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Slow structural changes shown by the 3-nitrotyrosine-237 residue in pig heart [Tyr($3NO_2$)^{237}] lactate dehydrogenase

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1. The pK_a of the phenolic hydroxy group of the Tyr($3NO_2$)-237 residue in pig heart [Tyr($3NO_2$)^{237}]lactate dehydrogenase is 7.2 in the apoenzyme, 7.4 in the enzyme–NADH complex and 7.8 in the enzyme–NADH–oxamate complex. The alkaline shift from apoenzyme to ternary complex is ascribed to the approach of the Glu-107 residue during the movement of the polypeptide loop residues 98–110. 2. The affinities of the nitrated enzyme for NADH and for oxamate (in the presence of NADH) are slightly less than those of the native enzyme. The turnover number for the nitrated enzyme in the pyruvate-to-lactate direction is about 0.75 of the value for the native enzyme. 3. Temperature-jump relaxation experiments of the enzyme saturated with NADH but fractionally saturated with oxamate are interpreted to show that the pK_a of the nitrotyrosine residue responds to a protein rearrangement after oxamate binds to the binary enzyme–NADH complex. 4. Transient-kinetic experiments show the environment of the Tyr($3NO_2$)-237 residue in the enzyme–NADH–pyruvate complex of the steady state to be similar to that in the enzyme–NADH–oxamate inhibitor complex.

There are as yet few enzymes in which the rate of a catalytic step can be assigned to a defined change in the three-dimensional structure of the enzyme protein. Lactate dehydrogenase crystallizes in two distinct structures (the apo- and ternary structures), which differ by up to 1.3 nm in the position of a loop of polypeptide chain (residues 98–110) over the entrance to the active site. In the apo-structure the loop extends out into the solvent; in the ternary complex it covers the active site and prevents the entry or release of the coenzyme (for the original references see Holbrook *et al.*, 1975). Since the enzyme binds coenzyme in one redox state and releases it in the other, it follows that a change in the conformation of the loop of polypeptide chain must take place twice for each molecule of substrate that is transformed by the enzyme.

Abbreviations used: symbols used for modifications of lactate dehydrogenase and of amino acid residues are in accordance with IUPAC–IUB Recommendations [*Biochem. J.* (1967) **104**, 17–19; *Biochem. J.* (1972) **126**, 773–780]; *S*-Lac–NAD$^+$, oxidized (3*S*)-5-(3-carboxy-3-hydroxypropyl)nicotinamide–adenine dinucleotide (Grau *et al.*, 1978).

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It would be desirable if the rate of movement of the polypeptide chain could be directly detected and correlated with a known rate of a step in the catalytic mechanism. Since these rates are rapid, it is necessary to introduce a reporter group into the protein. The loop itself has so far defied specific chemical modification, but Jeckel *et al.* (1971) have described the specific nitration of a tyrosine residue, now known to be Tyr-237 (Knitl, 1977; Kiltz *et al.*, 1977). This residue is at the surface of the protein in the region that is covered by the loop in the ternary complex. This residue, and the Glu-107 residue (on the loop), are conserved in all known sequences of the enzyme. The three-dimensional structures show the distance between the phenolic hydroxy group of the tyrosine residue and the carboxy group of the Glu-107 residue decreases from 0.85 nm in the structure of the dogfish apoenzyme to 0.45 nm in that of the pig H_4 enzyme–*S*-Lac–NAD$^+$ complex (Fig. 1). We expected that the increase in acidity due to the approach of the Glu-107 residue would raise the pK_a of the Tyr($3NO_2$)-237 residue to give yellow absorbance changes and thus rapidly respond to the position of the loop and enable kinetically observed isomerization rates to be compared to rates of change between defined structures. The structure of pig H_4 enzyme–NADH–oxamate complex is at

0.25nm resolution similar to dogfish M_4 enzyme–NAD–pyruvate complex (W. Eventoff & M. G. Rossmann, unpublished work cited by Eventoff *et al.*, 1977).

