Biochem. J. (1973) **131**, 739–748
Printed in Great Britain

The Use of Ternary Complexes to Study Ionizations and Isomerizations During Catalysis by Lactate Dehydrogenase*

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(*Received* 12 *September* 1972)

1. The binding of oxamate to pig heart and pig muscle isoenzymes of lactate dehydrogenase in the presence of NADH was studied by fluorescence titration. The dissociation constant of oxamate from the heart enzyme complex is 3 μM and from the muscle isoenzyme 25 μM at pH 5. These values quantitatively increase with pH as predicted if oxamate can bind only to the enzyme–NADH complex if a group with pK 6.9 is protonated. There are four non-interacting oxamate-binding sites per tetramer. 2. *o*-Nitrophenylpyruvate is a poor substrate for both isoenzymes but has a reasonable affinity to the heart isoenzyme. Initially, it forms an enzyme–NADH–substrate complex, which can be detected either by protein-fluorescence quenching or by NADH-fluorescence quenching. The pH-dependence of the dissociation constant of nitrophenylpyruvate also shows that this ternary complex can only form if a group with pK 6.8 is protonated. Taken with the results of chemical-modification experiments, these results allow the pK of 6.8 to be assigned to a system probably involving the imidazole side chain of histidine-195. Formation of a ternary complex from a binary one at pH 8 is predicted to result in a proton being taken up from solution. 3. Isotope-effect studies with NADH and its deuterium analogue show that the rapidly formed ternary complex with *o*-nitrophenylpyruvate slowly isomerizes to give an active ternary complex, which then rapidly decomposes to NAD^+. The isomerization is pH-independent, and it is suggested that histidine-195 is still protonated in the activated ternary complex, which is present before hydride transfer. 4. All four subunits of the enzyme are kinetically equivalent with respect to the oxidation of bound NADH by *o*-nitrophenylpyruvate. 5. A partial mechanism for the enzyme is described which emphasizes the isomerizations and ionizations involved in forming the reduced ternary complex at pH 6 and 8.

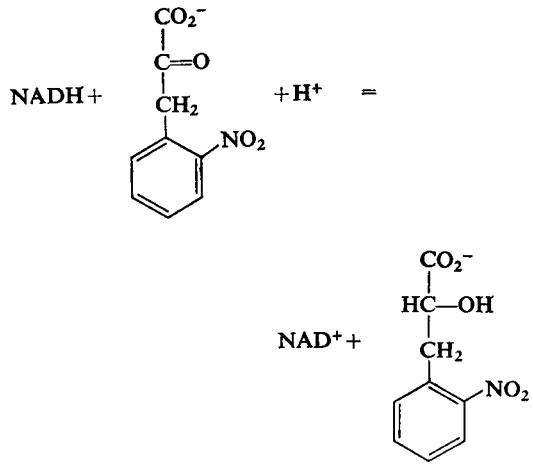
Stinson & Holbrook (1973) showed that there were no large changes in the affinity of lactate dehydrogenases for either the reduced or oxidized nicotinamide nucleotides in the pH range 5.5–8.5. The enzyme contains an essential histidine residue (Woenckhaus *et al.*, 1969). Chemical studies (Holbrook & Ingram, 1973) suggest that a reactive histidine residue (probably His-195) in the enzyme is protonated at pH 5.5 and unprotonated at pH 8.5. In this present paper we attempt to link changes in the state of protonation of this essential histidine residue with the ability of the enzyme to form various active and inactive reduced ternary complexes with oxamate and a new substrate, *o*-nitrophenylpyruvate. Schwert *et al.* (1967) found that binding of oxamate to ox heart lactate dehydrogenase was controlled by a system with an apparent pK of about 7. The direct measurements of oxamate binding are extended to higher pH values in an attempt to discover whether oxamate has even a weak affinity for the unprotonated enzyme–NADH complex.

Studies of the active ternary complex of lactate dehydrogenase are made possible by using rapid kinetics (Gutfreund, 1971). By using poor substrates it is possible to separate binding reactions from catalytic reactions on a slower time-scale and a study of the reaction has been undertaken in an attempt to determine which of the transient intermediates is responsible for the uptake and release of the proton from the solvent shown in the reaction. *o*-Nitrophenylpyruvate is a poor substrate (low $V_{max.}$) but is nevertheless most useful in that it binds to the enzyme tightly (low K_m).

Experimental

The sources of the pig heart lactate dehydrogenase, NADH, pyruvate and most other chemicals have

* Dedicated to Professor Dr. Theodor Wieland on the occasion of his sixtieth birthday.



been given, as have the methods used to characterize them (Stinson & Holbrook, 1973). *o*-Nitrophenylpyruvic acid was obtained from Kodak Ltd., London W.C.2, U.K. Oxamic acid was from BDH Chemicals Ltd., Poole, Dorset, U.K.

Enzyme assay by proton uptake

A solution (6ml) of *o*-nitrophenylpyruvate (15mM) and NADH (5mM) in water was adjusted to the required pH with 5M-NaOH in a vessel under N_2. The reaction was started by adding pig heart enzyme (about 0.5mg/ml) and the pH value was maintained constant at 25°C by the addition of 0.01M-HCl from a SBU1a syringe burette controlled by a titrator TTT1c (Radiometer, Copenhagen, Denmark). The enzyme activity was the zero-order rate of acid uptake expressed as μmol·min^{-1}.

