Thermodynamic studies of the activation of rabbit muscle lactate dehydrogenase by phosphate

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(Received 7 June 1983/Accepted 16 August 1983)

In an attempt to trace the source of phosphate activation of the enzyme-catalysed pyruvate–lactate interconversion by rabbit muscle lactate dehydrogenase, equilibrium constants were measured to examine the effects of phosphate on interactions pertinent to the enzymic process. Frontal gel-chromatographic studies of the binding of NADH to the enzyme established that the intrinsic association constant is doubled in the presence of 50 mM-phosphate in the buffer (pH 7.4, *I* 0.15). From kinetic studies of the competition between NAD⁺ and NADH for the coenzyme-binding sites of the enzyme it is concluded that the binding of oxidized nicotinamide nucleotide is also doubled in the presence of 50 mM-phosphate. Competitive-inhibition studies and fluorescence-quenching measurements indicated the lack of a phosphate effect on ternary-complex formation between enzyme–NADH complex and oxamate, a substrate analogue of pyruvate. The equilibrium constant for the interaction between enzyme–NAD⁺ complex and oxalate, an analogue of lactate, was also shown, by difference spectroscopy, to be insensitive to phosphate concentration. Provided that the effects observed with the substrate analogues mimic those operative in the kinetic situation, the equilibrium constant governing the isomerization of ternary complex is also independent of phosphate concentration. It is concluded that enhanced coenzyme binding is the source of phosphate activation of the rabbit muscle lactate dehydrogenase system.

From a kinetic study of the effect of phosphate on the enzyme-catalysed conversion of pyruvate into lactate by rabbit muscle lactate dehydrogenase (Ward & Winzor, 1982), the phenomenon of phosphate activation (Lovell & Winzor, 1974) was linked to enhancement of the coenzyme-binding process. Indeed, preliminary estimates of equilibrium constants from the kinetic parameters were reported (Ward & Winzor, 1982), which indicated that the binding of phosphate to the enzyme gives rise to a 2–4-fold increase in the association constant for its interaction with NADH. In the present investigation, part of which has appeared in thesis form (Ward, 1983), more direct thermodynamic approaches are used to provide further characterization of the effects of phosphate on the rabbit muscle lactate dehydrogenase system under the conditions (pH 7.4, *I* 0.15) used previously (Ward & Winzor, 1982).

Materials and methods

Rabbit muscle lactate dehydrogenase was obtained as a suspension in (NH₄)₂SO₄ solution from Boehringer–Mannheim, who also supplied NADH and NAD⁺. Sodium pyruvate was obtained from Sigma Chemical Co., the sodium oxamate being an Aldrich product. Other chemicals were of reagent grade. Before use, enzyme solutions were dialysed for 24 h at 4°C against the appropriate buffer, pH 7.4, *I* 0.15, which contained 30 mM-imidazole and 6.9 mM-HCl plus sufficient sodium phosphates and/or NaCl to provide the remainder of the ionic strength. In accordance with the previous protocol (Ward & Winzor, 1982), Na₂HPO₄ and NaH₂PO₄ were added in a 3.99:1 molar ratio, EDTA (1 mM), dithiothreitol (0.1 mM) and bovine serum albumin (20 µg/ml) being included in all buffers. An absorption coefficient (*A*_{1cm}^{1%}) of 14.4 at 280 nm (Jaenicke & Knof, 1968) was used for the spectrophotometric determination of the concentration of the dialysed lactate dehydrogenase solutions, and a value of 140 000 for the *M_r* (Jaenicke & Knof, 1968)

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was used to convert these concentration values into molar ones.

Studies of the enzyme–NADH interaction

The binding of NADH to rabbit muscle lactate dehydrogenase was studied by frontal gel chromatography (Nichol & Winzor, 1964) on a column (1.1 cm × 6.1 cm) of Sephadex G-25, pre-equilibrated with the appropriate buffer and thermostatically maintained at 25°C. Reaction mixtures (10 ml) containing enzyme (2.5 μM) and NADH (3–30 μM) were applied to the column, the flow rate of which was 0.16 ml/min. Throughout the subsequent elution with pre-equilibrating buffer the column effluent was monitored continuously at 340 nm. A value of 6220 M⁻¹·cm⁻¹ was used for the molar absorption coefficient of NADH at this wavelength to determine the equilibrium concentration of the coenzyme from the absorbance of the protein-free plateau region in the trailing elution profile. The difference between this concentration and the total NADH concentration in the mixture was then divided by the total enzyme concentration to obtain *r*, the binding function (Klotz, 1946).

