Direct Measurement of Proton Binding to the Active Ternary Complex of Pig Heart Lactate Dehydrogenase

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With the use of a glass electrode it is shown that one proton is taken up from the solvent when *o*-nitrophenylpyruvate forms a ternary complex with lactate dehydrogenase and NADH at pH 8 and before the complex has rearranged to give products.

o-Nitrophenylpyruvate is such a poor substrate for lactate dehydrogenase that it is possible to form the active ternary complex enzyme–NADH–nitrophenylpyruvate and observe its properties before an appreciable fraction of the complex is converted into NAD⁺ and nitrophenyl-lactate. The formation of the complex has been deduced from the fact that the fluorescence of NADH is quenched to about one-third of that of free NADH before there is any loss of extinction at 340 nm. The apparent dissociation constant for *o*-nitrophenylpyruvate determined in this way depends on H⁺ concentration in such a manner as to suggest that a stable active ternary complex can form only if a group in the binary complex of pK 6.8 is protonated (Holbrook & Stinson, 1973). Chemical studies (Woenckhaus *et al.*, 1969; Holbrook & Ingram, 1973) identified this group as equivalent to histidine-195 of the dogfish enzyme numbering sequence (Rossmann *et al.*, 1971). During the isomerization of initially formed ternary complex and before the chemical transformation of NADH into NAD⁺ this group remains protonated, and this supports the suggestion made by Schwert *et al.* (1967) that a protonated imidazole is the source of the proton that is derived from the solvent and is added to the carbonyl oxygen atom of pyruvate.

The present evidence for the compulsory protonation of histidine-195 in the active ternary complex with NADH rests only on the pH-dependence of the dissociation constant for *o*-nitrophenylpyruvate (Holbrook & Stinson, 1973). In the present paper direct measurements of uptake of a proton from the solvent are reported to occur when nitrophenylpyruvate binds to the enzyme–NADH complex at pH 8.

Pig heart lactate dehydrogenase was purchased from Whatman Biochemicals, Maidstone, Kent, U.K. The specific activity of the enzyme was determined, as described by Stinson & Holbrook (1973), to be 360 μ mol of NADH oxidized/min per mg of protein. Before the experiments buffer salts were removed by filtering the enzyme solution through a column of Sephadex G-50 equilibrated with 0.1 M-K₂SO₄ in 0.4 mM-potassium phosphate adjusted to pH 8.0 with 0.1 M-NaOH. The apparatus for measur-

ing proton uptake consisted of an open beaker (2 cm $\times$ 3 cm high) maintained at 12–13°C by circulation of tap water through a water jacket. The pH of the contents of the beaker was measured by a standard combined glass–calomel electrode (Pye–Ingold type 401 E07) connected to a pH-meter (Pye–Unicam model 292). The electrode was standardized at pH 7 with a standard phosphate buffer (supplied by BDH Chemicals Ltd., Poole, Dorset, U.K.). The output from the pH-meter was displayed on a chart recorder (Bryans model 27000) with the sensitivity adjusted such that a change of 0.1 pH unit gave about a full-scale deflexion of the pen. Each experiment was separately calibrated in terms of proton concentration by observing the pen deflexion when 0.01 ml of 10 M-HCl was added at the end of the experiment.

In most experiments the beaker contained 6 ml of a solution containing 3.2 mg of enzyme, 0.2 mM-NADH, 0.1 M-K₂SO₄ and 0.4 mM-potassium phosphate. At the start of the experiment the pH was adjusted to the value desired with 0.1 M-NaOH. The rate of drift in pH after thermal equilibration was equivalent to about 3 nmol of H⁺/min.

Results from a typical experiment are shown in Fig. 1. An addition of *o*-nitrophenylpyruvate to a solution of the enzyme–NADH complex results in an initial rapid uptake of H⁺ followed by a steady-state rate of uptake of H⁺. Subsequent additions of substrate result in progressively diminished rapid uptakes of H⁺, and the steady-state rate approaches a maximum value. The form of the experimental curve is the same as that reported by Holbrook & Stinson (1973) for the same experiment when NADH fluorescence was monitored. The initial rapid uptake of H⁺ does not appear to be as fast as the initial rapid quench of the fluorescence of bound NADH because of the long response time of the electrode assembly. The instantaneous uptake of H⁺ (Δ H⁺) was evaluated by extrapolating the steady-state rate back to the point at which the substrate was added.