Measurement of oxamate binding

The fluorescence of NADH bound to lactate dehydrogenase is enhanced threefold. The fluorescence of NADH in the ternary complex with oxamate is quenched to 20% of that of free NADH (Winer & Schwert, 1959). Quenching of NADH fluorescence was measured in a split-beam differential fluorimetric titrator as described by Holbrook (1972). A solution of sodium oxamate of appropriate pH value was added continuously at 5μl·min^{-1} to the stirred contents of two cuvettes. One contained only NADH and the other NADH and the enzyme (about 5μM binding sites). For the pig heart enzyme the NADH was 20μM (pH5–8) or 80μM (pH8–11). For the muscle enzyme the NADH was 80μM at pH5.5–8 and 400μM above pH8.5. These concentrations are sufficient to ensure that the enzyme was present as the binary complex with NADH at the start of the titration (Stinson & Holbrook, 1973). Fluorescence was excited at 320nm and measured as the radiation transmitted by a combination of Kodak Wratten filters 98 and 2B. The system was balanced at the start of the titration such that equal fluorescence changes in both cuvettes gave no difference in the signal recorded. The fractional saturation (α) of the total concentration of oxamate-binding sites in the cuvette ($[E]_0$) was equated to $\Delta F/\Delta F_{max.}$, where ΔF and $\Delta F_{max.}$ are the decrease in fluorescence and the limiting decrease in fluorescence approached at saturating concentrations of oxamate. The value of $\Delta F_{max.}$ could easily be estimated visually from the titrations at low pH. At high pH several values of $\Delta F_{max.}$ were tried until a plot of $1/(1-\alpha)$ against $[\text{oxamate}]_{total}/\alpha$ was linear. The value of $\Delta F_{max.}$ at constant enzyme concentration was approximately constant with changed pH. $\Delta F_{max.}$ was directly proportional to enzyme concentration in the range tested (5–100μM) at pH6.5. At high enzyme concentrations with respect to the oxamate-dissociation constant, the concentration of oxamate-binding sites in the solution was obtained as the extrapolated value of $[\text{oxamate}]_{total}/\alpha$ when $1/(1-\alpha)=0$. These plots for NADH were described in detail by Stinson & Holbrook (1973). The buffers were sodium pyrophosphate (I 0.15M) at the pH value desired. There was no loss of enzyme activity during the titration within the pH range studied.

Binding of o-nitrophenylpyruvate to enzyme–NADH complex

Although a substrate for both the heart and muscle isoenzymes, nitrophenylpyruvate binding could only be studied easily with the pig heart isoenzyme. The affinity of the muscle isoenzyme–NADH complex for this substrate was too weak to enable measurements to be made without unacceptably large absorption of the exciting radiation by the high concentrations of this yellow substrate necessary to form a complex. Binding to the heart isoenzyme was followed either by observing the apparent increase in the affinity of the enzyme for NADH (by using protein-fluorescence quenching) in the presence of added substrate, or by titrating the substrate directly into the enzyme–NADH complex and following the binding directly from the quenched fluorescence of the bound NADH (this latter method was analogous to that used for oxamate above).

By using protein fluorescence a solution (3ml) of the enzyme (0.1μM) in 67mM-sodium phosphate buffer of known pH containing up to 1.17mM-*o*-nitrophenylpyruvate was rapidly (within 2min) titrated with NADH (1.2mM) at 5μl·min^{-1} at 20°C. The quenching of protein fluorescence excited at 305nm and measured as transmission through a Kodak Wratten filter 18A was monitored by using

the titrating fluorimeter as described by Holbrook (1972). In these early experiments it was not realised that there was a non-linear relationship between fractional quenching of protein fluorescence by non-radiative energy transfer and the fraction of substrate-binding sites occupied. However, even under these non-linear conditions the concentration of NADH required to quench the protein fluorescence by 50% is still proportional to the apparent dissociation constant for NADH if the enzyme concentration is small compared with the dissociation constant for NADH. If the NADH concentration for half-maximal quenching is R and the dissociation constant for *o*-nitrophenylpyruvate from the binary complex is $K_{E-NADH,NPP}$ then the value of R ($R_{app.}$) in the presence of nitrophenylpyruvate will be related to its value without substrate (R_0) by eqn. (1):

$$\frac{1}{R_{app.}} = \frac{1}{R_0} \cdot \left(1 + \frac{[\text{nitrophenylpyruvate}]}{K_{E-NADH,NPP}}\right) \quad (1)$$

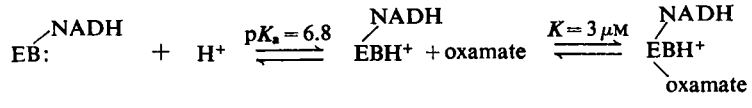
This equation assumes that there is a compulsory order of addition of substrates to the enzyme, with NADH binding first (as is the case for pyruvate). It is also necessary to assume that the concentration of bound nitrophenylpyruvate is small compared with that added and that the difference between the absolute protein fluorescence of the binary and ternary complexes is small (it is about 14%) compared with the protein fluorescence of the unliganded enzyme. There was no appreciable turnover of NADH during the experiments.

