Porcine Heart Lactate Dehydrogenase

OPTICAL ROTATORY DISPERSION, THERMODYNAMICS, AND KINETICS OF BINDING REACTIONS*

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Henry d'A. Heck

From the Department of Chemistry, University of California, Berkeley, California 94720, and Max-Planck-Institut für physikalische Chemie, Göttingen, West Germany

SUMMARY

The kinetics of NADH, 3-thionicotinamide adenine dinucleotide (TNAD), and oxamate binding to the H4 isoenzyme of lactate dehydrogenase from the pig has been investigated by temperature jump techniques. The dissociation rate constant for TNAD is considerably larger than for NADH, whereas the recombination rate constant is smaller for the oxidized coenzyme than for the reduced molecule. The kinetics of oxamate binding agrees satisfactorily with a simple binding mechanism. The optical rotatory dispersion spectrum of the protein, which indicates α-helical content, is unperturbed by binding of NADH and oxamate, or by changing the pH from 6 to 8. Both the enthalpy and entropy of oxamate binding are strongly pH-dependent. The interaction of this inhibitor with the protein cannot be explained simply in terms of an electrostatic attraction. Other effects, primarily entropic, are of large importance. Evidence is presented which indicates that TNAD in solution exists in two conformational forms which are in rapid equilibrium.

The isoenzymes of lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27) differ from one another in numerous ways (1). Some of the most prominent dissimilarities are in their catalytic mechanisms. The results from steady state kinetics indicate that structural changes play a significant role in catalysis by the M2 isoenzyme (3) but not by the H4 isoenzyme (4). In view of efforts to determine the structure of this enzyme by x-ray crystallography (5), it is important to ascertain by other types of evidence whether substrate-dependent conformational changes occur in the various isoenzymes.

In the case of the M2 isoenzyme, the results seem clear. Evidence from fluorometric titrations (6), optical rotatory dispersion (7), and chemical relaxation (8) points to differences in conformation between apoenzyme, binary complex with NADH, and ternary complex with oxalate. In the H4 isoenzyme, however, the information is less conclusive.

Hathaway and Criddle (9) have shown with bovine H2 lactate dehydrogenase that, at low enzyme concentrations, pyruvate can affect the quaternary structure of lactate dehydrogenase. Stopped flow experiments with the H4 isoenzyme of pig suggest that a structural change occurs either in the enzyme-NAD complex or in the enzyme-NAD-pyruvate complex during catalysis (10). Di Sabato and Ottesen (11) have interpreted their experiments on hydrogen-deuterium exchange as evidence for a structural change in the complex of NADH with the chicken heart H4 isoenzyme.

In order to obtain further data regarding structural changes in the H4 isoenzyme of lactate dehydrogenase and to clarify certain questions regarding its mechanism, the present investigations were undertaken.

EXPERIMENTAL PROCEDURE

Materials—Pig heart lactate dehydrogenase was a product of C. F. Boehringer und Soehne, Mannheim, West Germany. The enzyme isolated from pig heart is known to contain all five isoenzymes (12), but the proportion of H4 isoenzyme is very high (over 90%) (13). That the Boehringer enzyme does contain a large fraction of a single, rapidly migrating anodic component at pH 6.8 was shown by vertical polyacrylamide gel electrophoresis. It has been established that this component is the H4 isoenzyme (12). The appearance of the electrophoreograms was very similar to that in a published report on pig heart lactate dehydrogenase electrophoresis which was carried out in starch gel (14). The molecular weight and other physical properties of the H4 isoenzyme of pig heart were recently determined to a high degree of accuracy (15).

Because a small proportion of isoenzymes other than the H4 isoenzyme is present in the Boehringer preparation, it was necessary to determine whether these other isoenzymes would affect the kinetic and equilibrium investigations. The enzyme was purified by fractionation on a column of DEAE-Sephadex (16). Key kinetic experiments were then checked with the purified H4 component. No differences were found from the results obtained with unresolved lactate dehydrogenase.

For the preparation of enzyme stock solutions, the suspension...
of crystals was centrifuged at 30,000 × g, and the precipitate was dissolved in the appropriate cold phosphate buffer. Washed, activated charcoal (approximately 1 mg of charcoal per mg of enzyme) was added to the solution; and the suspension was swirled gently for 10 min at room temperature, then centrifuged. The supernatant solution, filtered through a Millipore filter, was dialyzed for several hours against the desired buffer. The resulting enzyme stock solutions had A280:A260 ratios of 1.98 to 2.04 and a specific activity of approximately 360 units per mg, when tested according to Biochemica Informationen, Boehringer. When kept cold, enzyme solutions were stable for a few days.