Interaction of enzyme with NAD⁺

The binding of oxidized coenzyme to rabbit muscle lactate dehydrogenase was measured by the ability of NAD⁺ to compete with NADH for the nucleotide-binding site and hence to inhibit the enzymic conversion of pyruvate into lactate. Kinetic measurements were carried out at 25°C in a Cary 14 spectrophotometer. The oxidation of NADH in the presence of NAD⁺ (0.77 mM) was followed by measuring the decrease in absorbance at 340 nm, the NADH concentration in these mixtures being in the range 10–110 μM and the pyruvate concentration being maintained constant at 2.77 mM; the enzyme concentration was 0.28 mM. Best-fit values of the two Michaelis parameters describing the observed dependence of the initial velocity, *v*, on NADH concentration were obtained by a non-linear regression analysis of the untransformed results in terms of a rectangular-hyperbolic relationship (Duggleby, 1981).

Binding of oxamate to the enzyme–NADH complex

Initial studies of the binding of oxamate to the binary enzyme–NADH complex were based on the ability of this substrate analogue to compete with pyruvate for the active sites of the enzyme. The conversion of pyruvate into lactate was again followed by observing the decrease in the concentration of NADH from its initial value of 0.27 mM in all assay mixtures; this concentration was selected to ensure that the coenzyme-binding sites were essentially saturated with NADH. The pyruvate concentration ranged from 0.42 to 2.77 mM in

experiments without inhibitor, and from 0.67 to 5.32 mM in experiments where oxamate (0.48 mM) was included in the assay mixtures. An enzyme concentration of 0.45 mM pertained to all assays.

The interaction of oxamate with the enzyme–NADH complex was also investigated by means of its ability to quench the fluorescence of the binary complex (Winer & Schwert, 1959) in mixtures containing 5.5 μM-NADH and 0.71 μM rabbit muscle lactate dehydrogenase in the appropriate buffer, pH 7.4, *I*0.15. Since imidazole could not be included in the buffers because of its strong fluorescence, these studies were performed in 5 mM-, 20 mM- and 50 mM-phosphate buffers, pH 7.4, the ionic strengths of which were adjusted to 0.15 by inclusion of NaCl. Solutions were subjected to an excitation wavelength of 340 nm and the emission intensities were measured at 450 nm in an Aminco SPF-500 spectrofluorimeter, the cell holder being thermostatically maintained at 25°C. Portions (1–400 μl) of concentrated (15 mM) oxamate were added to the enzyme/coenzyme mixtures (3 ml), the fluorescence being measured after each addition. Values of the fluorescence coefficients (molar emitances) *β* and *β'* (Winer *et al.*, 1959) for NADH and enzyme–NADH complex respectively were obtained by titrating 0.71 μM lactate dehydrogenase with portions of 0.7 μM-NADH and combining the measured fluorescence at 450 nm after each addition with the mixture composition deduced on the basis of the equilibrium constant governing binary-complex formation in the appropriate medium. Values of 10 500 and 28 500 were obtained for the respective values of *β* and *β'*, these being the means of values from titrations performed in buffers (pH 7.4, *I*0.15) containing 5 mM-, 20 mM- and 50 mM-phosphate. In the analysis of mixtures containing enzyme, coenzyme and oxamate, the fluorescence coefficient of ternary complex was taken as zero (Winer *et al.*, 1957), a check having been made that the fluorescence of a mixture of enzyme, NADH and a saturating concentration of oxamate could be accounted for by the concentration of free NADH present.

Interaction of oxalate with the enzyme–NAD⁺ complex

The binding of oxalate to the lactate dehydrogenase–NAD⁺ complex was measured by difference spectroscopy (Schmid *et al.*, 1976*a,b*). Solutions (3 ml) containing enzyme (1.9 μM) and NAD⁺ (83 μM) in the appropriate buffer were placed in both the reference and the sample cuvettes of an Aminco, DW-2a spectrophotometer and thermostatically maintained at 25°C. The sample cuvette was then supplemented with portions (1–2 μl) of oxalate solution (0.75 or 0.073 M), and the peak-to-trough difference in the absorbance at 280 nm and 302 nm

was taken as a measure of the degree of ternary-complex formation (Schmid *et al.*, 1976a,b).