Experimental

Pig heart H_4 lactate dehydrogenase was prepared by affinity chromatography on oxamate–agarose as modified by Parker & Holbrook (1981). The enzyme was nitrated with tetranitromethane (Serva, Heidelberg, Germany), and the degree of nitration was determined as described by Jeckel *et al.* (1971) with the exception that any inactive over-nitrated enzyme was removed by repeating the affinity chromatography on oxamate–agarose. The degree of nitration was 0.9–1 mol of nitrotyrosine/mol of subunits (M_r 36000). The very simple ^1H n.m.r. difference spectrum in the aromatic region (Parker *et al.*, 1981) shows that only one proton is lost and confirms that the modification does not alter the interaction of any other side chains in either the apoenzyme or the binary complex with NADH. The specific activity of the nitro-enzyme with 0.13mM-NADH and 1mM-pyruvate at pH7.2 at 25°C was 275μmol/min per mg (compared with 360μmol/min per mg for the native enzyme with 0.3mM-pyruvate). NADH and NAD^+ were from Boehringer, Mannheim, Germany, and were purified by column chromatography as described by Holbrook & Wolfe (1972).

The modified dipeptide Boc-Glu-($3NO_2$)Tyr was prepared from the non-nitrated dipeptide (E. Merck, Darmstadt, Germany) by treatment with a 2-fold excess of tetranitromethane for 30min in 0.1M-sodium pyrophosphate buffer at 25°C. The yellow peptide was recovered by filtering the reaction mixture through a column of Sephadex G-25 in 20mM-NaCl.

The tryptic-digest peptide containing the nitrotyrosine residue from the nitro-enzyme (used for pK determinations) was prepared by incubating the denatured nitro-enzyme (20mg) at 25°C with trypsin (2mg) added as 1mg portions at 0 and 3h in a 21h digestion. The solution was centrifuged at 20000**g**, and the yellow peptide was isolated by filtering the supernatant through a column of Sephadex G-50 in 5mM-sodium phosphate/20mM-NaCl buffer, pH6.4.

The equilibrium binding of NADH to the nitro-enzyme was followed from the decrease in protein fluorescence measured, corrected for geometric quenching, and analysed for a single dissociation constant by the method of Holbrook (1972). The buffers were 20mM-sodium phosphate buffer, pH6.5, and 20mM-sodium pyrophosphate buffer, pH8.5.

The equilibrium binding of oxamate to E–NADH (enzyme–NADH complex) was evaluated by determining the apparent dissociation constant ($K_{app.}$) for NADH binding (as described for the binary complex above) in solutions containing 0–22μM-oxamate at pH6.5 and 0–20mM-oxamate at pH8.5. The plots of $1/K_{app.}$ versus [oxamate] were linear, and $K_{E-NADH,oxamate}$ was obtained from the equation (Holbrook & Stinson, 1973):

$$1/K_{app.} = 1/(K_{E-NADH}) \cdot \{1 + [\text{oxamate}]/(K_{E-NADH,\,oxamate})\}$$

For the van't Hoff plot to determine ΔH, the binding of oxamate was directly measured by titrating an oxamate solution into a solution of the nitro-enzyme (18μM) and NADH (4.6μM) while the decrease in fluorescence of the NADH was monitored (excitation wavelength 340nm; emission measured with Kodak no. 98 filter, as described by Holbrook & Stinson, 1973). The temperature was varied from 25 to 10.5°C and the buffer was 0.1M-sodium phosphate, pH7.0. The plot was used to interpolate the dissociation constant of oxamate from the ternary complex at the temperature of the temperature-jump experiments.

The relaxation of the transmission of the nitro-enzyme at 428nm was measured in a temperature-jump apparatus designed by L. DeMaeyer and constructed by Messanlagen (Göttingen, Germany) in which a 0.05μF capacitor at 17kV was discharged through 2ml of solution of enzyme, NADH and oxamate in 0.1M-sodium phosphate buffer, pH7.0. The temperature increased by 70% of its final value in less than 10μs. The solutions were initially at 19°C and the temperature increase was about 4°C. The enzyme concentration varied from 20 to 150μM, the NADH concentration was 0.3mM and the oxamate concentration was varied from 15 to 300μM. The high concentration of NADH ensured that the enzyme was saturated with NADH at all times. The initial concentrations of the enzyme–NADH complex and the concentrations of free oxamate were calculated from the dissociation constants reported in the present paper. In all 1024 values of the transmission at 428nm were acquired at 5μs intervals by a Datalab DL905 transient recorder. The time constant was usually 20μs. After each discharge the solution was cooled and the discharge was repeated, and the results from 10–20 experiments were averaged. The results were transferred via magnetic tape to a Cromemco Z-2 computer and were analysed by a non-linear least-squares fit (Marquardt, 1963) to either a single-exponential or a double-exponential decay. Under the conditions described for Fig. 3 neither the apoenzyme nor the enzyme–NADH complex showed relaxation rates less than 10000s^{-1}.