Inorganic buffers and salts were reagent grade. NADH, NAD+, TNAD, sodiutn ljuuvate were Boehringer products. Sodium oxamate, obtained from Aldrich, was reccrystallized from ethanol-water.

Activated animal charcoal was washed by boiling twice with 12% HCl, after which it was rinsed thoroughly with water, 2 M KCl, and water, then dried. Dialysis tubing was washed according to the method of Anderson and Weber (6).

Apparatus—Fluorimetric titrations were carried out in a thermostated Amico-Bowman spectrophotometer. Additions of NADH to enzyme test solutions, or of oxamate to enzyme-NADH solutions, were made with an Agla microosyringe assembly (Burroughs Wellcome). Spectrophotometric titrations with TNAD were done in a thermostated Zeiss PMQ II spectrophotometer. Optical rotatory dispersion measurements were performed in a Cary model 60 spectropolarimeter containing a thermostated cell holder.

The kinetics of NADH and oxamate binding to lactate dehydrogenase was followed in a temperature jump apparatus by observing the changes in protein or NADH fluorescence following the temperature perturbation. A high pressure Osram lamp, either 100 watt mercury or 75 watt xenon, was used for excitation.

The desired excitation wave length was selected by means of a Bausch and Lomb high intensity grating monochromator.

Fluorescence was observed at 90° with an RCA 1P28 photomultiplier. The temperature jump cell contained four quartz windows. Concave mirrors were placed at locations facing the directions of excitation and emission, so that the intensities of exciting and emitted light were essentially doubled. Filters (Jenser Glaswerk Schott und Genossen, Mainz, West Germany) were used to select the desired emission bands. Except for these optical modifications, the temperature jump apparatus was constructed as previously described (18).

Temperature jump investigations of TNAD binding to lactate dehydrogenase were performed by observing the changes in TNAD absorption at 360 nm that were associated with enzyme binding.

Enzyme activity determinations were made in either a Cary model 11 or an Eppendorf recording spectrophotometer equipped with thermostated cell holders. Measurements of pH were made with a Radiometer 22 pH meter. The high voltage electrofee rotation apparatus was produced by the E-C Apparatus Corporation, Philadelphia, Pennsylvania.

Methods—The concentration of active sites in enzyme stock solutions was measured before each day's runs by titration with NADH and sulfite according to the method of Holbrook (19).

The value of oxamate binding, which is pH-dependent. The results of the titration experiments are compiled in Table I.

The thermodynamics of oxamate binding, determined from van't Hoff plots (Fig. 1), are listed in Table II. Throughout the temperature range investigated, the effect of pH on the dissociation constants is small. It is evident, however, particularly at lower temperatures, that the affinity of the enzyme for oxamate is greater at pH 7 than at pH 6. These findings suggest that the interaction of oxamate with the protein is not solely an electrostatic attraction. In contrast, the results of Winer and Schwert (24) indicate that in bovine H4 lactate dehydrogenase the binding of oxamate is greatest at pH 6 and decreases at all higher pH values.

A further difference from the bovine H4 isoenzyme is in the enthalpy of oxamate binding, which is pH-dependent. Winer and Schwert (24) stated that in the bovine enzyme the value of ∆H‡ is unchanged in Tris buffers between pH 7.10 and 9.51. The value of ∆H‡ determined in the present experiments at pH 7 is within experimental error identical with the value of Winer and Schwert for pHi 6.80 in 0.1 m phosphate (24).

The absolute magnitudes of the oxamate binding constants in phosphate buffer for the bovine (24) and pig enzymes nearly coincide at pH 6, but at higher pH the pig enzyme appears to
have a greater affinity for this substrate analogue. Thus, despite several kinetic similarities (10, 20), bovine and pig lactate dehydrogenase differ in several ways with respect to oxamate binding.

Approximate values for the enthalpies of coenzyme binding were obtained from titration data and chemical relaxation experi-

**Table I**

Microscopic dissociation constants for pig heart lactate dehydrogenase in 0.1 M sodium phosphate, with ionic strength of 0.3 M with NaCl, from fluorometric and spectrophotometric titrations.

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_{\text{NADH}}$</th>
<th>$K_{\text{oxamate}}$</th>
<th>$K_{\text{KNAD}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>1.2 ± 0.4</td>
<td>17.8 ± 2.6</td>
<td>(350 ± 59)$^{+d}$</td>
</tr>
<tr>
<td>7.0</td>
<td>0.56 ± 0.25</td>
<td>25.7 ± 3.8</td>
<td></td>
</tr>
</tbody>
</table>

$^{a}$ 20°; excitation and emission wave lengths, 295 and 440 nm, respectively.

