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Malate Dehydrogenase of the Cytosol

IONIZATIONS OF THE ENZYME–REDUCED-COENZYME COMPLEX AND A COMPARISON WITH LACTATE DEHYDROGENASE

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1. The pH-dependencies of the binding of NADH and reduced nicotinamide–benzimidazole dinucleotide to pig heart cytoplasmic malate dehydrogenase and lactate dehydrogenase are reported. 2. Two ionizing groups were observed in the binding of both reduced coenzymes to lactate dehydrogenase. One group, with pK_a in the range 6.3–6.7, is the active-site histidine residue and its deprotonation weakens binding of reduced coenzyme 3-fold. Binding of both coenzymes is decreased to zero when a second group, of pK_a 8.9, deprotonates. This group is not cysteine-165. 3. Only one ionization is required to characterize the binding of the two reduced coenzymes to malate dehydrogenase. The group involved appears to be the active-site histidine residue, since its ethoxycarbonylation inhibits the enzyme and abolishes binding of reduced coenzyme. Binding of either reduced coenzyme increases the pK_a of the group from 6.4 to 7.4, and deprotonation of the group is accompanied by a 10-fold weakening of coenzyme binding. 4. Two reactive histidine residues were detected per malate dehydrogenase dimer. 5. A mechanism which emphasizes the homology between the two enzymes is presented.

In seeking to understand the catalytic mechanism of lactate dehydrogenase it has been helpful to use the simplification that the protonation state of the active-site histidine residue does not change when coenzymes bind to the apoprotein (Holbrook & Stinson, 1973; Parker & Holbrook, 1977). This conscious simplification ignores possible changes of 0.5pK unit in the ionization of the active-site histidine residue, in comparison with the perturbation by 5pK units in the histidine ionization, which has been detected when the ternary complex forms (Holbrook & Ingram, 1973; Holbrook & Stinson, 1973). Although helpful for lactate dehydrogenase, the simplification could not apply to horse liver and yeast alcohol dehydrogenases (Dalziel, 1963; Dickenson & Dickinson, 1977). In the present paper we set out to examine the ionizations that are expressed in the formation of the complex between cytoplasmic malate dehydrogenase and NADH, on the one hand, because this enzyme, like lactate dehydrogenase, contains an essential histidine residue, and on the other hand, because the two dehydrogenases are reported to have superimposable three-dimensional structures (in both the catalytic domain and the nucleotide-binding fold) (Hill *et al.*, 1972; Rao & Rossman, 1973; Holbrook *et al.*, 1974). Since we have now developed a very sensitive method for the detection of ionizations in some enzymes, we report not only that the ionization of the essential histidine residue of MDH is partially changed on interaction with NADH but also that there is an analogous, but small, change in the ionization of histidine-195 when NADH binds to lactate dehydrogenase [the change was too small for us to detect previously (Stinson & Holbrook, 1973), but should be observed if, as we suppose, the two dehydrogenases have homologous structures and mechanisms]. In seeking structural explanations for the ionizations that we observe, it has been useful to compare the binding of NADH to the binding of reduced nicotinamide–benzimidazole dinucleotide, an NADH analogue which lacks the ability to form hydrogen bonds to the pyrimidine portion of the 'adenine' ring.

Abbreviations used: LDH, lactate dehydrogenase (L-lactate–NAD^+ oxidoreductase, EC 1.1.1.27); MDH, malate dehydrogenase (malate–NAD^+ oxidoreductase, EC 1.1.1.34).

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Materials and Methods

NADH was purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, German Federal Republic and was purified as described by Holbrook & Wolfe (1972). Nicotinamide–benzimidazole dinucleotide was synthesized as described by Woenckhaus (1964) and was reduced by incubating for 30min a solution of the analogue (30mM) in ethanol (0.5M), yeast alcohol dehydrogenase (2mg/ml), semicarbazide hydrochloride (50mM) and glycine (0.5M) adjusted to pH9.3 with 10M-NaOH. It was purified by

filtering 1.5ml of the incubation mixture through a column (1.5cm×150cm) of Sephadex G-10 equilibrated with 50mM-glycine adjusted to pH9.3 with 10M-NaOH. The purified reduced coenzymes were usually used on the day of their preparation, but in one case a sample was stored frozen for 2 days without detectable decomposition.

Pig heart cytoplasmic MDH was prepared by method 2 of Lodola *et al.* (1978) and was determined and assayed as described by those authors. Pig heart LDH was prepared as usual in the laboratory and assayed as described by Stinson & Holbrook (1973). Before the experiments the crystalline suspension of the enzyme in $(NH_4)_2SO_4$ was centrifuged in a bench centrifuge and the crystals were redissolved in and dialysed for 24h against 1000vol. of 10mM-NaH_2PO_4/10mM-NaCl at pH7. For fluorescence measurements at very low protein concentrations the solutions were also filtered through a Millipore disc (10μM pore size). Glass-distilled water which had not been in contact with plastic tubing was used. Diethyl pyrocarbonate was obtained and assayed as noted in Holbrook & Ingram (1973).

pH-dependence of NADH binding to MDH and LDH

In a preliminary experiment the binding of NADH to MDH was followed by measuring the difference in NADH fluorescence when the coenzyme was simultaneously added to enzyme solution (3ml) and the same volume of buffer. The dissociation constant was obtained from a plot of $1/(1-\bar{v})$ against $[NADH]/\bar{v}$ exactly as described by Lodola *et al.* (1978). By repeating the determination in a series of buffers (see the legend to Fig. 4) of constant ionic strength ($I=0.1$) K_d was obtained as a function of pH. A comparable result for LDH has been published (Stinson & Holbrook, 1973).

