ISOMERIZATION OF THE BINARY COMPLEX OF REDUCED ACETYLPYRIDINE ADENINE DINUCLEOTIDE AND CHICKEN H_4 LACTATE DEHYDROGENASE

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1. Introduction

Some time ago we reported [1,2] that the binding of the reduced form of 3-acetylpyridine adenine dinucleotide (AcPy)ADH to chicken-heart lactate dehydrogenase (LDH) may be a two-step reaction, and that the actual binding of the coenzyme to the enzyme appears to trigger the occurrence of a conformational change in the enzyme–coenzyme complex. This statement is based on the observations that the quenching of the protein fluorescence, that occurs as a result of the binding of the coenzyme, displays second-order kinetics, whereas the enhancement of the coenzyme fluorescence follows first-order kinetics. The observed half-times of the reactions were 1.5 ms and 7 ms, respectively, under the conditions used in our experiments.

Shortly thereafter Holbrook and Gutfreund published a paper [3] in which they questioned the validity of this interpretation of our results. They pointed out that 'a reversible process, such as

$$E + \mathrm{NADH} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E.\mathrm{NADH}$$

(with k_1 a second-order rate constant and k_{-1} a first-order rate constant) proceeds towards equilibrium as an apparently first-order reaction if the concentrations are such that the reaction reaches less than 80% completion', suggesting that the conditions of our experiments were such that an unequivocal interpretation of the results is not possible. These views were recently reinforced by Holbrook in a review article [4].

It is the purpose of this paper to show that the criticism of Holbrook and Gutfreund [3] is unfounded, and that our original observations, as well as results from subsequent experiments, are consistent with the interpretation that we have previously advanced [1].

2. Results and discussion

In our previous paper we showed that the enhancement of the coenzyme fluorescence of (AcPy)ADH that occurs upon binding of the reduced coenzymes to chicken H_4 LDH follows first-order kinetics. A plot of $\log C_0/(C_0-x)$ as a function of time yielded linear plots, whereas a plot of the reciprocal of C_0-x as a function of time, was decidedly non-linear. An apparent rate constant of 95 s^{-1} was calculated from the changes in coenzyme fluorescence that were obtained when a solution of 2 μM enzyme was mixed with an equal volume of a solution containing 8 μM (AcPy)ADH in a stopped-flow apparatus (final concentrations in the observation chamber: 1 μM enzyme, 4 μM (AcPy)ADH). We also showed that this value did not change when the final concentration of the

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reduced coenzyme was reduced to 1 μM or 2 μM. The same apparent rate constant was also obtained when a solution of 4 μM reduced coenzyme was mixed with an equal volume of a solution containing 2 μM enzyme and 4 μM reduced coenzyme (final concentrations: 1 μM enzyme, 4 μM (AcPy)ADH). In the latter experiment we observed the apparent rate constant of the fluorescence enhancement by adding two moles of reduced coenzyme to a mole of LDH tetramer that already contained two moles of (AcPy)ADH.

On the basis of these observations, we concluded that the enhancement of the coenzyme fluorescence represents a unimolecular reaction with a half-time of about 7.5 ms, which results from an internal rearrangement of the enzyme–coenzyme complex that occurs following the binding. This interpretation was further substantiated by the fact that the rate of the reaction was about three times slower than the rate of quenching of the protein fluorescence under the same conditions.

This interpretation was questioned by Holbrook and Gutfreund [3] on the basis that the reaction reaches less than 80% completion. This criticism is unfounded. The dissociation constant for (AcPy)ADH and chicken H_4 LDH has been estimated as 0.15 μM [5]. Calculations based on this constant reveal that the reaction of a 1 μM solution of enzyme (4 μM in subunits) with a 4 μM solution of (AcPy)ADH is about 80% complete at equilibrium, whereas the reaction of a 1 μM solution of enzyme with a 1 μM solution of (AcPy)ADH reaches about 95% completion. Hence, our data were obtained under conditions that were well above the requirements stated by Holbrook and Gutfreund [3].

Furthermore, the rate of a bimolecular reaction, even if the reaction does not proceed to completion is dependent on the concentrations of the reactants. The data shown in table I illustrate that the half-time of the increase in coenzyme fluorescence is independent of both the enzyme and the reduced coenzyme concentration. These data clearly refute the argument by Holbrook and Gutfreund [3].

It should also be mentioned that our data were obtained with the H_4 LDH from chicken, whereas the experiments of Gutfreund et al. were done with the pig H_4 LDH. It is possible that such an isomerization as we have observed with the chicken enzyme does not occur with the pig enzyme, but this explanation appears unlikely (see below). It could be that the rate of isomerization with the pig enzyme is much faster than that observed with the chicken enzyme, which would make its detection much more difficult.

Table 1
Rates of enhancement of coenzyme fluorescence at various concentrations of LDH and (AcPy)ADH[a]

LDH subunit concentration	(AcPy)ADH concentration	Half-time of reaction
μM	μM	ms
1	1	7.7
1	4	7.5
1	10	7.6
2	1	6.5
2	10	7.1

[a]Data were obtained using an Aminco-Berger stopped-flow apparatus, as described in ref. [9] and [10].

Several other lines of evidence indicate that the binding of the reduced coenzyme to lactate dehydrogenase results in a conformational change of the complex. X-ray crystallographic data indicate the occurrence of conformational changes in the dogfish M_4 LDH upon binding of NAD^+ or NADH [6,7]. In fact, crystals of the apoenzyme shatter when they are soaked in solutions of the reduced or oxidized coenzyme. Furthermore, the fact that values for the dissociation constants for NAD^+ and NADH obtained from equilibrium measurements are often significantly smaller than the values obtained from initial rate measurements [8] could also be indicative of the occurrence of a two-step process. There appears therefore to be little doubt that an isomerization of the enzyme–reduced coenzyme complex takes place. Whether or not this isomerization is an obligatory step in the reaction mechanism of lactate dehydrogenase remains to be established.

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References

[1] Everse, J., Berger, R. L. and Kaplan, N. O. (1972) in: Structure and Function of Oxidation Reduction Enzymes (Akeson, A. and Ehrenberg, A. eds) pp. 691–708, Pergamon Press, New York.
[2] Everse, J. and Kaplan, N. O. (1973) Advances in Enzymology (Meister, A. ed) pp. 61–133, Academic Press, New York.
[3] Holbrook, J. J. and Gutfreund, H. (1973) FEBS Lett. 31, 157–169.
[4] Holbrook, J. J., Liljas, A., Steindel, S. J. and Rossmann, M. G. (1975) in: The Enzymes, Third Ed. (Boyer, P. ed) Vol. XI, pp. 191–292, Academic Press, New York.
[5] McKay, R. H. and Kaplan, N. O. (1964) Biochim. Biophys. Acta 79, 273–283.
[6] Adams, M. J., McPherson, A., Rossmann, M. G., Schevitz, R. W. and Wonacott, A. J. (1970) J. Mol. Biol. 51, 31–38.
[7] Chandrasekhar, K., McPherson, A., Adams, M. J. and Rossmann, M. G. (1973) J. Mol. Biol. 76, 503–518.
[8] Dalziel, K. (1975) in: The Enzymes, Third Ed. (Boyer, P. ed) Vol. XI, pp. 1–60, Academic Press, New York.
[9] Berger, R. L., Balko, B., Borcherdt, W., and Friauf, W. (1968) Review of Scientific Instr. 39, 486–493.
[10] Berger, R. L., Balko, B., and Chapman, H. F. (1968) Review of Scientific Instr. 39, 493–498.