Fig. 2 is the analysis of results, from experiments such as that just described above, by double-reciprocal plots. The same dissociation constant for nitrophenylpyruvate is obtained whether the steady-state rate of

Fig. 1. *Compulsory protonation of the ternary complex lactate dehydrogenase-NADH-nitrophenylpyruvate at pH 8*

Records of small changes in pH are shown as a function of time. Six 25 μ l portions of 0.2M-nitrophenylpyruvate were added at 0.5min intervals to 6ml of solution containing (trace A) 3.2mg of enzyme, 0.2mM-NADH, 0.1M-K₂SO₄ and 0.4mM-sodium phosphate buffer at 12°C adjusted to pH8.0. At the end of the experiment the trace was calibrated by the addition of 100nmol of HCl. The trace B was obtained under the same conditions except that the NADH was omitted.

H⁺ uptake ($K_s = 1.7$ mm), the sum of the rapid initial uptakes of H⁺ (1.9mm) or the sums of the initial rapid quench in NADH fluorescence (2.2mm) is taken as the fraction of the enzyme in the active ternary complex. This experiment was at pH8 where, with a pK of 6.8, the enzyme-NADH complex would be expected to be only about 6% protonated. The point at which the plot in Fig. 2 cuts the axis shows that 90nmol of enzyme subunits (relative molecular mass 36000) would rapidly take up 83nmol of H⁺ when an infinite concentration of nitrophenylpyruvate was added. The maximum turnover number at 13°C (0.02s⁻¹) is similar to that derived from the fluorescence experiments.

In a separate series of experiments the initial rapid uptake of H⁺ was monitored when a single addition of 2.5mM-nitrophenylpyruvate was made at a series of different pH values. The initial rapid uptakes (mol of H⁺/mol of subunits) were 0.61 at pH8, 0.42 at pH7.5, 0.35 at pH7 and 0.19 at pH6. The values of the dissociation constant at each pH (Holbrook & Stinson, 1973) suggest that 2.5mM-nitrophenylpyruvate would result in the formation of ternary complex equivalent to 53, 69, 82 and 90% of the enzyme protein at pH8, 7.5, 7 and 6 respectively. These values and the measured proton uptakes are

Fig. 2. *Double-reciprocal plots of the sums of the rapid proton uptake (▲) and of the steady-state rate of proton uptake (●)*

Results are from experiments similar to that in Fig. 1 at pH8. The enzyme-NADH complex is largely unprotonated at pH8. Addition of nitrophenylpyruvate (NPP) results in protonation of the complex and subsequent turnover according to:

The rate of formation of products is slow compared with the preceding equilibria. The concentration of nitrophenylpyruvate for half-maximum rapid protonation was 1.9mM and for half-maximum steady-state rate of proton uptake was 1.7mM. The intercept with the axis indicates that 83nmol of H⁺ is consumed by 89nmol of subunits at saturating substrate concentrations.

consistent with the group in the binary complex having a pK of 6.8 and a very high pK in the ternary complex.

These results confirm the conclusion of Holbrook & Stinson (1973) that thermodynamically stable ternary complexes are formed only when a group with pK6.8 (in the enzyme-NADH complex) is protonated. The pK of this group in the ternary complex is much greater than 6.8 and may be even as high as that when oxamate is bound (pK greater than 11). The results also confirm that protonation of the ternary complex precedes the chemical conversion of NADH into NAD⁺, but do little to determine

whether the protonation rapidly (less than 1 ms) precedes or rapidly follows the binding of substrate to the enzyme-NADH complex (Holbrook & Gutfreund, 1973).

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Holbrook, J. J. & Gutfreund, H. (1973) *FEBS Lett.* **31**, 157-169

Holbrook, J. J. & Ingram, V. A. (1973) *Biochem. J.* **131**, 729-738

Holbrook, J. J. & Stinson, R. A. (1973) *Biochem. J.* **131**, 739-748

Rossmann, M. G., Adams, M. H., Buehner, M., Ford, G. C., Hackert, M. L., Lentz, P. J., McPherson, A., Schevitz, R. W. & Smiley, I. E. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 179-191

Schwert, G. W., Millar, B. R. & Peanasky, R. J. (1967) *J. Biol. Chem.* **242**, 3245-3252

Stinson, R. A. & Holbrook, J. J. (1973) *Biochem. J.* **131**, 719-728

Woenckhaus, C., Berghäuser, J. & Pfeleiderer, G. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 473-483