Results and discussion

The effects of phosphate on the thermodynamics of various steps of the enzyme-catalysed pyruvate-lactate interconversion were examined separately in order to test the tentative conclusion (Ward & Winzor, 1982) that the observed activation is primarily, if not solely, the consequence of phosphate-enhanced interaction between enzyme and coenzyme.

Enzyme-NADH interaction

Results of a gel-chromatographic study of the interaction between rabbit muscle lactate dehydrogenase and reduced coenzyme are summarized in Fig. 1, about which several points are noted. First, the linearity of each Scatchard (1949) plot clearly allows an estimate to be made of an intrinsic association constant (Klotz, 1946) for the interaction of NADH with the four equivalent and independent coenzyme-binding sites. Because of the shape change that accompanies binary-complex formation between lactate dehydrogenase and nicotinamide nucleotides (Jaenicke *et al.*, 1979), the parameter so determined is effectively the product of equilibrium constants reflecting isomerization and ligand binding. Secondly, the magnitude of the association constant deduced from the slope of the plot for the binding of NADH to rabbit muscle lactate dehydrogenase in 50 mM-phosphate buffer (pH 7.4, $I_0.15$) is $3.0 \times 10^5 \text{ M}^{-1}$, which is in good agreement with the value of $2.9 \times 10^5 \text{ M}^{-1}$ obtained by Stinson & Holbrook (1973) from fluorescence studies of the enzyme-NADH interaction in 67 mM-phosphate buffer, pH 7.2, and also with the values of 4.2×10^5 – $4.8 \times 10^5 \text{ M}^{-1}$ and $3.0 \times 10^5 \text{ M}^{-1}$ reported by Fromm (1963) on the basis of NADH and protein fluorescences respectively of the system in 50 mM-phosphate buffer, pH 6.9.

A third point to note from Fig. 1 is the dependence of the equilibrium constant for the enzyme-NADH interaction on phosphate concentration, $[P]$, the values for the mean intrinsic association constant $\bar{k}_{E \cdot NADH}$ and its experimental uncertainty (twice the S.E.M.) being as follows: $1.5(\pm 0.2) \times 10^5 \text{ M}^{-1}$ in the absence of phosphate; $2.2(\pm 0.4) \times 10^5 \text{ M}^{-1}$ in the presence of 20 mM-phosphate; $3.0(\pm 0.4) \times 10^5 \text{ M}^{-1}$ in the presence of 50 mM-phosphate. As noted previously (Ward & Winzor, 1982), this variation reflects the coexistence in equilibrium of phosphate-modified and unmodified enzymic states with different affinities for NADH, the relevant expression for $\bar{k}_{E \cdot NADH}$ being:

$$\bar{k}_{E \cdot NADH} = (k_{E \cdot NADH} + k_{E \cdot P} k_{EP \cdot NADH} [P]) / (1 + k_{E \cdot P} [P]) \quad (1)$$

Fig. 1. Scatchard plots of binding data for the interaction of NADH with rabbit muscle lactate dehydrogenase

Results were obtained by frontal gel chromatography (Nichol & Winzor, 1964) of mixtures (10 ml) containing enzyme ($2.5 \mu\text{M}$) and NADH (3 – $30 \mu\text{M}$) on a Sephadex G-25 column ($1.1 \text{ cm} \times 6.1 \text{ cm}$) pre-equilibrated with buffers (pH 7.4, $I_0.15$) in which the phosphate concentration was zero ($\bullet$), 20 mM ($\square$) and 50 mM ($\blacksquare$).

where $k_{E \cdot NADH}$ and $k_{EP \cdot NADH}$ denote the intrinsic association constants for interaction of reduced coenzyme with the unmodified (E) and phosphate-modified (EP) forms respectively of rabbit muscle lactate dehydrogenase, and $k_{E \cdot P}$ is the corresponding association constant for the enzyme-phosphate interaction. From the above results $k_{E \cdot NADH} = 1.5 \times 10^5 \text{ M}^{-1}$, and solution of the two simultaneous equations so generated yields values of 10 M^{-1} and $6.0 \times 10^5 \text{ M}^{-1}$ for the respective magnitudes of $k_{E \cdot P}$ and $k_{EP \cdot NADH}$. We note that a similar conclusion, namely $k_{E \cdot P} = 20 \text{ M}^{-1}$ and $k_{EP \cdot NADH} = 4k_{E \cdot NADH}$, was deduced from the preliminary investigation of these interactions by kinetic studies (Ward & Winzor, 1982).