The rapid-mixing experiments used a dual-beam stopped-flow spectrophotometer of light-path 1cm with dead time 2.4ms (Shore *et al.*, 1975) at room temperature.

The views of lactate dehydrogenase in Fig. 1 were

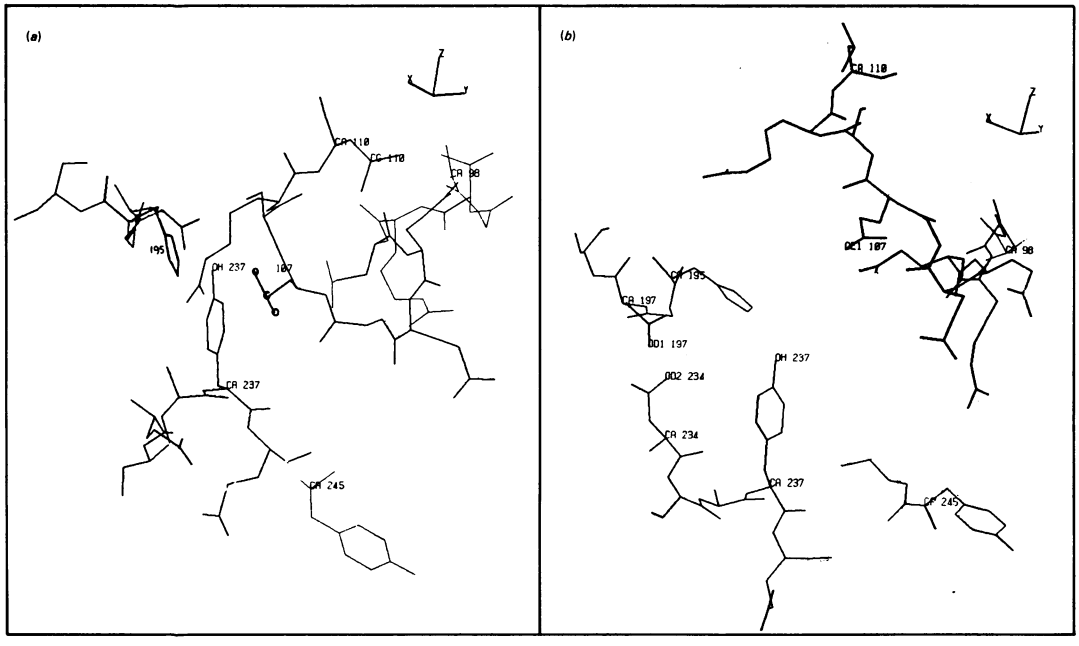

Fig. 1. *Views of the environment of the Tyr-237 residue in the apo- and ternary crystal structures of lactate dehydrogenase*

At the left (*a*) is a section of pig H_4 enzyme in a complex with the coenzyme–substrate compound *S*-Lac–NAD^+. The loop residues (98–110) are close to the Tyr-237 residue hydroxy group, to the active site (His-195 residue) and to the Asp-197 residue. The coenzyme–substrate compound is not shown. On the right (*b*) is a similar view of dogfish M_4 apoenzyme. The loop now extends into solution (to the right) opening the cleft to the active centre. The distance between the Tyr-237 hydroxy group and the δ-carbon atom of the Glu-107 residue increases from 0.45 nm to 0.85 nm from the ternary complex to the apoenzyme. The Asp-197 residue is also further away. The symbols CA 237 etc. on the Figure refer to the position of the α-carbon atom of residue 237 etc. Similarly OD1 is the 1-oxygen at the δ-carbon atom and OE1 107 is the 1-oxygen at the ε-carbon atom of glutamate residue 107.

kindly prepared by Dr. Graeme Wistow using the Evans & Sutherland Picture System II at the Department of Crystallography, Birkbeck College, University of London, London, U.K., by using the algorithm FRODO (Jones, 1978; modified and extended by A. Jones & I. J. Tickel, unpublished work) operating on the co-ordinates distributed by The Protein Data Bank (Bernstein *et al.*, 1977: magnetic tape version of July 1981) but originally collected by Professor M. Rossmann's group in Purdue University, W. Lafayette, IN, U.S.A.