Binding of o-nitrophenylpyruvate measured by decreased NADH fluorescence

The split-beam differential fluorimetric titrator was arranged to measure the difference in the fluorescence through a Kodak Wratten filter 98 (maximum transmission at about 435nm) of two solutions excited at 370nm. The system was balanced such that equal changes of fluorescence in each cuvette gave no difference signal at the recorder: 370nm was chosen as the excitation wavelength rather than the 320nm usually used with this system (Holbrook, 1972), because the yellow substrate had much less absorption there. Each solution contained at 15°C NADH (40μM) in sodium pyrophosphate buffer (I 0.15M), at the required pH value. Portions (1 or 2μl) of *o*-nitrophenylpyruvate (0.5M) were added every 12s to the contents of each cuvette. One cuvette contained pig heart enzyme (5μM). The fluorescence from the solution without enzyme was compared with a reference voltage. If it decreased owing to absorption of the exciting light by the added substrate, the photomultiplier voltage and thus the gain of both channels was automatically increased to cancel out the decrease. The extinction coefficient at 370nm of nitrophenylpyruvate was only 22.5 litre·mol^{-1}·cm^{-1} and the correction used was never more than 5%. Each addition of substrate gave an instantaneous decrease in fluorescence (ΔF) before a steady-state rate of decrease was established (this is best seen from Fig. 5). The fraction of substrate-binding sites occupied (α) was proportional to $\Sigma\Delta F$. Since the concentration of added substrate was always much higher than the enzyme concentration, the dissociation constant of the *o*-nitrophenylpyruvate from the ternary complex was obtained from a double-reciprocal plot of $1/\Sigma\Delta F$ against 1/[nitrophenylpyruvate]. The maximum steady-state rate at each pH value was obtained as the limiting value to which the rate between each addition tended at saturating concentrations of substrate. The kinetics of the rate of NADH oxidation were also observed at 3°C by measuring extinction changes at 366nm by using a Hilger–Gilford reaction-kinetics spectrophotometer (this wavelength was chosen to avoid problems that arose from nitrophenylpyruvate absorption at 340nm).

Results

The pH-dependence of the logarithm of the dissociation constant of oxamate from the lactate dehydrogenase complex with NADH is shown in Fig. 1. Oxamate binds some sevenfold more tightly to the heart isoenzyme complex at all pH values than to the muscle isoenzyme complex. Below about pH6 the affinity to both systems approaches a low, constant value. At over about pH7.5 the affinity to both systems begins to decrease 10-fold for every increase in pH of 1 unit. This behaviour suggests that oxamate can bind only to the protonated heart enzyme–NADH complex according to the model:



The enzyme–NADH complex is shown as a non-protonic acid to emphasize the similarity in the pK_a to that of the essential histidine residue in the enzyme–NADH complex (Holbrook & Ingram, 1973). No oxamate binding to the unprotonated pig heart enzyme complex is shown as the affinity continues to decrease 10-fold per pH unit at pH values 3.7 units above the pK. Should there be any such binding it must be at least 10000-fold weaker than to the protonated complex. One cannot be so

sure that there is no oxamate binding to unprotonated pig muscle enzyme complex because the results do not continue so far above the pK. Nevertheless, there is no indication of binding to the unprotonated complex in the pH range measured (Fig. 1).

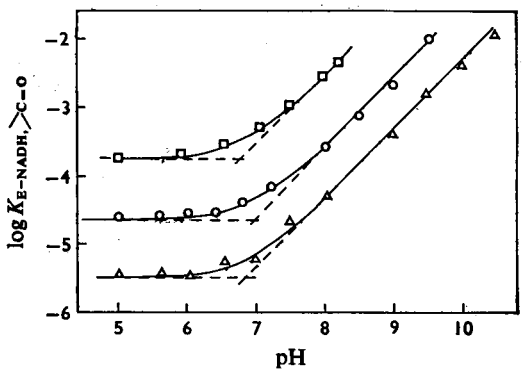


Fig. 1. *Variation with pH in the affinity of reduced binary complex of lactate dehydrogenase for carbonyl compounds*

All results are plotted as if the substrate or inhibitor could only bind if a group on the reduced binary complex with p$K_a = 6.8 \pm 0.2$ was protonated. Oxamate binding to the pig heart isoenzyme–NADH complex (△) and to the pig muscle isoenzyme–NADH complex (○) was measured as described in the text at 21°C. The rapid formation of the ternary complex of pig heart enzyme–NADH with *o*-nitrophenylpyruvate was measured at 15°C before sufficient time had elapsed for NADH to be appreciably oxidized (□). Binding was followed by quenching of NADH fluorescence as described in the text. The lines are drawn to indicate groups with p$K = 6.9$ (△), p$K = 7.0$ (○) and p$K = 6.8$ (□). $>C{=}O$ symbolizes the carbonyl group in oxamate and nitrophenylpyruvate.

The affinity of the binary complex for oxamate is independent of protein concentration (Table 1). The high affinity at low pH values made it possible to determine the concentration of oxamate-binding sites in a solution of enzyme of known protein concentration with fair accuracy. The results in Table 1 are clearly as expected for one oxamate binding to each lactate dehydrogenase subunit (molecular weight 36000). Plots of oxamate-binding results are shown in Fig. 2. The experiment for the muscle isoenzyme demonstrates that the concentration of oxamate-binding sites expected from the enzyme concentration and a single dissociation constant are sufficient to describe the binding. Plots according to Brown & Hill (1922–23) were straight lines of slope 1.0. There was thus no interaction between the oxamate-binding sites.

o-Nitrophenylpyruvate is a substrate for both isoenzymes of pig lactate dehydrogenase. Double-reciprocal plots of steady-state kinetic experiments (Fig. 3) are linear and show no trace of substrate inhibition at concentrations up to 2mM in the range pH6.4–8.6. The K_m value for nitrophenylpyruvate at pH6.4 (0.135mM) is only about 10-fold greater than that for pyruvate, although the $V_{max.}$ is about one two-thousandth of that for pyruvate. This $V_{max.}$ was so small that it was possible to titrate NADH into solutions containing enzyme and nitrophenylpyruvate and observe the formation of a ternary complex without all the NADH being oxidized. When the binding of NADH was monitored by the quenching of protein fluorescence it was noted that the apparent affinity of the enzyme for NADH was increased if nitrophenylpyruvate was present and it was concluded that a ternary complex had formed. The ternary complex could form with either NADH or the pyruvate analogue binding to the enzyme first. A pathway in which the pyruvate analogue binds first seems