A continuous record of the degree of saturation ($\bar{v}$) of the coenzyme-binding sites of an enzyme as a function of pH will be a far more sensitive method than that described above of detecting whether coenzyme binding responds to ionizations of the protein, coenzyme, or both. The apparatus described in Fig. 1 was used to record the fluorescence of the two dehydrogenases as a continuous function of pH. When NADH is bound to these dehydrogenases the intrinsic fluorescence of the proteins is quenched. The degree to which the fluorescence is quenched at each pH can be directly observed from records, such as that shown in Fig. 5. For MDH, the fraction of maximum quench may be directly equated to $\bar{v}$ (Lodola *et al.*, 1978). For LDH, $\bar{v}$ was obtained by using the transformation $\bar{v}=(1-F^{\frac{1}{2}})/0.4$ made by a simple analogue circuit operating on the fluorescence of the test solution relative to that of the enzyme blank (F) (Holbrook, 1972). When appreciable concentrations of reduced coenzyme are added to a protein solution the intrinsic fluorescence is also decreased (*a*) by the NADH decreasing the intensity of the exciting radiation and (*b*) by the NADH decreasing the intensity of the emitted radiation in a trivial process normally designated the 'inner-filter effect'. The magnitude of the inner-filter effect was determined by titrating a solution of bovine serum albumin with NADH (or the reduced benzimidazole analogue) and, again using an analogue circuit, estimating the magnitude of the molar absorption coefficient ε to be used in the equation $1=F_{measured}\cdot 10\exp(0.5\varepsilon c)$ as c (the nucleotide concentration) was varied from 0 to 70μM. Although the value of the absorption coefficient that we determined will be slightly affected by the geometry of the optics of our fluorimeter, it was gratifying that the values determined (10900 for NADH and protein fluorescence excited at 297nm; 8000 for reduced nicotinamide–benzimidazole dinucleotide and protein fluorescence excited at 292nm) were those expected from the sum of the absorption coefficients of the reduced coenzyme at the excitation and emission wavelengths and the effective path-length of the fluorescence cell of 0.5cm. An example of the effectiveness of correction made for inner-filter effects by this method is shown in Lodola *et al.* (1978). The absolute magnitude of corrected fluorescence signals does become increasingly uncertain at coenzyme concentrations over 5μM (when $10\exp(0.5\varepsilon c)>1.07$), but fortunately it is the relative, not the absolute, variation of these signals with pH that contains information on the ionizing groups of the system.

Number of essential histidine residues in MDH

The formation of ethoxycarbonylhistidine during the reaction at 25°C of 37μM-MDH (mol.wt. 70000) with 87μM-diethyl pyrocarbonate in 20mM-sodium phosphate buffer, pH6.5, was continuously monitored from the ΔA_{250} recorded in a spectrophotometer. The concentration of acylimidazole was calculated by using $\varepsilon=1.9$litre·mmol^{-1}·cm^{-1} at 250nm (Holbrook & Ingram, 1973). At timed intervals samples of the incubation mixture were removed and immediately assayed for enzyme activity (malate oxidation).

Effect of ethoxycarbonylhistidine formation on the ability of MDH to bind NADH

MDH (55μM) was incubated with various concentrations of diethyl pyrocarbonate and after various timed intervals one sample of the incubation mixture was diluted 1:100 in ice-cold 20mM-sodium phosphate buffer, pH6, and then a 10μl sample was assayed, whereas a second sample of the incubation mixture was diluted 1:30 into 20mM-sodium phos-

