KINETICS OF BINDING OF OXIDIZED COENZYMES IN TERNARY COMPLEXES WITH GLUTARATE BY L-GLUTAMATE DEHYDROGENASE

Yoshiaki UMEMIYA* and Takao NAKAMURA**
Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka, Japan

Received 17 June 1974

1. Introduction

$NADP^+$ and NAD^+ are known to bind to L-glutamate dehydrogenase (EC 1.4.1.3) in ternary complexes with glutarate, which is a substrate analogue [1,2]. The modes of binding of $NADP^+$ and NAD^+ in the complexes seem to be different. Formation of the enzyme–$NADP^+$–glutarate complex is accompanied by perturbation of the absorption spectrum of the nicotinamide moiety of the coenzyme [1]. On the other hand, it has been suggested that binding of NAD^+ in the ternary complex is accompanied by a large perturbation of the adenine moiety of the coenzyme in addition to that of the nicotinamide moiety, though only the difference spectrum of the NADH complex has been published [3,4].

In the present work the binding of NAD^+ in the ternary complex with glutarate was titrated spectrophotometrically, and the kinetic characteristics of the bindings of $NADP^+$ and NAD^+ in ternary complexes were compared by rapid spectrophotometric measurements. The kinetics of $NADP^+$ binding were consistent with a reversible, single step reaction while those of NAD^+ indicated a two step reaction.

2. Materials and methods

Crystalline L-glutamate dehydrogenase was prepared from beef liver by the method of Kubo et al. [5]. The molar concentration of the enzyme was determined spectrophotometrically [6], and calculated on the basis of the polypeptide chain, taking the molecular weight as 56 100 [7]. The difference spectrum of the $NAD(P)^+$ complex was obtained as described by Cross, McGregor and Fisher [1], and the stoichiometry and association constant of the NAD^+ binding were estimated by the method of Stockell [8]. For rapid spectrophotometric measurements the flow apparatus described previously [9] was used. All measurements were carried out at 20°C in 0.1 M phosphate buffer, pH 7.5.

3. Results and discussion

Fig. 1 shows the difference spectra of L-glutamate dehydrogenase obtained by adding NAD^+ in the presence and absence of excess (3 mM) glutarate. The spec-

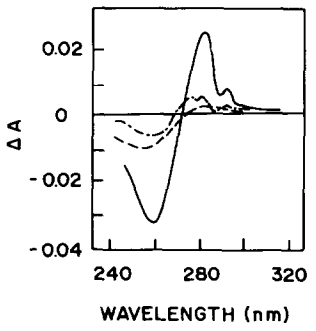

Fig. 1. Difference spectra of complexes of L-glutamate dehydrogenase. Concentration of enzyme, 17 μM. (——) With 80 μM NAD^+ and 3 mM glutarate; (– – –) with 80 μM NAD^+, no glutarate; (–·–) with 60 μM $NADP^+$ and 3 mM glutarate.

* Present address: Fruit Tree Research Station, Ministry of Agriculture and Forestry, Hiratsuka, Kanagawa, Japan.
** To whom reprint request should be addressed.

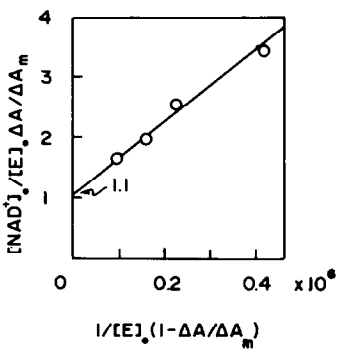

Fig. 2. Binding of NAD^+ to L-glutamate dehydrogenase. $[E]_0$ and $[NAD^+]_0$ represent the total concentrations of the enzyme and NAD^+, respectively. ΔA_{max}, ΔA extrapolated to $1/[NAD^+]_0 = 0$. Measurements made at 283 nm. Data are plotted by the method of Stockell [8].

trum obtained in the presence of glutarate was much larger than that in the absence of glutarate with the same concentration of NAD^+, showing that this spectrum is caused by the formation of a ternary complex of the enzyme with NAD^+ and glutarate. The spectral features in the region of 260–290 nm were essentially the same as those reported for the enzyme–NADH complex [3,4], and the latter have been ascribed to perturbations of the adenine and nicotinamide moieties of bound NAD^+.

The absorption difference of the 283 nm peak in the spectrum obtained with different NAD^+ concentrations was used to estimate the stoichiometry and association constant (K) of NAD^+ binding in the ternary complex (fig. 2), and values of 1.1 ± 0.1 mol NAD^+/mol polypeptide chain and $K(K_{app})$ of 1.7×10^5 M^{-1} were obtained.