Results and discussion

When NADH is added at pH7.2 to lactate dehydrogenase nitrated at the Tyr-237 residue there is a decrease in the absorbance at 428 nm (difference molar absorbance coefficient $300\,M^{-1}\cdot cm^{-1}$). When oxamate is added to convert this binary complex into a ternary complex there is a further absorbance decrease (an additional $300\,M^{-1}\cdot cm^{-1}$). The difference spectra show no wavelength shifts and appear to reflect the proportion of the nitrotyrosine residue that exists as nitrotyrosinate (Jeckel, 1976). The inhibitor oxamate is an isoelectronic and isosteric analogue of the substrate pyruvate. In a similar experiment we observed that the absorbance at 428 nm of a 42 μM enzyme solution at pH6.4 decreased 0.03 in a titration in which the enzyme was converted into E–NAD^+–oxalate complex by the addition of 87 μM-NAD^+ and 120 μM-oxalate.

Differences in acidic dissociation constant were directly observed by varying the pH of a solution of the nitro-enzyme or its complexes and recording the change in absorbance at 428 nm (Fig. 2). Under these conditions the dissociation constants of the nitro-enzyme for NADH and for oxamate (see below) predict that the enzyme remains saturated at all pH values and the pH for half-maximum absorbance change will correspond to the p*K* of the nitrotyrosine residue. Indeed the same p*K* was observed when the oxamate concentration in the

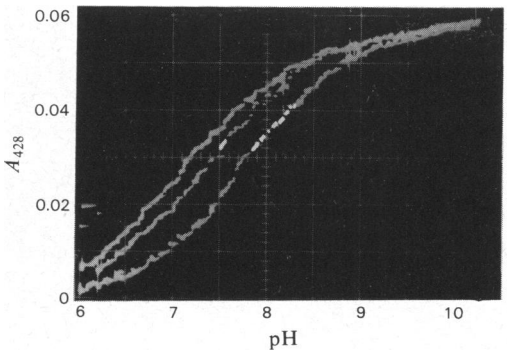

Fig. 2. *pK_a of the phenolic hydroxy group in the Tyr(3NO_2)-237 residue of pig H_4 lactate dehydrogenase*
The three curves are from left to right 22 μM enzyme sites with no addition, with 222 μM-NADH and with 222 μM-NADH plus 10 mM-oxamate. The support medium was 5 mM-sodium phosphate/20 mM-NaCl buffer initially at pH 6. The pH was increased by adding 2 M-NaOH to the stirred solution. The ordinate shows the increase in absorbance at 428 nm. The pK_a values are 7.2 (apoenzyme), 7.4 (binary complex) and 7.8 (ternary complex).

cuvette was 2 mM or 10 mM. The pK_a of the apoenzyme (7.2) is very close to the pK_a of the same residue in its tryptic-digest peptide (7.1 ± 0.1), and suggests that the environment of this residue in the apoprotein is largely aqueous (in agreement with the n.m.r. results obtained by Parker *et al.*, 1981). The increase in pK_a from 7.2 to 7.8 on going from apoenzyme to ternary complex is as would be expected from the closure of the loop bringing the acidic Glu-107 residue close to the Tyr-237 residue. The increase in pK_a of the phenolic hydroxy group when close to a stronger acid was experimentally demonstrated with model compounds: the pK_a of the phenolic hydroxy group in 3-nitrotyrosine is about 7.0, whereas in Boc-Glu-Tyr(3NO_2) the pK_a is 7.5. Comparison of the crystal structure of the dogfish apo-(lactate dehydrogenase) and that of the pig enzyme–*S*-Lac–NAD^+ ternary complex shows that the Glu-107 residue (on the loop) is about 4 nm closer in the ternary complex than in the apoenzyme.