Table 1. *Binding of oxamate to pig lactate dehydrogenases*

The concentrations of oxamate-binding sites in solutions of known protein concentration were determined from plots of $1/(1-\alpha)$ against $[\text{oxamate}]_{total}/\alpha$ as described in the text. For the heart isoenzyme the buffer was 25mM-sodium phosphate, pH6.5 at 21°C, containing 150μM-NADH. For the muscle isoenzyme, the buffer was 50mM-sodium phosphate, pH7, containing 200μM-NADH.

Enzyme	Concentration of enzyme protein (μM)	Concentration of oxamate-binding sites from fluorescence titration (μM)	Dissociation constant for oxamate (μM)
Pig heart	20	20	3.0
	40	35	5.7
	98	108	3.5
Pig muscle	133	116	34

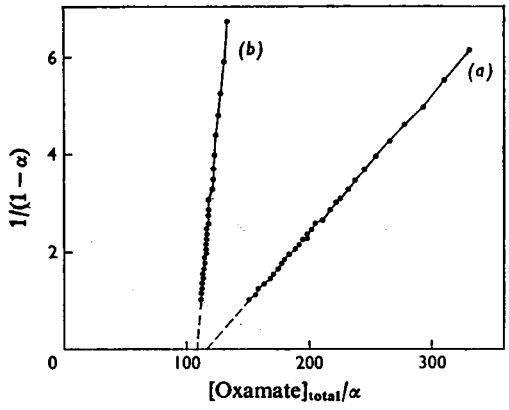


Fig. 2. *Determination of the number of oxamate-binding sites on the lactate dehydrogenase molecule*

The fractional saturation (α) of the oxamate-binding sites was measured from the decrease in fluorescence when oxamate was added to (*a*) 133μM pig muscle isoenzyme and 200μM-NADH in 50mM-sodium phosphate buffer, pH7 at 21°C, or (*b*) 98μM pig heart isoenzyme in 25mM-sodium phosphate buffer, pH6.5, containing 150μM-NADH. The protein concentration is calculated by assuming a subunit weight of 36000. The concentration of oxamate-binding sites was the intercept with the $[\text{oxamate}]_{total}/\alpha$ axis when $1/(1-\alpha) = 0$. The dissociation constant was the reciprocal slope of the graph.

unlikely since: (*a*) NADH can bind in the absence of the pyruvate analogue; (*b*) the affinity of the enzyme for NADH continues to increase with increasing concentration of nitrophenylpyruvate and does not reach a constant value in the range examined (Fig. 4); (*c*) pyruvate and its competitive inhibitors bind in an ordered manner.

A far more convenient means of following the formation of the ternary complex with nitrophenylpyruvate and NADH was to titrate the nitrophenylpyruvate directly into a solution of the binary complex of the enzyme with NADH. There was a rapid decrease in the enhanced fluorescence of the bound NADH to a value less than that for free NADH (Fig. 5) before NADH was appreciably oxidized to NAD^+. This fluorescence behaviour is reminiscent of the quenching of enzyme–NADH fluorescence when oxamate binds and when pyruvate binds (R. A. Stinson, unpublished work), and indicates that the environment of the bound NADH is changed in the same manner by the two substrates or by the competitive inhibitor oxamate. Thus the structure of the enzyme–NADH–oxamate complex may be analogous to that of the inactive enzyme–NADH–substrate complex (form 3) and not to the active

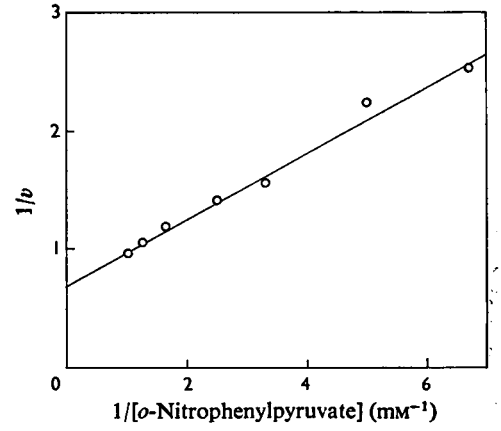


Fig. 3. *o-Nitrophenylpyruvate as a substrate for lactate dehydrogenase*

The rate of decrease in the extinction at 340nm (v) of solutions of NADH (0.18mM) containing various concentrations of *o*-nitrophenylpyruvate in 67mM-sodium phosphate buffer, pH6.4 at 23°C, was measured in the presence of pig heart lactate dehydrogenase (about 40μg/ml).