Solutions of enzyme and $NADP^+$ or NAD^+, each containing 6 mM glutarate, were mixed in the flow apparatus, and the time course of optical density change at 283 nm was recorded by the stopped flow method. The time course of the reaction (increase of optical density) was found to follow apparent first order kinetics (fig. 3), and k(apparent binding velocity constant) of the reaction was estimated from the recorder trace at different $NAD(P)^+$ concentrations. In fig. 4 values of k are plotted against the coenzyme concentration. With $NADP^+$, a straight line relationship was obtained between k and $[NADP^+]_0$ (total concentration of $NADP^+$). Under the present conditions of $[NADP^+]_0 \gg [E]_0$ and $[G]_0 \gg K_i$ (inhibition constant of glutarate, 0.58 mM [10]), the simplest interpretation of this relationship is that the binding of $NADP^+$ in the ternary complex is a one step, reversible reaction

$$E(G) + NADP^+ \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E(G)NADP^+$$

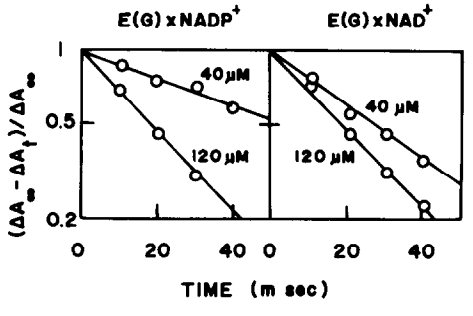

Fig. 3. Semi-log plot of the time course of $NAD(P)^+$ binding in ternary complexes with glutarate by L-glutamate dehydrogenase at various $NAD(P)^+$ concentrations. Coenzyme concentrations are indicated in the figure. Enzyme concentration, 8.5 μM; glutarate concentration, 6 mM. ΔA_t and ΔA_∞ ΔA at time t and at termination of the reaction.

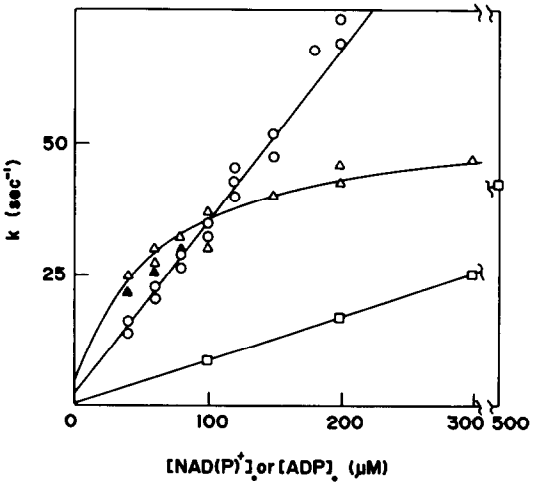

Fig. 4. Plot of k(apparent velocity constant) of the reaction of coenzyme binding against the coenzyme concentration. (○—○) $NADP^+$; (△—△) NAD^+ (283 nm); (▲—▲) NAD^+ (266 nm); □—□, ADP (283 nm). Solid lines represent theoretical curves obtained from the equations and numerical values in the text.

where E and G denote the enzyme and glutarate, respectively and k_{+1} and k_{-1} the rate constants and k is given by

$$k = k_{-1} + k_{+1}\,[NADP^+]_0,$$

since in these experiments it is reasonably assumed that [$NADP^+$] and [G] are kept constant during the course of a reaction. From this equation and the data in fig. 4, k_{-1} and k_{+1} were determined independently as 2.5 sec^{-1} (intercept on the ordinate) and 3.2×10^5 M^{-1} sec^{-1} (slope of the straight line), respectively. Then $k_{+1}/k_{-1} = 1.3 \times 10^5$ M^{-1}, which is in good agreement with the published K value of 1.0×10^5 M^{-1} [1].