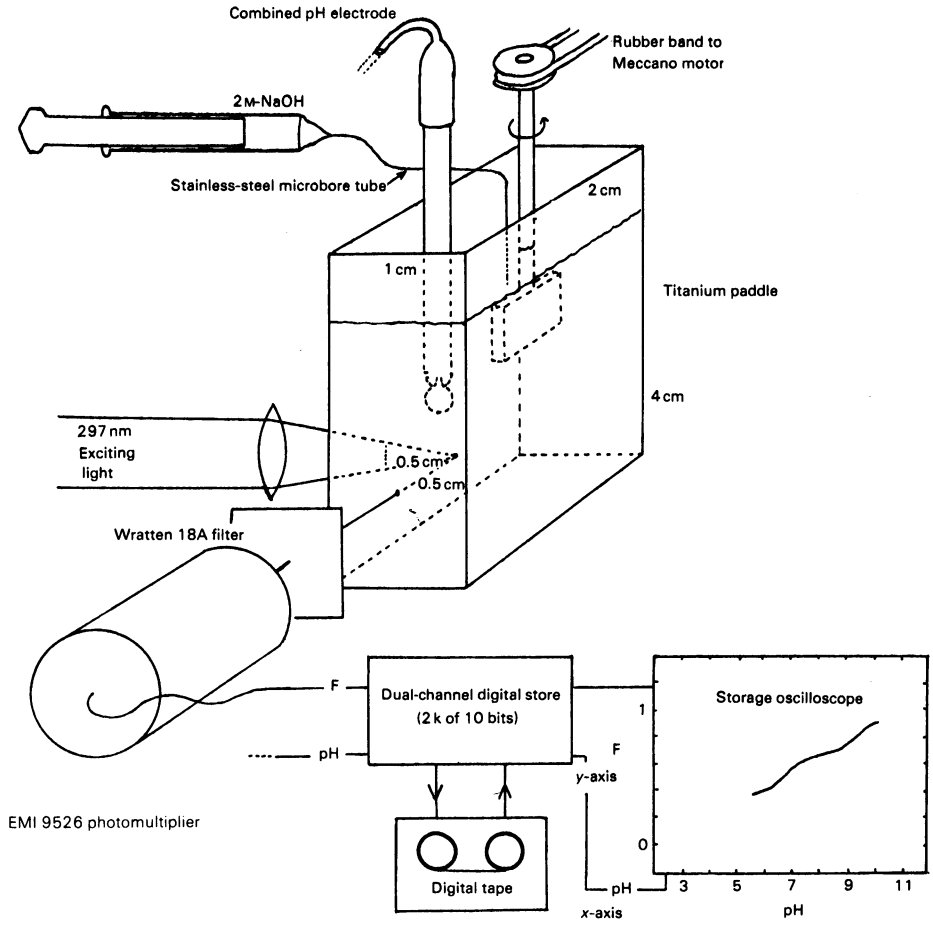


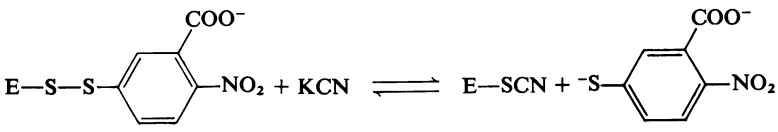
Fig. 1. *Apparatus used to record the pH-dependence of the intrinsic fluorescence of proteins*

A solution (6ml) of protein (0.1 μM-NADH-binding sites) in 5mM-NaH_2PO_4 and 10mM-NaCl was placed in a 2cm×1cm×4cm quartz fluorescence cuvette equipped with an overhead titanium stirring paddle (driven from a Meccano motor 6V/1000 with a reduction gear box on speed setting 3:1 via an elastic band), combined calomel/glass pH electrode (Pye–Unicam type 401, E7, M5) and a fine stainless-steel tube connected to a motor-driven syringe through which 2M-NaOH could be added at rates between 0.1 and 100 μl/min. The protein fluorescence was excited at 297nm over an effective path-length of 0.5cm with light from an Osram XBO 250 xenon arc and was measured as light transmitted through a Kodak–Wratten no. 18A filter over an effective path-length of 0.5cm by an E.M.I. no 9256 photomultiplier. The pH was caused to rise from 5.7 to 10.7 over 200s by addition of 2M-NaOH and the measurements were digitized every 0.2s and stored in a dual-channel recycling store (Transidyne General, 2k of 10-bit bytes). After each experiment the results were archived on magnetic tape in records of 0.5k 8-bit bytes and it was then possible to recall the data and superimpose the results of any number of experiments by using an oscilloscope in the *x*–*y* mode. Photographs of the results are shown in this paper.

phate buffer, pH6, and then rapidly titrated with NADH as described by Lodola *et al.* (1978). The concentration of NADH-binding sites in the solution was calculated from the trace of the nucleotide fluorescence (excitation at 360nm, emission via a Kodak Wratten filter 98).

S-Cyano-LDH

A solution of the thionitrobenzoyl derivative of cysteine-165 of pig heart lactate dehydrogenase in 0.1M-glycine/70mM-Na_2HPO_4 adjusted to pH9 with 10M-NaOH was prepared as described by Holbrook & Jeckel (1967) (2.5mg of protein/ml) and was made



0.1 M in KCN by addition of the solid reagent at 25°C (Jeckel, 1976). After incubation for 10min, the solution was passed through a column of Sephadex G-50 to separate the colourless *S*-cyano-LDH from the yellow thionitrobenzoate and excess KCN. The enzyme activity in the standard assay of the *S*-cyano-LDH was 40% of that of an untreated control (E is the enzyme). We estimate that all the thionitrobenzoyl groups had been removed from the enzyme with KCN, since the protein fluorescence of the *S*-cyano-LDH was identical in magnitude with that of LDH.