$^{b}$ At 20°; excitation and emission wave lengths, 340 and 445 nm, respectively. The concentration of free NADH in these experiments exceeded $K_{\text{NADH}}$ by 20-fold.

$^{c}$ Probable error.

$^{d}$ 20°; spectrophotometric titration at 360 nm.

$^{e}$ At 3°

**Figure 1.** Plots of the logarithms of the dissociation constants for oxamate from the lactate dehydrogenase-NADH-oxamate complex against reciprocal absolute temperature (T). The measurements were made in 0.1 M sodium phosphate, with ionic strength of 0.3 M with NaCl. Lines were drawn by a least squares analysis.

**Figure 2.** Plots of the reciprocal relaxation times against concentrations for the system lactate dehydrogenase + NADH in 0.1 M sodium phosphate, with ionic strength of 0.3 M with NaCl. Lines were drawn by the method of least squares.

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**Table II**

Thermodynamics of oxamate dissociation at 20° in 0.1 M sodium phosphate, with ionic strength of 0.3 M with NaCl.

<table>
<thead>
<tr>
<th>pH</th>
<th>$\Delta G^\circ$</th>
<th>$\Delta H^\circ$</th>
<th>$\Delta S^\circ$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>6.37 ± 0.20$^{a}$</td>
<td>4.60 ± 0.29$^{a}$</td>
<td>-6.0 ± 1.5$^{a}$</td>
</tr>
<tr>
<td>7.0</td>
<td>6.54 ± 0.20$^{a}$</td>
<td>7.40 ± 0.80</td>
<td>2.9 ± 3.4</td>
</tr>
<tr>
<td>8.0</td>
<td>6.15 ± 0.20$^{a}$</td>
<td>12.1 ± 0.7</td>
<td>20.3 ± 3.1</td>
</tr>
</tbody>
</table>

$^{a}$ Probable error.

**Figure 3.** Plots of the reciprocal relaxation times against concentrations for the system lactate dehydrogenase-NADH + oxamate at 20°, 0.1 M sodium phosphate, with ionic strength of 0.3 M with NaCl. Lines were drawn by the method of least squares.
FIG. 4. Difference absorption spectrum of pig heart lactate dehydrogenase in 0.1 M sodium phosphate, with ionic strength of 0.3 M with NaCl, 20°C. Four 1-ml cuvettes with 0.5-cm path lengths were used. Enzyme solutions were 2.5 X 10^{-3} M in sites. TNAD in sample and reference buffer cuvettes was 3 X 10^{-2} M.

FIG. 5. Relaxation experiment for the interaction of lactate dehydrogenase with TNAD in 0.1 M sodium phosphate, with ionic strength of 0.3 M with NaCl, 20°C, at pH 6.00. [Sites] = 1.6 X 10^{-4} M; [TNAD] = 5.3 X 10^{-4} M; scanning rate, 0.1 msec per large division; sensitivity, 20 mV per cm; total signal, 4 volts.

not always give a quantitative measure of helix content. The value of \( \alpha \) is in good agreement with a value reported by Jaenicke (30) for the pig heart enzyme, and by Bolotina et al. (7) for the pig skeletal muscle enzyme.

Neither NADH nor oxamate, at concentrations sufficient to saturate the binding sites, has any detectable effect on the optical rotatory dispersion spectrum of the protein, and no extrinsic Cotton effects are observed in the 340 nm region of NADH absorption. The former results are in contrast with the findings of Bolotina et al. (7), who reported large changes in both \( \beta \) and \( \alpha \) when NADH and oxamate are bound to pig skeletal muscle lactate dehydrogenase.

The observation that \( \beta \) and \( \alpha \) are essentially independent of pH in the region of neutrality corroborates Jaenicke’s measurements (30), but differs considerably from results obtained with pig skeletal muscle enzyme (7).

The difference spectrum obtained on TNAD binding (Fig. 4) was used to investigate the binding kinetics of this oxidized coenzyme to lactate dehydrogenase. In temperature jump experiments with this molecule, two relaxation times can be clearly discerned (Fig. 5). The shorter of these is observed in the absence of enzyme, and is evident both at pH 6 and at pH 8. Its estimated value is less than 3 msec. The determination of the actual relaxation time is instrument-limited. Even at 3°C, this relaxation effect is complete within the heating time of the solution. There was no apparent effect on the velocity of this reaction over a 20-fold range in coenzyme concentration.