On binding of NAD^+ a plot of increase of k against $[NAD^+]_0$ did not give a straight line but a saturation value was attained at higher NAD^+ concentrations (fig. 4). This saturation occurred in a range where little substrate inhibition of NAD^+ was observed [11]. This is not consistent with a simple one step mechanism and may most simply be ascribable to a two step, reversible reaction in which the first step is in rapid equilibrium and the second step is a slower isomerization process:

$$E(G) + NAD^+ \overset{K_1}{\rightleftharpoons} E(G)\,NAD^+ \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E(G)\,NAD^{+\prime}$$

$$K_2 = k_{+2}/k_{-2},$$

where E(G) + NAD^+ and E(G)NAD^+ exhibit the same spectrum. Then k is given by

$$k = k_{-2} + \frac{k_{+2} \cdot K_1\,[NAD^+]_0}{1 + K_1\,[NAD^+]_0}$$

[12] since $[NAD^+]_0 \gg [E]_0$ and $[G]_0 \gg K_i$, and $[NAD^+]$ and [G] stay constant in a reaction. The association constant of NAD^+ binding, K_{app}, which was previously calculated assuming one step reversible reaction, can be written using the intrinsic equilibrium constants of each step K_1 and K_2 as

$$K_{app} = K_1\,(1 + K_2)$$

Assuming the two step reaction the values of k_{-2} and k_{+2} for the reaction of NAD^+ binding were estimated to be 5 sec^{-1} and 50 sec^{-1}, respectively, as the best fit for the experimental values (fig. 4). The values of K_1 and K_2 were calculated as 1.5×10^4 M^{-1} and 10, respectively. Perturbation of the adenine moiety of ADP was also observed on binding to the enzyme [3,4]. However, the kinetic characteristics of this spectral change were compatible with a one step reaction (fig. 4), and a value of $k_{+1} = 8.4 \times 10^4$ M^{-1} sec^{-1} was obtained. Since $K = 7.7 \times 10^5$ M^{-1} [3], it was calculated that $k_{-1} = k_{+1}/K = 0.11$ sec^{-1}.

An appreciable absorption change was observed at 283 nm when the nicotinamide moiety of $NADP^+$ was perturbed on binding to the enzyme. Thus the observed absorption difference at 283 nm caused by NAD^+ binding may be the sum of the perturbations of the adenine and nicotinamide moieties of the coenzyme, even though the amplitude of the former seems to be much larger than that of the latter at this wavelength [3,4]. The k values of the change in absorption upon NAD^+ binding at 266 nm, which is the isosbestic point of the nicotinamide perturbation (fig. 1), tended to be in accordance with that observed at 283 nm (fig. 4). This indicates that the absorption change on NAD^+ binding at 283 nm is mainly due to adenine perturbation of NAD^+, even though the time course at 266 nm could be followed only over a restricted range of NAD^+ concentrations, because of the high basal absorption of NAD^+ at this wave length.

It has been reported that the fluorescence spectrum of the nicotinamide moiety of NADH is perturbed upon binding to L-glutamate dehydrogenase, and the kinetic characteristics of this change are compatible with those of a simple reversible bimolecular reaction [13]. All these facts indicate that when only a single moiety of $NADP^+$ or ADP is perturbed upon binding to L-glutamate dehydrogenase the interaction occurs as a single step reaction, but that when both moieties are perturbed in NAD^+ that of adenine has a two step mechanism.

Acknowledgements

The authors express their gratitude to Professors Y. Tonomura and H. Watari for advice. This work was supported in part by grant from the Ministry of Education of Japan.

References

[1] Cross, D. G., McGregor, L. L. and Fisher, H. F. (1972) Biochim. Biophys. Acta 289, 28–36.
[2] Dalziel, K. and Egan, R. R. (1972) Biochem. J. 126, 975–984.
[3] Cross, D. G. and Fisher, H. F. (1970) J. Biol. Chem. 245, 2612–2621.
[4] Pantaloni, D. and Iwatsubo, M. (1967) Biochim. Biophys. Acta 132, 217–220.
[5] Kubo, H., Iwatsubo, M., Watari, H. and Soyama, T. (1959) J. Biochem. 46, 1170–1185.
[6] Olson, J. A. and Anfinsen, C. B. (1952) J. Biol. Chem. 197, 67–79.
[7] Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, T. J. and Melamed, M. D. (1970) Proc. Natl. Acad. Sci. U. S. 67, 724–730.
[8] Stockell, A. (1958) J. Biol. Chem. 234, 1286–1292.
[9] Nakamura, T. (1967) J. Japan, Biochem. Soc. 39, 855–866.
[10] Caughey, W. S., Smiley, J. D. and Hellerman, L. (1957) J. Biol. Chem. 224, 591–607.
[11] Barton, J. S. and Fisher, J. R. (1971) Biochemistry 10, 577–585.
[12] Gutfreund, H. (1972) Enzymes: Physical Principles, p. 205, John Wiley and Sons, New York.
[13] Malcolm, A. D. B. (1972) Eur. J. Biochem. 27, 453–461.