$K_{E,NADH}$ was 1.8 μM at pH 6.5 and 3.6 μM at pH 8.5. The decrease in affinity with pH of 2-fold is similar to the 2.5-fold decrease observed with the native enzyme, although the absolute affinities are about 3-fold weaker (Lodola *et al.*, 1978). From an experiment with the nitro-enzyme similar to that of Fig. 10 in Lodola *et al.* (1978) (where the protein fluorescence of solutions of enzyme and NADH are measured as the pH is increased from 5.9 to 10.5), the pK_a of the His-195 residue in the E–NADH complex was observed to be 7, similar to that with the native enzyme. As with the native enzyme, the affinity of the nitro-enzyme for NADH decreased rapidly with an alkaline pK of 9.7, i.e. well above the pK of the nitrotyrosine-to-nitrotyrosinate dissociation.

$K_{E-NADH,oxamate}$ was 6 μM at pH 6.5, 30 μM at pH 7.2 and 1.7 mM at pH 8.5. The very great decrease in affinity above the pK of the His-195 residue is similar to that with the native enzyme. The change in $K_{E-NADH,oxamate}$ with temperature was used to construct a van't Hoff plot, and this suggested that it might be possible to use relaxation of equilibria perturbed by a jump in temperature to determine the rapid rate of change in the environment of the Tyr(3NO_2)-237 residue when oxamate binds to the E–NADH complex.

Fig. 3 shows the change in absorbance at 428 nm after the equilibrium

$$\text{E–NADH} + \text{oxamate} \rightleftharpoons \text{E–NADH–oxamate}$$

was perturbed by a rapid jump in temperature from 19°C. The conditions of the experiment are such that the enzyme is saturated with NADH, and the observed increase in absorbance (decrease in percentage transmission) is due to the above equilibrium shifting to a new position further to the left. Most of the curve is described by a single exponential with a relaxation time of 1860 s^{-1}. Fig. 3 relates the relaxation time to $[\text{E–NADH}]_f + [\text{oxamate}]_f$. The concentrations of free E–NADH complex and oxamate were calculated from $K_{E-NADH,oxamate}$

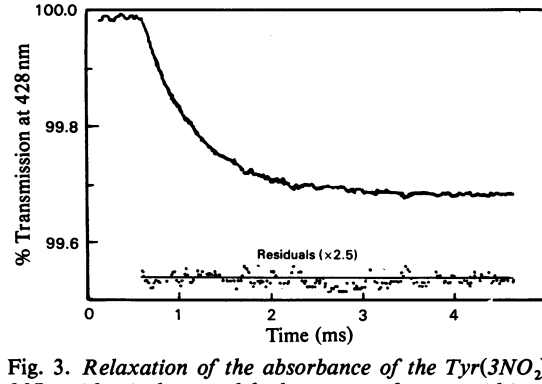

Fig. 3. *Relaxation of the absorbance of the Tyr(3NO_2)-237 residue in lactate dehydrogenase after a rapid jump in temperature*
The solution contained 60 μM enzyme, 0.3 mM-NADH and 0.1 mM-oxamate, initially at 19°C. The curve is the average of 11 transients observed after a rapid jump in temperature of about 4°C. The residuals (on a scale 2.5 × the original) are those after subtracting an exponential of rate constant 1860 s^{-1}.

(30 μM). The relaxation time does not increase linearly with concentration of free reactants, as would be expected if the binding of oxamate were a simple bimolecular collision process or if a slow bimolecular step precedes a unimolecular step. A model in which a rapidly formed bimolecular complex undergoes a subsequent unimolecular rearrangement (giving rise to the pK_a change):

$$\text{E–NADH} + \text{oxamate} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{E–NADH–oxamate} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{E*–NADH–oxamate}$$

was used to describe the results and predicts (Czerlinski, 1966) the slower of two relaxation times to be:

$$1/\tau = \frac{k_{+2}}{1 + k_{-1}/(k_{+1}\{[\text{E–NADH}]_f + [\text{oxamate}]_f\})} + k_{-2}$$

In some experiments the faster relaxation was observed (in the range 4000–20000 s^{-1}), but was too close to the electronic disturbance caused by the high-voltage heating transient, and to the time constant, to be evaluated reliably. The fit of the experimental points to the model was obtained with the restriction that the overall equilibrium constant was the experimental value of 30 μM and was $k_{-1}/k_{+1} = 0.156$ mM, $k_{-2} = 580\,s^{-1}$ and $k_{+2} = 3020\,s^{-1}$.