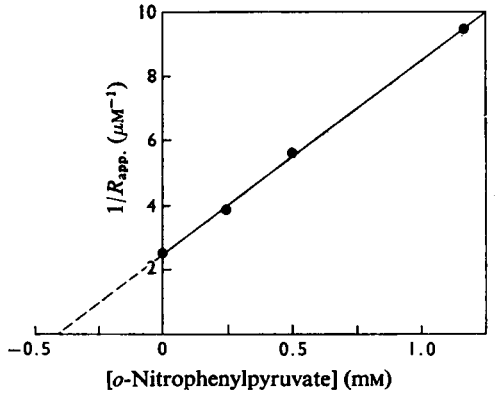


Fig. 4. *Effect of o-nitrophenylpyruvate on the apparent affinity of lactate dehydrogenase for NADH*

The concentration ($R_{app.}$) of NADH required to quench by 50% the protein fluorescence of 0.1μM pig heart lactate dehydrogenase in 67mM-sodium phosphate buffer, pH6.35, was measured at 0°C in solutions containing the added concentrations of *o*-nitrophenylpyruvate shown. During the time (less than 2min) required for the titration with NADH ($2\mu M \cdot min^{-1}$) there was no appreciable turnover of the NADH. The line is drawn through the points with $K_{E-NADH,NPP} = 0.4$mM. With buffer adjusted to pH6.35, 7.2 and 8.5 the dissociation constants were 0.4mM, 0.8mM and 2mM respectively.

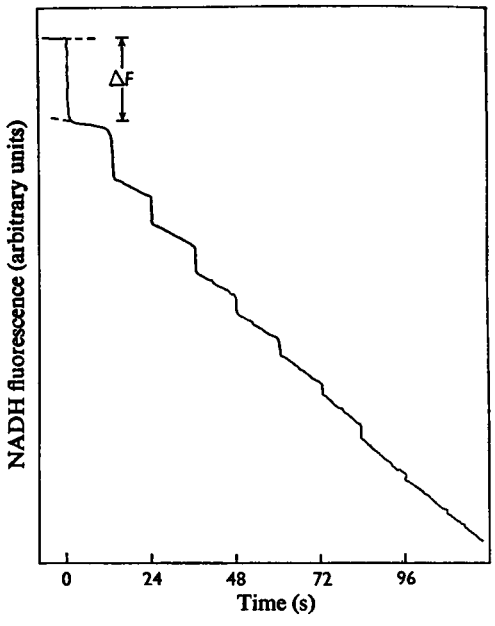


Fig. 5. *Formation of the lactate dehydrogenase–NADH–o-nitrophenylpyruvate ternary complex*

The fluorescence of NADH excited at 370nm in a solution of 40μM-NADH and 5.3μM pig heart enzyme in 67mM-sodium phosphate buffer, pH7.2 at 15°C, is shown. Every 12s a portion of *o*-nitrophenylpyruvate was added to increase the concentration by 0.167mM. An electrical device corrected for any decrease in the fluorescence owing to absorption of the incident light by the added yellow substrate (see the Experimental section). The increase in concentration of the ternary complex was proportional to the instantaneous decrease in the fluorescence after each addition (ΔF). This value was obtained by extrapolating the steady states before and after the addition to the point of addition.

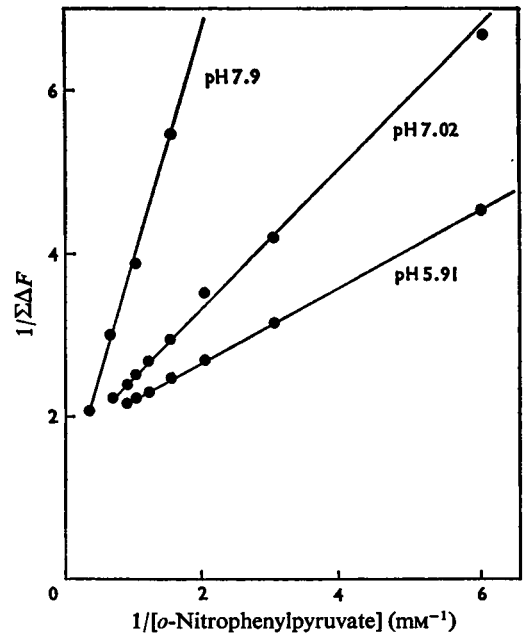


Fig. 6. *Determination of the affinity of the enzyme–NADH–complex for o-nitrophenylpyruvate*

The reciprocal of the sum of the instantaneous decreases in NADH fluorescence (such as those shown in Fig. 5) is plotted against the reciprocal of the *o*-nitrophenylpyruvate concentration. The solutions at 15°C contained 40μM-NADH and 5.3μM pig heart enzyme in sodium pyrophosphate buffers of *I* 0.15M and the pH shown.

isomer, form 4 (Scheme 1). A record of a binding experiment is shown in Fig. 5. The immediate decrease in the fluorescence after each addition of nitrophenylpyruvate is followed by the establishment of a new steady-state rate of NADH oxidation. As increasing concentrations of nitrophenylpyruvate are added the immediate decreases in fluorescence are decreased, and the slow zero-order rate of decrease of fluorescence approaches a maximum value. An excess of NADH is present in solution and thus the enzyme is saturated in either the binary or ternary complex throughout. Since the turnover is so slow, the amount of substrate used up between additions (0.167mM) is small. Under these conditions $\Sigma\Delta F$ is proportional to the fraction of the enzyme in the ternary complex, and the dissociation constant of nitrophenylpyruvate from this complex (K) can be obtained from a double-reciprocal plot based on eqn. (2):

$$\frac{K}{[\text{nitrophenylpyruvate}]} = \frac{\Sigma\Delta F_{max.}}{\Sigma\Delta F} - 1 \qquad (2)$$

An equivalent plot replaces $\Sigma\Delta F$ by v, the steady-state velocity at each nitrophenylpyruvate concentration. The double-reciprocal plots are linear (Fig. 6), indicating that a single macroscopic dissociation constant characterizes all the nitrophenylpyruvate-binding sites of the enzyme. The variation in the apparent affinity of the enzyme for nitrophenylpyruvate with pH may be described accurately by a model that assumes that the substrate can only bind when a group on the enzyme with pK6.8 is protonated (Fig. 7). The pK of nitrophenylpyruvate is 2.3. Because of the light absorbed by high concentrations of the yellow substrate it is not possible to test the