Results and Discussion

Number of reactive histidine residues in MDH

In an earlier paper (Holbrook *et al.*, 1974) we reported that there was only one histidine residue in MDH that was reactive towards diethyl pyrocarbonate. Since that time we have redetermined the absorption coefficient of the enzyme at 280nm (Lodola *et al.*, 1978). Using this new value we have re-measured the relationship between the enzyme activity of the cytoplasmic enzyme and the number of histidine residues that react with diethyl pyrocarbonate (Fig. 2). These new results, which after taking into account the changed absorption coefficient, are very similar to those we previously (Holbrook *et al.*, 1974) presented, and indicate that inhibition of 37 μM-enzyme (mol.wt. 70000) results from the formation of 65 μM-ethoxycarbonylhistidine, i.e. two histidine residues for the MDH dimer. This should remove some of the apparent need to speculate that MDH is a 'half-of-the-sites' enzyme.

We have previously shown that the ethoxycarbonyl-enzyme cannot form a ternary complex with NADH and hydroxymalonate (Holbrook *et al.*, 1974). By analogy with LDH, we had assumed, but not checked, that this enzyme with blocked essential histidine would easily form a complex with NADH alone. As shown in Fig. 3 this is not so: as the enzyme is progressively inhibited with the histidine-specific reagent, the ability to form a binary complex with NADH is decreased to a value indistinguishable from zero. This result parallels that obtained by Dickenson & Dickinson (1977) with yeast alcohol dehydrogenase. We interpret the result to indicate that the 'loop' of polypeptide chain in the MDH binary complex has moved to a different position from the loop of polypeptide chain in the LDH

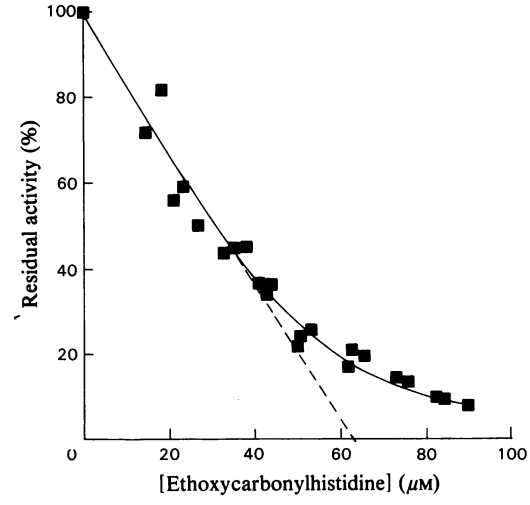


Fig. 2. *Number of rapidly reacting histidine residues in cytoplasmic MDH*

MDH (37 μM; mol.wt. 70000) was allowed to react with 87 μM-diethyl pyrocarbonate in 0.02 M-phosphate buffer, pH 6.5 at 21°C. The concentration of ethoxycarbonylhistidine formed was calculated from the change in A_{250}. At timed intervals samples were removed and assayed for enzyme activity.

binary complex (this can exist in the apoenzyme conformation, i.e. extending out into the solvent). This point has been emphasized by Weininger *et al.* (1977), although it should be mentioned that it is by no means clear whether the MDH–NAD^+–SO_4^{2-} complex that they crystallized is to be taken as an analogue of the binary or the ternary complex of the enzyme. The suggestion that there is a much larger rearrangement when NADH binds to MDH than when it binds to LDH agrees with results from other experimental approaches (Cassman, 1967) and would certainly agree with the demonstration below that there is a tighter coupling between NADH-binding and the essential histidine pK in MDH than in LDH.

pH-dependence of reduced-cofactor binding to MDH

Discrete measurements of the dissociation constant for NADH binding to MDH showed that the affinity of the enzyme for the coenzyme is decreased with

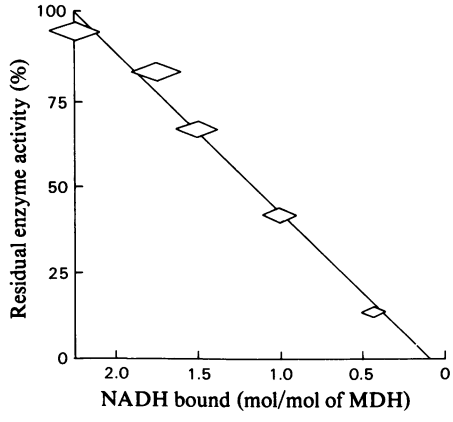


Fig. 3. *Decrease in the NADH-binding capacity of MDH caused by inhibition after reaction of the essential histidines with diethyl pyrocarbonate*

The NADH-binding capacity of samples of MDH was measured from the enhancement of NADH fluorescence in samples of the enzyme which had been treated with diethyl pyrocarbonate and had the residual enzyme activity shown. Although the concentration of NADH-binding sites decreased with increasing inhibition, there was no change in the dissociation constant of the residual sites. The shape of the symbols reflect the 10% uncertainty (horizontal) in the residual-site values and the 5% uncertainty (vertical) in the enzyme-activity determination.

