five-fold excess) binds to lactate dehydrogenase. The points were measured from enhanced nucleotide fluorescence (Φ) and from 'double transfer' (Φ) experiments. The line is drawn for a bimolecular reaction using $k_{-1} = 50\ s^{-1}$ [3] and $k_{+1} = 8 \times 10^7\ M^{-1} . s^{-1}$ according to $k_{obs} = k_{-1} + k_{+1}$ [NADH].

Geraci and Gibson [9] from their experiments on the association of NADH with liver alcohol dehydrogenase.

As pointed out by Everse et al. there is good reason to believe that the protein structures in the apo- and holo-enzymes will be different. With the pig heart enzyme any such rearrangement process must be faster than 1000 s^{-1}. It would be interesting to know whether the re-arrangement for the system of Everse et al. is slower and can be resolved by kinetic analysis. We should like to summarise, however, why we believe that the experiments reported by Everse et al. are insufficient to warrant the conclusion that there is a kinetically distinguishable rearrangement.

Everse et al. have ignored the fact that calculations of rate constants from uncorrected records of protein fluorescence experiments and from nucleotide fluorescence would give different results even if they represent the same process [2]. First half-times of reactions as listed by Everse et al. [6] are useful for planning further experiments, but not for establishing a reaction order. This is particularly important when the original traces provide no conclusive evidence for the total amplitude of the process (second-order reactions have very long tails) and the amplitudes at different concentrations are not considered. The experimental records presented by Everse et al. [4] can not be regarded as sufficient evidence for the authors' claim. Only one uncorrected record of each of the two signals is shown. The start- and end-point of the reaction records are in doubt and the derived plots cover only about 60% in one, and less than 50% in the other, of the total experimental record shown. Rates derived in such a manner are grossly misleading.

Acknowledgement

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