Our results are not the same as those reported by Heck (1969). In a temperature-jump study of the binding of oxamate, but monitored by the decrease in the fluorescence of NADH on going from the binary to the ternary complex in 0.3 M-NaCl, Heck (1969) concluded that the binding was bimolecular. An explanation of the difference might be that the nitrotyrosine residue was specifically designed to monitor polypeptide loop closure, whereas Heck (1969) monitored a change in the electronic structure of bound NADH. Alternatively, it might be noted that Heck (1969) reported that there was only a small decrease in the affinity of the E–NADH complex for oxamate between pH6 and pH8, whereas others (Winer & Schwert, 1959; Holbrook & Stinson, 1973), with the same system, observe a 10-fold decrease over that same pH range. The abscissae of plots such as Fig. 4 require an accurate value of $K_{\text{E–NADH,oxamate}}$ for their calculation. Equally, Heck (1969) did not make measurements at rates above 1000 s^{-1}: the approach to rate saturation in Fig. 4 is not visible at such low rates.

Trommer & Gloeggler (1979) have observed, by using saturation transfer e.s.r., that the rotational correlation time of a spin label on the adenine 6-N atom in NAD^+ and NADH in inhibitory ternary complexes with pig H_4 lactate dehydrogenase varied between 10^{-5} and 10^{-6} s. This variation was interpreted to reflect a much faster equilibrium between open and closed loop positions, which varied from largely open with NADH to largely closed with E–NAD^+–oxalate complex. Stopped-flow experiments (Shore *et al.*, 1975) show that loop closure is faster than $10^3\,s^{-1}$, whereas the n.m.r. experiments performed by Parker *et al.* (1981) show that the loop is not in rapid motion on an ^1H n.m.r. time scale. These and our present results all indicate motion around the active centre with rates 10^6 to $5 \times 10^2\,s^{-1}$. It is possible that the various regions probed by the different techniques sense motions of separate segments of the loop residues 98–110.

The environment around the Tyr-237 residue becomes more acid under conditions that are predicted to stabilize inhibitor complexes in which the loop (residues 98–110) is close to the enzyme surface. Does that residue experience the same environment in the active ternary complex of the enzyme? The steady-state complex of the native enzyme in the reverse direction is a ternary complex E–NADH–pyruvate, which slowly isomerizes to the active ternary complex before being rapidly con-

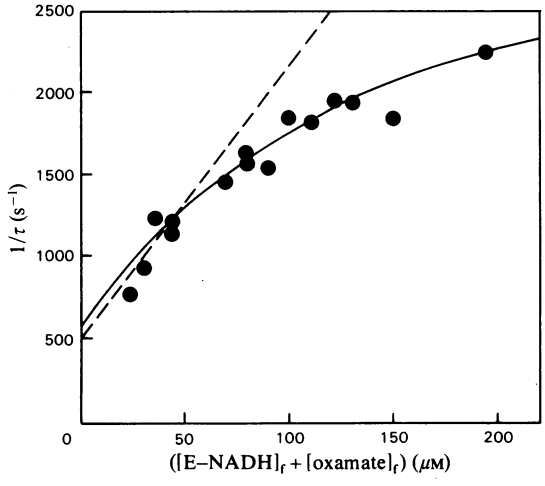

Fig. 4. *Variation in the slow relaxation time at 428 nm with the free concentration of reactants*

The points are from experiments similar to that shown in Fig. 3. The continuous line is drawn with $k_{-1}/k_{+1} = 0.156$ mM, $k_{-2} = 580\,s^{-1}$ and $k_{+2} = 3020\,s^{-1}$. The broken line is calculated for bimolecular binding with $K_{eq.} = 30\,\mu$M (the experimentally measured value) and $k_{-1} = 500\,s^{-1}$.