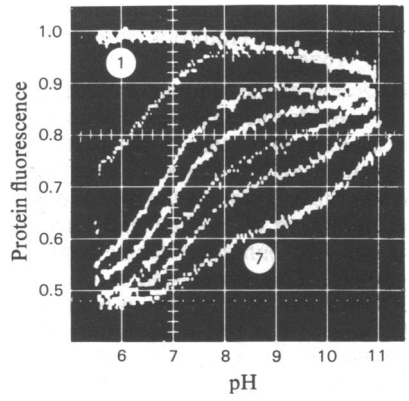


Fig. 5. *Variation in the protein fluorescence of MDH with pH*

The pH of a stirred solution of cytoplasmic MDH (0.05 μM, mol.wt. 70000) in 5mM-NaH_2PO_4 and 10mM-NaCl was steadily increased from 5.5 to 11 by the addition of 2M-NaOH from a syringe driven by a variable-speed motor. The pH of the solution monitored with a glass combined electrode and the protein fluorescence (297nm excitation, Wratten 18A emission) were continuously measured and stored. From the top (curve 1) to the bottom (curve 7) the solutions contained 0, 0.1, 0.4, 0.8, 1.6, 3.2 and 10 μM-purified NADH. The photographed results had been corrected for the inner filter effects caused by the added NADH. Fluorescence in this and other Figures is referred to the fluorescence of the same concentration of apoenzyme set = 1.0 at pH7.

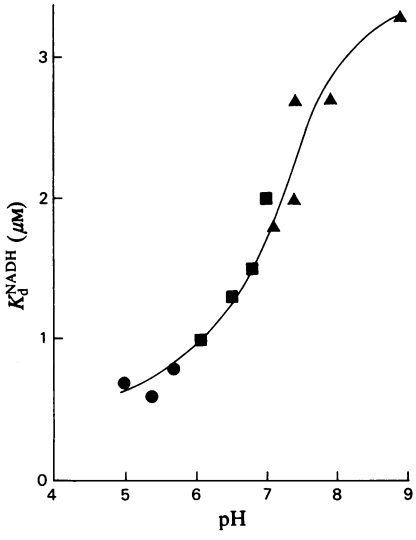


Fig. 4. *Variation in the dissociation constant of NADH from MDH with pH*

The results are from nucleotide fluorescence equilibrium titrations of 0.125 μM-MDH with NADH in a series of buffers of $I = 0.1$. ●, Sodium acetate; ■, potassium phosphate; ▲, Tris/HCl.

increasing pH and that the process appears to depend on an ionization with pK between 6 and 7 (Fig. 4). The nature of the ionization is the more easily discerned from the continuous traces of protein fluorescence (F) as a function of pH shown in Fig. 5. Between pH5 and 9 the traces resemble titration curves and at each NADH concentration they can be characterized by values of $pH_{\frac{1}{2}}$, i.e. pH values at which dF/d(pH) goes through a maximum. These maxima were either estimated visually or by displaying electrically the first derivative of the curve. We observed that with increasing concentrations of NADH the amplitude of the protein fluorescence change between pH5 and 9 is decreased (corrections for inner-filter effects by added NADH are already made) and we also observed that $pH_{\frac{1}{2}}$ increased from 6.4 and tended to an asymptote to $pH_{\frac{1}{2}} = 7.4$ (Fig. 6). These results can be qualitatively and quantitatively explained by the mechanism shown in Scheme 1. An obvious candidate for the protein group whose pK is shifted from 6.4 to 7.4 on binding NADH is the active-centre histidine. This suggestion is made because modification of that residue blocks NADH binding and because, by using NAD–SO_3^- as a

probe, the pK of that residue is shown to be 6.3 (D. M. Parker & J. J. Holbrook, unpublished work; Shore *et al.*, 1975).

A comparison of the binding to MDH of NADH and the benzimidazole analogue is shown in Fig. 7. In both cases the ionization in the pH6–7 region is readily apparent and we therefore conclude that the group responsible is not in the pyrimidine portion of the adenine ring of NADH and that the group is not in the hydrophobic adenine-binding pocket of the protein. This conclusion is of course fully consistent with the ionizing group being the active-centre histidine, since this residue must be close to (although not in direct contact with) the dihydronicotinamide ring and distant from the adenine ring. We have also obtained results analogous to those shown in Figs. 5 and 6 for reduced nicotinamide–benzimidazole dinucleotide. These again show that the histidine pK in the apoenzyme is 6.3 and that reduced benzimidazole analogue perturbs that pK to 7.3. The limiting dissociation constants for NADH from the protonated and unprotonated enzyme are 0.3 μM and 3 μM respectively.

A close examination of the curve at 10 μM-NADH in Fig. 7 reveals that NADH bound to MDH continues to decrease above pH10 in a manner which is not accounted for by Scheme 1 and which suggests that there may be a second ionizing group with p$K = 10\frac{1}{2}$. We have not attempted to characterize the possible group because pH11 is close to the limit that we can study and because the magnitude of the change in NADH bound suggests that the possible group will be remote from the cofactor-binding site. The form of the curve for reduced nicotinamide–benzimidazole dinucleotide is markedly different from that for NADH above pH9, and this suggests that the putative group with p$K10\frac{1}{2}$ may be closer to the adenine than to the nicotinamide end of the cofactor.