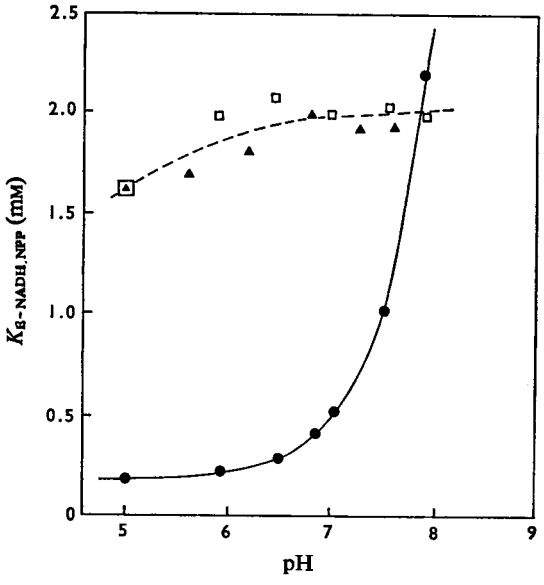


Fig. 7. *Variation of the apparent affinity of the pig heart lactate dehydrogenase–NADH complex for o-nitrophenylpyruvate with pH*

Dissociation constants for *o*-nitrophenylpyruvate (●) were determined from plots such as Fig. 6. The buffer was sodium pyrophosphate, *I* 0.15M, at 15°C. ---- shows the trend in the maximum velocity of NADH oxidation when observed either by the uptake of H^+ (▲) at 15mM-*o*-nitrophenylpyruvate in unbuffered solution or by the rate of decrease of NADH fluorescence at saturation with the substrate (□). —— is the dissociation constant for substrate calculated by assuming that the dissociation constant was 0.2mM at low pH values and that the substrate cannot bind when a group with apparent p*K*6.8 is protonated.

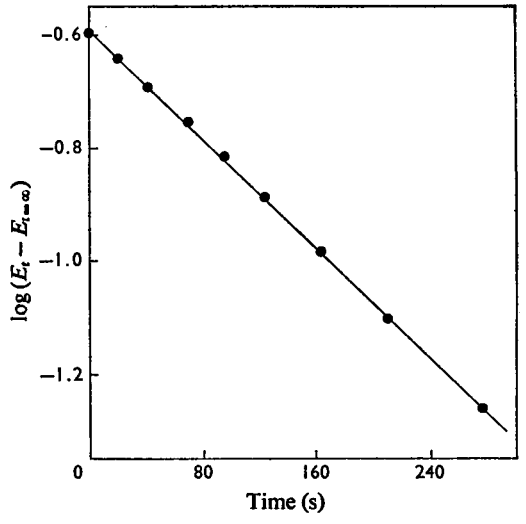
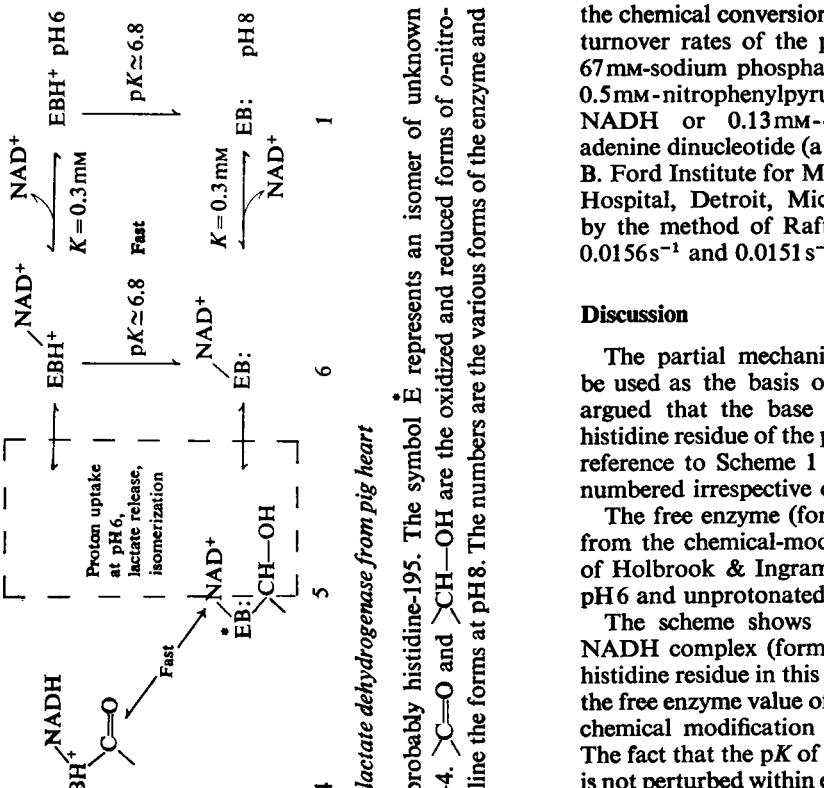


Fig. 8. *Single turnover of lactate dehydrogenase–NADH complex with o-nitrophenylpyruvate*

A solution containing 0.1mM-NADH and 0.105mM pig heart lactate dehydrogenase in sodium pyrophosphate buffer, pH6.0, *I* 0.15M, at 3°C, was mixed with *o*-nitrophenylpyruvate to give a final concentration of 1.67mM. The decrease in extinction at 366nm was recorded. The amplitude of the extinction decrease (0.26) was slightly less than expected for free NADH (0.33). The first-order rate constant obtained from the plot was $5.5\times10^{-3}s^{-1}$. E_t and $E_{t=\infty}$ are the extinctions of the solution at time *t* (s) and infinite time.

model at very high pH values, and the present results could not exclude the possibility that the affinity of nitrophenylpyruvate for the unprotonated binary complex was more than 25-fold less than that for the protonated complex. The slow steady-state turnover of the ternary complex is almost independent of pH (Fig. 7) but does depend on temperature. At 3°C the first-order rate constant is $5\times10^{-3}s^{-1}$ and increases to about $5\times10^{-2}s^{-1}$ at 25°C. The dissociation constant of nitrophenylpyruvate also depends on temperature, and at pH6 it increases from 0.2mM at 15°C to about 0.45mM at 23°C.