The second relaxation phenomenon, which gives rise to the larger optical density change, is associated with the binding of coenzyme (Fig. 6).

The dissociation (\( k_D \)) and recombination (\( k_R \)) rate constants for the various reactions can be obtained from the plots of Figs. 2, 3, and 6. These values are summarized in Table IV. The ratios of these rate constants, \( k_D/k_R \), are in good agreement with
The observation that the optical rotatory dispersion spectrum of pig heart lactate dehydrogenase is unchanged by NADH binding is in agreement with the findings of Liskowsky et al. (31) with bovine heart and rabbit muscle lactate dehydrogenases. We have extended these measurements to include oxamate binding. The combination of these experiments shows no observable change in the helical content of lactate dehydrogenase when complexed with NADH and oxamate. It should be noted that, in contrast, significant changes in the optical rotatory dispersion parameters were found with pig skeletal muscle lactate dehydrogenase (7), which contains a considerable fraction of M type subunits (32). The purity of the preparation of Bolotina et al. (7) was not, however, reported.

The pH dependence of the oxamate binding enthalpy is of interest. If the interaction of oxamate with the protein were purely electrostatic, one might expect that increasing the pH would lead to decreased enthalpies of oxamate dissociation, since the total charge on the protein would become more negative. Exactly the reverse is found, however. This unexpected effect of pH on the binding enthalpy might result from an electrostatic interaction with the active site. This would result in a conformational adjustment of the active site, which is the rate-limiting step, and that a slow conformational change does not occur (4).

The dissociation rate constant for TNAD is considerably smaller for the oxidized coenzyme. Velick (25) has suggested that the mechanism of coenzyme replacement is dissociative, i.e., that at high concentrations of NAD+, the replacement rate of TNAD by NAD+ at the active site is independent of NAD+ concentration.

TABLE IV

| Rate constants for binding reactions of pig heart lactate dehydrogenase in 0.1 M sodium phosphate, with ionic strength of 0.8 M with NaCl, from temperature jump experiments |
|---|---|---|---|---|
| Kinetics | pH | $k_D$ | $k_R$ | $k_D/k_R$ |
| NADH | 6.00 | 32 ± 0 | 33 ± 1 | 0.96 ± 0.17 |
| Oxamate† | 6.00 | 4.1 ± 1.5 | 16 ± 3 | 0.25 ± 0.14 |
| 8.00 | 39 ± 2 | 54.6 ± 0.6 | 0.70 ± 0.05 |
| Oxamate‡ | 6.00 | 128 ± 7 | 8.00 ± 0.13 | 37.2 ± 0.9 |
| 8.00 | 127 ± 5 | 4.54 ± 0.09 | 28.0 ± 1.2 |
| TNAD | 6.00 | 2900 ± 10 | 5.8 ± 0.5 | 410 ± 60 |
| 6.00 | 3800 ± 100 | 2.5 ± 0.3 | 153 ± 57 |

* At 20°C.
† Probable error.
‡ The concentration of free NADH was 10-fold larger than $K_{NADH}$.

values for the dissociation constants obtained by equilibrium titrations (Table 1).

**Discussion**

The results of the optical rotatory dispersion measurements can be summarized as follows. (a) No new Cotton effects are observed when NADH and oxamate are bound to pig heart lactate dehydrogenase; (b) no change in the parameters $\lambda_2$ or $\delta_3$ for the protein are found when these molecules are bound in the enzyme; (c) there is no evidence of change in the helical content between pH 6 and pH 8.

The theory of the optical rotatory dispersion spectrum of pig heart lactate dehydrogenase is not valid. The dissociation rate constant for TNAD is considerably larger than for NADH, whereas the recombination rate constant is smaller for the oxidized coenzyme. Velick (25) has suggested a reasonable mechanism to account for the differences in the affinities of lactate dehydrogenase for oxidized and reduced coenzymes. According to this view, the effect of oxidizing NADH is to cause a change in bond angles on the nitrogen of the pyridinium ring. This would result in a conformational adjustment of the protein which could lead to decreased enthalpies of oxamate dissociation.

In contrast to the results reported here, Czerlinski and Schreck (8) reported that the binding of NADH to rabbit muscle lactate dehydrogenase, which consists almost exclusively of the M type isoenzyme (33), occurs in at least two steps, one of which is apparently a structural rearrangement. The various relaxation experiments therefore indicate that the dissociation of NADH is the rate-limiting step, and that a slow conformational change does not occur (a very rapid conformational change is, of course, still possible). The optical rotatory dispersion measurements indicate that, as far as helical content is concerned, no measurable structural changes take place.