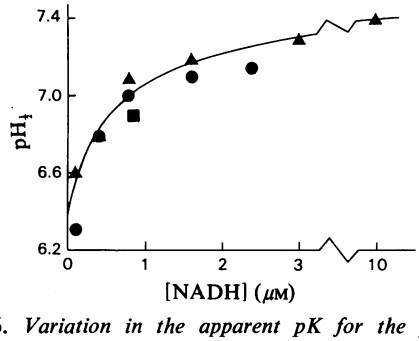
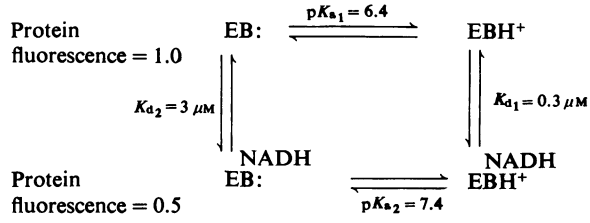


Fig. 6. *Variation in the apparent pK for the protein fluorescence transition of MDH with NADH*
The values of $pH_{\frac{1}{2}}$ taken from results similar to Fig. 5 are plotted against the [NADH] in the system. The different symbols are from different series of experiments. Note the change of scale on the x-axis.

pH-dependence of reduced-coenzyme binding to LDH

The pH-dependences of the binding of NADH and the benzimidazole analogue to LDH are so similar that it was necessary to displace one curve artificially in order to display the results (Fig. 8). The benzimidazole analogue binds to the enzyme with about one-third of the affinity of NADH at all pH values. As with NADH (Holbrook, 1972) the relationship between the fluorescence of the LDH–reduced benzimidazole analogue complex (F) and the fraction of the nucleotide-binding sites occupied ($\bar{v}$) is non-linear (Fig. 9). $\bar{v}$ may, however, be obtained from F by using the transformation $\bar{v} = (1-F^{\frac{1}{2}})/0.3$. The untransformed non-linear titration curves (Fig. 8) show that two ionizations are needed to explain the pH-dependences of the binding of NADH and the benzimidazole analogue to LDH, one ionization between pH6 and 7, the second between pH9 and 10. In our own previous discrete determinations of K_{NADH} as a function of pH we (and others) had only



Scheme 1. *Mechanism to explain the pH-dependences of NADH binding to MDH*
The titrations of Fig. 5 with [NADH] < K_{d1} have $pH_{\frac{1}{2}}$ just greater than pK_{a1} (Fig. 6). The titrations of Fig. 5 with [NADH] > K_{d2} have values of $pH_{\frac{1}{2}}$ which asymptotically approach pK_{a2} (Fig. 6). K_{d2} is taken as the average of eight experiments with $\bar{v}$ chosen at pH9 and pH9.5. E is the enzyme and B: is the imidazole residue of the essential histidine of the enzyme.

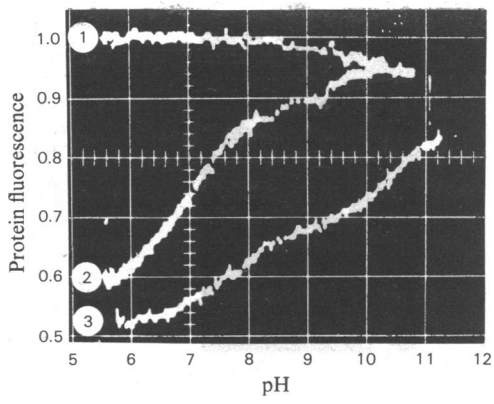


Fig. 7. *Comparison of the pH-dependences of the binding of NADH and benzimidazole analogue to MDH*
The experiments were similar to those in Fig. 5. Curve 1 is a 0.05 μM-MDH blank. Curve 2 is with 9.3 μM-reduced benzimidazole analogue added and curve 3 is with 10 μM-NADH.

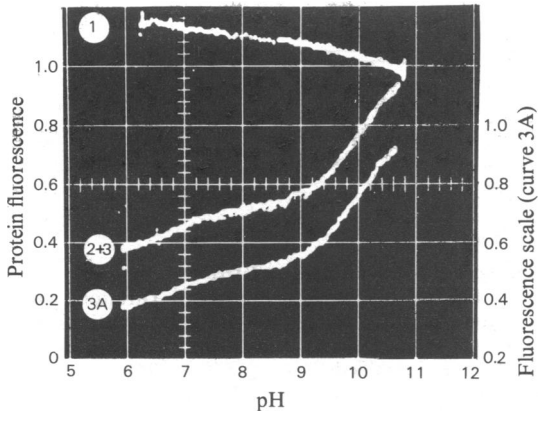


Fig. 8. *Comparisons of the binding of NADH and the benzimidazole analogue to LDH*
The experimental conditions were similar to those of Fig. 5. Curve 1 is with 0.025 μM-LDH alone. Curve 2 was with 5.2 μM-reduced benzimidazole analogue added and curve 3 was with 3 μM-NADH. Curve 3A is curve 3 displaced on the fluorescence axis by −0.2.

observed the second alkaline ionization (Stinson & Holbrook, 1973).