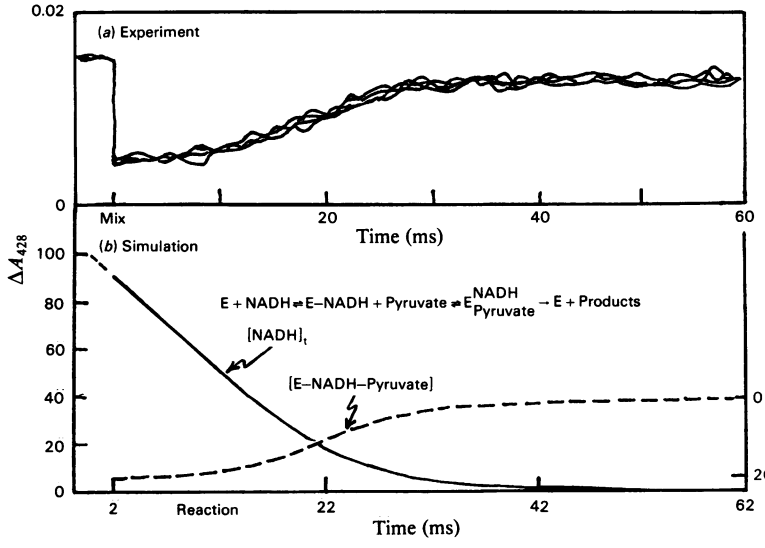

Fig. 5. *Absorbance of the Tyr(3NO$_2$)-237 residue in lactate dehydrogenase in its steady-state complex with NADH and pyruvate*

(*a*) Nitro-enzyme (66 μM sites) and NADH (0.2 mM) were rapidly mixed in a stopped-flow apparatus with an equal volume of 0.6 mM-sodium pyruvate at 20°C in 20 mM-sodium phosphate buffer, pH 7.0. The absorbance at 428 nm was monitored with a time constant of 0.6 ms. The curves from four identical experiments are superimposed. (*b*) Simulation of the decrease in [NADH]$_t$ and of [E–NADH–pyruvate] by using eqn. (1) as a model, $K_{E-NADH} = 3\,\mu M$, $K_{E-NADH,pyruvate} = 170\,\mu M$ and $k = 200\,s^{-1}$ with the total concentrations of enzyme, NADH and pyruvate of (*a*) obtained by using the program FACSIMILE (Chance *et al.*, 1977) as described by Hollaway *et al.* (1980).

verted into NAD^+ and lactate (since these present experiments give no information about the His-195 residue the state of protonation of that group is not specified):

$$E^{NADH} + \text{Pyruvate} \overset{\text{Fast}}{\rightleftharpoons} E^{NADH}_{Pyruvate} \overset{\text{Slow}}{\rightleftharpoons} E^{*NADH}_{Pyruvate} \overset{\text{Fast}}{\rightleftharpoons} \text{Products} \qquad (1)$$

Thus, when the enzyme (final concentration 33 μM) and excess NADH (final concentration 100 μM) are rapidly mixed with pyruvate (final concentration 0.3 mM), there is a zero-order rate of oxidation of NADH (rate = 4 $\mu M \cdot ms^{-1}$) until all unbound NADH is used up, and then a first-order decrease in [NADH] to zero. The excess 67 μM-NADH is used up in 67/4 = 17 ms, during which time the enzyme is present as its steady-state complex (a detailed simulation is shown in Fig. 5*b*). When that experiment is monitored by absorbance at 428 nm with the nitro-enzyme (Fig. 5*a*) we observe a decrease in A_{428}, within the mixing time of the apparatus, due to the formation of the steady-state complex, a lag time of about 15 ms while the enzyme is in a low-absorbance steady-state complex and then an increase in absorbance as the steady state decays (in this case to free enzyme). We thus conclude that the environment around the Tyr(3NO$_2$)-237 residue in the steady-state complex is similar to that in the E–NADH–oxamate compound.

The experiments reported in the present paper establish that after oxamate binds to the E–NADH complex the Tyr-237 residue undergoes a slow rearrangement in which its pK is raised by about 0.5. Although designed in the expectation that this increase in pK would occur as a loop of polypeptide chain bearing the Glu-107 residue approached the tyrosine residue (the loop closing at 3020 s^{-1} and opening at 580 s^{-1}), the observations are consistent with any slow change in structure in which the distance between the tyrosine residue and nearby acidic residues is decreased (e.g. the Asp-197 or Glu-238 residue; we thank Professor M. Rossmann for describing the environment of the Tyr-237 residue to us). The rates (580 s^{-1} and 3020 s^{-1}) are far too slow to represent proton-binding equilibria alone.

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