All the substrate-binding sites of the lactate dehydrogenase tetramer are kinetically identical when pyruvate is the substrate (Stinson & Gutfreund, 1971; Gutfreund, 1971). The same is true when nitrophenylpyruvate is the substrate. When 0.1mM-enzyme–NADH complex (formed from a slight excess of enzyme over NADH, since the affinity of the enzyme for NADH at pH6 is great; Stinson & Holbrook, 1973) is mixed with an excess of nitrophenylpyruvate there is a clean first-order decrease in the concentration of NADH until all the NADH is oxidized to NAD^+ (Fig. 8). From this experiment it is concluded that the catalytic activity per site of lactate dehydrogenase is the same whether an average of four sites per tetramer or only one site per tetramer is occupied by NADH. The extinction change per mol of bound NADH converted into NAD^+ was less than that expected from free NADH, but no allowance was made for the difference in extinction of bound NADH or for any change at the observing wavelength owing to the conversion of the coloured pyruvate into the lactate analogue. In similar experiments with excess of NADH (0.5mM), there was an initial zero-order rate of oxidation of NADH, indicating that the rate-limiting step was not after



Scheme 1. *Partial mechanism for lactate dehydrogenase from pig heart*

The base B: represents the imidazole portion of a histidine residue, probably histidine-195. The symbol $\overset{\bullet}{E}$ represents an isomer of unknown structure. The words 'Fast' and 'Slow' are relative to the slow step 3→4. $\rangle C{=}O$ and $\rangle CH{-}OH$ are the oxidized and reduced forms of *o*-nitrophenylpyruvate. The upper line has enzyme forms at pH6 and the lower line the forms at pH8. The numbers are the various forms of the enzyme and are referred to in the text.

the chemical conversion of NADH into NAD^+. The turnover rates of the pig heart enzyme assayed in 67mM-sodium phosphate buffer, pH7.2, containing 0.5mM-nitrophenylpyruvate and either 0.2mM-NADH or 0.13mM-4-[^{2}H]dihydronicotinamide-adenine dinucleotide (a gift from Dr. J. Shore, Edsel B. Ford Institute for Medical Research, Henry Ford Hospital, Detroit, Mich. 48202, U.S.A.) prepared by the method of Rafter & Colowick (1957) were $0.0156\,s^{-1}$ and $0.0151\,s^{-1}$ respectively.

Discussion

The partial mechanism given in Scheme 1 will be used as the basis of this discussion. It will be argued that the base B: is the reactive, essential histidine residue of the pig heart enzyme. To simplify reference to Scheme 1 each form of the enzyme is numbered irrespective of its state of protonation.

The free enzyme (form 1 of Scheme 1) is known from the chemical-modification and kinetic studies of Holbrook & Ingram (1973) to be protonated at pH6 and unprotonated at pH8. The pK is 6.8±0.2.

The scheme shows only one kind of enzyme–NADH complex (form 2). The pK of the essential histidine residue in this complex is not changed from the free enzyme value of 6.8±0.2 when monitored by chemical modification with diethyl pyrocarbonate. The fact that the pK of the essential histidine residue is not perturbed within experimental error by NADH binding is consistent with the facts that the reactivity towards diethyl pyrocarbonate is decreased by only 60% in the binary complex (Holbrook & Ingram, 1973) and that the dissociation constant for NADH increases only 2.5-fold between pH5.5 and 8.5 (Stinson & Holbrook, 1973). Lack of pH-dependence for NADH in this region was also observed by Winer (1963) for the ox heart enzyme, although the dissociation constant was larger (1.8μM). Formation of the enzyme–NADH complex is very rapid and may be diffusion-controlled (Heck, 1969; Stinson & Gutfreund, 1971).

Rapid formation (within 1s) of reduced ternary complexes (form 3) with oxamate or poor substrates has been observed. The apparent equilibrium constants are small and constant at low pH values and increase 10-fold per pH unit above an apparent pK of 6.8 (Fig. 1). Such pH-dependence with oxamate was observed by Winer & Schwert (1959) for the ox heart enzyme, although the limiting dissociation constant at low pH values was higher (70μM), as it is for the pig muscle enzyme. We conclude that formation of the stable initial ternary complex (form 3) can occur only when the essential histidine residue is protonated. Such a conclusion predicts that oxamate binding to the binary complex should be accompanied by the uptake of a proton at pH8 but not at pH6. The degree to which carbonyl binding to the

unprotonated binary complex can be rigorously excluded was discussed earlier in the present paper for the muscle isoenzyme and for nitrophenylpyruvate binding.