Characterization of the most acidic ionization is more difficult for LDH than for MDH, since LDH is rapidly denatured at pH values less than 5.5 in solutions which contain only traces of NADH at 25°C. In the presence of high concentrations of NADH it is readily observed that the acidic $pH_{\frac{1}{2}}$ reaches an asymptote of 6.7 (Fig. 10*b*). The lowest $pH_{\frac{1}{2}}$ that we

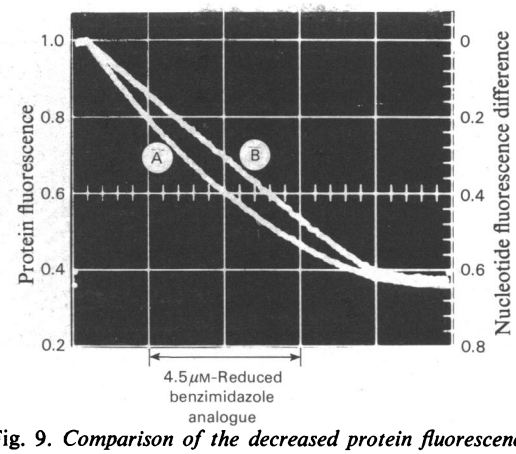


Fig. 9. *Comparison of the decreased protein fluorescence and the decreased NADH fluorescence when LDH is titrated with the benzimidazole analogue in oxamate buffer*
Curve A is the decreased protein fluorescence (excitation at 292nm and emission via Kodak–Wratten filter 18A). Curve B is the difference between the nucleotide fluorescence of the shown concentrations of reduced benzimidazole analogue added to 3ml of 2.5 μM-LDH (mol.wt. 144000) and to 3ml of a buffer solution which contained 20mM-NaH_2PO_4 and 0.1M-oxamate adjusted to pH6.5 with 10M-NaOH.

have observed (not shown by the curves in Fig. 10, which start at pH6) is 6.3, which is consistent with the original observation of Stinson & Holbrook (1973) that the dissociation constant for NADH increases 3-fold between pH5.5 and pH8.5. The corrected results in Fig. 10 show that the dissociation constant in the plateau region between pH8 and 8.5 is on average 1.3 μM and that the dissociation constant at pH6 is 0.59 μM. Thus the most acidic ionizing system expressed in the binding of NADH to LDH is best described with $pK_{a1} = 6.3$, $pK_{a2} = 6.7$, $K_{d1} = 0.5\,\mu M$ and $K_{d2} = 1.3\,\mu M$ (Scheme 2). For the reasons given in detail in Holbrook & Ingram (1973) we ascribe the group with p*K*6.3–6.7 to histidine-195. The smaller perturbation by NADH of the p*K* of the histidine in LDH compared with that in MDH is in agreement with the observation by Holbrook & Ingram (1973) that the ethoxycarbonyl derivative of histidine in LDH binds NADH almost normally, whereas the same derivative of MDH has lost the ability to bind NADH.

The ionization state of histidine-195 of LDH is only slightly altered when NADH binds. In contrast, the curves of Fig. 10 show that deprotonation of a second ionizing group between pH9 and 10 lowers the affinity of the enzyme for NADH (and the benzimidazole analogue, Fig. 8) to a value indistinguishable from zero. The corollary to this observation is that the alkaline $pH_{\frac{1}{2}}$ should increase without limit

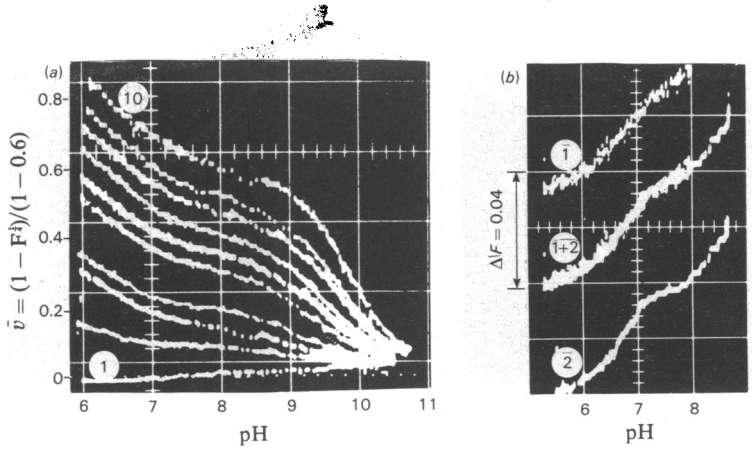
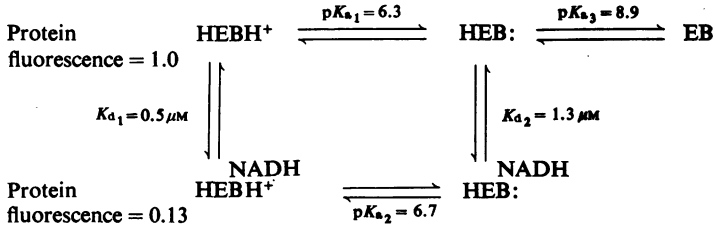