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entire dinucleotide, leading to weakened interactions with the protein at possibly several points.

It is not difficult to see how the weak forces of enzyme-substrate complex formation, many of which are short range (e.g., van der Waals-London forces, hydrogen bonds), could have a large effect on the rate constants of a "diffusion-controlled" reaction. The reaction of lactate dehydrogenase with coenzyme may be formally written

\[
E + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot C \quad \overset{k_{20}}{\underset{k_{21}}{\rightleftharpoons}} \quad EC
\]

where (2) is an encounter complex.

If the intermediate encounter complex is assumed to be in a steady state, there are two limiting cases for the diffusional rate constants (36): (a) the diffusion step (1-2) is rate-limiting, i.e., \( k_{21} \gg k_{20} \). Then

\[
k_R = k_{15}, \quad k_D = \frac{k_{21}}{k_{20}} \quad (2)
\]

(b) The transformation step (2-3) is rate-limiting, i.e., \( k_{21} \ll k_{20} \). Then

\[
k_R = \frac{k_{21}}{k_{20}}, \quad k_D = k_{20} \quad (3)
\]

For the explicit calculation of the rate constants from diffusion theory, Case a must be assumed.

If, however, Case b holds, the short range forces become extremely important. Assuming that \( k_{21} \) and \( k_{20} \) are identical for TNAD and NADH, we have from Equation 3 and Table IV that, at \( 20^\circ \) and \( pH 6 \),

\[
k_R, NAD = k_{21}, NADH, \quad k_D, TNAD = 6
\]

and

\[
k_R, NADH, \quad k_D, TNAD = 0.013
\]

Such effects are readily conceivable in terms of short range forces. It is plausible that such forces would strongly influence \( k_{20} \) but have a relatively small effect on \( k_{21} \). The model proposed by Velick (25) is thus consistent with the kinetic data which are observed.

Three possible hypotheses could explain the rapid chemical relaxation which is found with TNAD. One mechanism would involve a fast protonation-deprotonation equilibrium in the coenzyme. That this explanation is implausible is indicated by the following. (a) NADH does not have a pK in the pH range investigated (37), and (b) the amplitude and velocity of the effect are not perturbed by changing the pH from 6 to 8.

A second possible explanation is that the effect is a bimolecular binding reaction between 2 molecules of coenzyme. The observation that this effect is independent of coenzyme concentration renders this explanation unacceptable.

The most reasonable explanation, therefore, is that TNAD undergoes a temperature-dependent structural isomerization in solution. Evidence in support of this hypothesis has been obtained by Jardetzky and Wade-Jardetzky (38), who postulated two conformational forms of NADH, an elongated form and a collapsed form, with overlap of pyridine and adenine rings. In addition, Czerlinski and Hommes (39) reported that both NADH and NADPH undergo temperature-dependent isomerizations in solution, which occur in the 100 μsec range. Thus, the present experiments are in agreement with the postulate of Jardetzky and Wade-Jardetzky (38) and indicate that the relaxation time for the oxidized form is considerably shorter than for the reduced molecule. It is presumably only the elongated form which is bound to the enzyme (38).

The kinetics of oxamate binding provides evidence that no slow relaxation effects occur during oxamate binding. Recent experiments have shown, however, that during pyruvate binding, the initial enzyme-NADH-pyruvate complex undergoes a pH-dependent rearrangement to a catalytically active ternary complex, which is sufficiently slow to be measurable in the stopped flow (34). It is difficult to conceive of a better nonreactive structural analogue of pyruvate than is oxamate. Nevertheless, the latter is evidently unable to induce a similar conformational change in the protein.

The kinetic data provided in this paper and elsewhere (10, 34) are of significance for understanding the mechanism of catalysis by enzymes such as lactate dehydrogenase. Because this enzyme, like several other dehydrogenases, has a compulsory binding order (38), it is important that the velocities of the initial steps be known and their mechanisms understood. The mechanisms of NADH binding to liver alcohol dehydrogenase (40), pig heart malate dehydrogenase (41), and rabbit muscle lactate dehydrogenase (8) have already been studied by stopped flow or temperature jump techniques. Kinetic evidence for a coenzyme-induced structural isomerization has been reported only in the case of the rabbit muscle enzyme. Although the present results agree satisfactorily with simple schemes for coenzyme binding, they do not, of course, exclude the possibility of very rapid structural rearrangements nor, in the case of TNAD, of slow conformational changes with small amplitudes.

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