The group or system with pK6.8±0.2 that is associated with substrate binding involves the essential histidine residue of the pig heart enzyme. It is not normally possible to ascribe the apparent pK of a rate or equilibrium parameter to ionization of an individual group on an enzyme. The grounds for the identification in the present case are that: (*a*) chemical studies show that the pK of the reactive essential histidine residue is 6.8 in both the apoenzyme and the enzyme–NADH complex; (*b*) the essential histidine residue is unavailable for reaction with diethyl pyrocarbonate in the ternary complex; (*c*) enzyme in which the essential histidine residue is carbethoxylated and therefore has a decreased pK (the pK of the carbethoxy derivative is approximately 4) cannot bind the substrate to the reduced binary complex. Even so, it is not known whether the pK involving the histidine represents the pK of that group in an isolated aqueous environment or whether it represents the pK of a system in which the imidazole group is hydrogen bonded, in the unprotonated state, to some other amino acid side chain and not to water. As yet, there is no explanation of the unusually high reactivity of the histidine residue with pK6.8 towards diethyl pyrocarbonate. There is also no satisfactory structural explanation as to why the substrate can only bind to the protonated reduced binary complex.

The initially formed ternary complex (form 3) slowly isomerizes to form 4 before the rapid chemical oxidation of the NADH to NAD^+. The rapid quenching of bound NADH fluorescence occurs before the oxidation of NADH and is thus evidence for at least one reduced ternary complex. The oxidation of NADH by *o*-nitrophenylpyruvate is very slow and the rate-determining step could either be the oxidation itself, or occur after it or before it. If the rate-limiting step were after the chemical interconversion of the ternary complex, a rapid turnover of the first mol of NADH would occur. This is not observed; the rate with excess of NADH is zero order initially. If the rate-limiting step were the interconversion of the ternary complex it would involve C–H bond breakage and thus be slower by a factor of 5–10 with ^2H than with ^1H. There was no isotope effect with [^2H]NADH. When ^1H was replaced by ^2H decreases in rates of sevenfold were observed with alcohol dehydrogenase in partial reactions where C–H bond scission was rate-limiting (Shore & Gutfreund, 1970). Since the rate-limiting step was not the formation of the oxidized ternary complex nor after it, then it must have been before it. For this reason an isomer of the reduced ternary complex (form 4) is included in Scheme 1. What protein structure this isomer represents is unknown. An identical argument and method was used by Stinson & Gutfreund (1971) to justify the postulate of an isomer of the reduced ternary complex with pyruvate. The steady-state rate of nitrophenylpyruvate reduction is only slightly dependent on pH between pH 5.5 and 8.5. This pH-independence is observed whether the rate is monitored by proton uptake or by NADH oxidation (Fig. 7). This is analogous to the pH-independent $V_{max.}$ found by Schwert *et al.* (1967) for pyruvate. Since this slow steady-state rate is now identified as the isomerization of two ternary complexes, its pH-independence is interpreted to mean that there is no overall uptake or release of a proton to the solvent during the isomerization. Thus, since the first ternary complex is protonated, its isomer, form 4, is also protonated at the essential histidine residue. This last conclusion is speculative, since although the overall complex must remain protonated during the isomerization, there is no reason why the proton could not migrate during this step to another amino acid side chain if there was some compensating change in the pK of the histidine residue such that it remained unprotonated at pH 6. If the analogy to pyruvate noted above also holds, then this histidine residue may be protonated in the activated reduced ternary complex of pyruvate as well.

There is much uncertainty about the mechanism between the formation of the first enzyme–NAD^+–nitrophenyl-lactate complex and the enzyme–NAD^+ complex. It is assumed, by analogy with lactate, that there is an ordered release of products, i.e. lactate first and NAD^+ last (Takenaka & Schwert, 1956). Formation of the enzyme–NAD^+ complex (form 6) from form 5 must involve at least two events at pH 8 and three at pH 6. These are the reverse isomerization, lactate release and (at pH 6 only) proton uptake. There is no information on the sequence of these events.

In Scheme 1 the binding of NAD^+ to the enzyme is shown not to perturb the pK of the essential histidine residue. A direct study of the pK of this group in the enzyme–NAD^+ complex was not possible because the high concentrations of NAD^+ needed to saturate the enzyme would make observation of carbethoxyhistidine at 250nm very difficult. This problem might be avoided if the reaction of diethyl pyrocarbonate could be followed by CO_2 release. Indirect evidence that the pK of this histidine residue is not perturbed is that NAD^+ does not protect against diethyl pyrocarbonate inactivation and that the affinity of the enzyme for NAD^+ is not dependent on pH within the range pH 6–8.5 (Stinson & Holbrook, 1973).

Scheme 1 is consistent with the finding by Loewus *et al.* (1953) that the α-hydrogen atom of lactate is stereospecifically transferred to the A position in NADH without exchange with the solvent.

These findings are usually interpreted to mean that hydrogen transfer occurs in a rigid ternary complex without transient reduction or oxidation of an amino acid residue of the protein. However, it has been pointed out by Colowick *et al.* (1966) that transient reduction of the protein could occur as long as the reduced residue could not equilibrate with the solvent. Schellenberg (1967) claimed that lactate dehydrogenase can be specifically labelled at a tryptophan residue by [2-^{3}H]lactate. However, if the trichloroacetic acid precipitate of the protein is washed sufficiently all the radioactivity is lost from the protein (Holbrook *et al.*, 1970). There is no electron density characteristic of tryptophan near to the coenzyme–substrate binding site in the dogfish muscle enzyme (Rossmann *et al.*, 1971).

This work has been supported by grants from the Science Research Council. The fluorimeter was constructed with a grant-in-aid from The Royal Society (London). R. A. S. was supported in Bristol by a Post-Doctoral Fellowship of the Canadian Medical Research Council.

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