Fig. 10. *Variation with pH of (a) the degree of saturation with NADH ($\bar{v}$) and (b) the protein fluorescence of LDH* The experimental conditions were as for Fig. 5, except that 0.025 μM-LDH was used instead of MDH. In (*a*) $\bar{v}$ was calculated from the protein fluorescence as described by Holbrook (1972). The NADH concentrations in (*a*) were (from the bottom, curve 1, to the top, curve 10) 0, 0.1, 0.15, 0.2, 0.4, 0.6, 0.8, 1.2, 1.8 and 3 μM. In (*b*) the protein fluorescence of a solution of 0.025 μM-LDH and 10 μM-NADH was recorded in two different experiments. The results are shown on the same scale expansion, but vertically displaced to emphasize the original results (curve 1 and curve 2) and the fact that they superimpose (curves 1+2).



Scheme 2. *Mechanism to explain the pH-dependences of NADH binding to LDH*
Titrations with [LDH] < [NADH] < K_{d1} yield titration curves with the acid $pH_{\pm}$ approaching pK_{a1}. Titrations with [LDH] < [NADH] > K_{d2} yield curves with the acid $pH_{\pm}$ tending towards pK_{a2}. At all NADH concentrations the alkaline $pH_{\pm}$ will be greater than pK_{a3}, according to $pH_{\pm} = pK_{a3} + \log\{1 + ([NADH]/K_{d2})\}$. B: is the imidazole moiety of the histidine −195 of LDH. The alkaline ionizing group has not been identified.

according to $pH_{\pm} = pK_{a3} + \log\{1 + ([NADH]/K_{d2})\}$. This relationship is indeed obeyed within the limits to which we can measure $pH_{\pm}$: at low concentrations of NADH, $pH_{\pm} = 9.0$, with [NADH] = 10 μM $\{\log[1 + (10/1.3)] = 0.94\}$ the value of $pH_{\pm}$ is increased to pH 10.2. Kolb & Weber (1975) have emphasized that it is thermodynamically incorrect to write out compulsory-ordered pathways such as that at the right of Scheme 2. Although we have always accepted this theoretical point, we do not think it helpful to include in the scheme the species $EB:^{NADH}$ which has an infinite pK and infinitesimal affinity for NADH. Of course, for $E^{NADH}_{oxalate}$ (Kolb & Weber, 1975) the pathway is by no means as completely ordered as we observe in Scheme 2.

Although we ascribe the acidic ionization to histidine-195 in LDH, we have not been able to identify the group responsible for the alkaline ionization. There are six ionizing groups which are close to NADH bound to pig heart LDH (arginine-171, -109 and -101, tyrosine-85 and -237 and cysteine-165) (Eventoff *et al.*, 1977; Kiltz *et al.*, 1977). We have excluded cysteine-165 because when that residue is converted into its *S*-cyano derivative (Jeckel, 1976) the alkaline ionization is still clearly visible (Fig. 11) (the acid ionization is also visible, with pK displaced by +0.2). Further experiments are needed to distinguish between the other five groups.

We undertook a detailed study of the mechanism of pig heart cytoplasmic MDH in order to evaluate whether two enzymes (LDH and MDH) with homo-

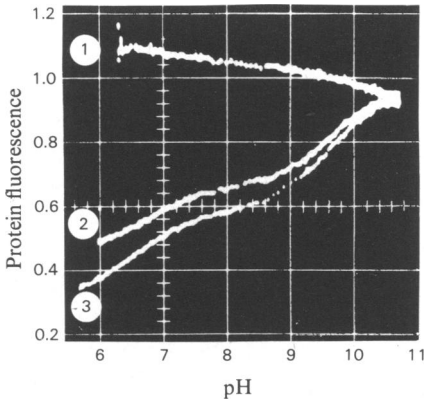


Fig. 11. *Comparison of the pH-dependences of the binding of NADH to LDH and S-cyano-LDH*

The experimental conditions were similar to those of Fig. 5. Curve 1 is for 0.025 μM-LDH (and is identical with that for 0.025 μM-*S*-cyano-LDH). Curve 2 is for 0.025 μM-LDH with 0.8 μM-NADH. Curve 3 is for 0.025 μM-*S*-cyano-LDH with 1 μM-NADH.

logous three-dimensional structures had homologous catalytic mechanisms. In this work we first detected a coupling between the ionization of histidine in MDH and NADH binding and then, on the basis of our working hypothesis, went on to detect a homologous but weaker coupling between these groups in LDH. The working hypothesis would not have predicted that there is an important alkaline ionization in LDH, which is either absent or remote from the active centre in MDH. When taken together with the results of Dickenson & Dickinson (1977) on yeast alcohol dehydrogenase, the present results suggest that the slight perturbation by NADH of the p*K* of the essential histidine towards the alkaline value may be a common feature of the simple histidine dehydrogenases.

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