Enzyme-NAD⁺ interaction

Results of a kinetic study of the effect of NAD⁺ on the conversion of pyruvate into lactate by rabbit muscle lactate dehydrogenase under the present

conditions (pH 7.4, $I0.15$) were first plotted in double-reciprocal format to establish the competitive nature of the inhibition in these experiments with NADH as the variable substrate. Non-linear-regression analysis (Duggleby, 1981) of the untransformed results yielded the apparent Michaelis constants shown in the first two lines of Table 1, K_m^1 being used to denote the quantity obtained in the presence of NAD^+ . Interpretation of these two parameters in terms of classical competitive inhibition, whereby $K_m^1/K_m = 1 + \bar{k}_{E \cdot I}[I]$, yields the apparent intrinsic association constants $\bar{k}_{E \cdot I}$ listed in the final column of Table 1. Consideration of these values as equilibrium constants for the interaction between enzyme and NAD^+ does, of course, require the assumption to be made that abortive ternary-complex formation contributes negligibly to the ratio K_m^1/K_m , which is rigorously defined as $\{1 + \bar{k}_{E \cdot I}[I](1 + \bar{k}_{EI \cdot S}[S])\}$, where $\bar{k}_{EI \cdot S}$ is the association constant describing the interaction of binary enzyme-inhibitor complex with pyruvate, present at concentration $[S]$ in the reaction mixtures. Justification for making this approximation is provided by earlier kinetic studies of the inhibition of rabbit muscle lactate dehydrogenase by NAD^+ at lower ionic strength (Zewe & Fromm, 1962). From their Table 1 a value of $1600 M^{-1}$ for $\bar{k}_{E \cdot I}$ may be inferred from the ratio of the complex kinetic constant ($K_{NAD^+ \cdot lactate}$) to the Michaelis constant for lactate (Alberty, 1953), whereas the ratios of K_m^1/K_m deduced from Fig. 13 of Zewe & Fromm (1962) lead to estimates of $1500\text{--}1900 M^{-1}$ for $\bar{k}_{E \cdot I}(1 + \bar{k}_{EI \cdot S})$. Furthermore, the magnitude of the ratio $\bar{k}_{E \cdot NADH}/\bar{k}_{E \cdot I}$ from the present study is 83, which compares favourably with the values of 82 and 75 obtained for the ratio of NADH- and NAD^+ -binding constants for pig heart lactate dehydrogenase (Schmid *et al.*, 1976b) and rabbit muscle enzyme (Zewe & Fromm, 1962) respectively. We shall therefore assume that the values of $\bar{k}_{E \cdot I}$ reported in the first two lines of Table 1 do approximate to the magnitudes of

$\bar{k}_{E \cdot NAD^+}$, the intrinsic association constant for the interaction between enzyme and oxidized coenzyme. Since the presence of 50 mM-phosphate gives rise to a 2-fold increase in $\bar{k}$ for this interaction, as well as for that between enzyme and NADH, it is evident that the attachment of phosphate to rabbit muscle lactate dehydrogenase exerts a similar influence on the binding of coenzyme, be it in the oxidized or the reduced state.

Interaction of oxamate with the binary enzyme-NADH complex

In order to gain insight into the equilibrium constants governing ternary-complex formation, the substrate analogues oxamate and oxalate were substituted for the substrates pyruvate and lactate respectively. For preliminary studies of the interaction between oxamate and the enzyme-NADH complex, advantage was taken of the competitively inhibitory action of this substrate analogue on the enzymic conversion of pyruvate into lactate in experiments with pyruvate as the variable substrate: the final two lines of Table 1 list the apparent Michaelis constants deduced from these kinetic studies. Since there is no interaction between free enzyme and oxamate (Novoa & Schwert, 1961), the inhibition constant $\bar{k}_{E \cdot I}$ obtained from the ratio K_m^1/K_m refers unambiguously to ternary-complex formation between enzyme-NADH complex and oxamate. Although this parameter should be denoted (Novoa *et al.*, 1959) by $\bar{k}_{E \cdot I}\{K_{pyruvate}[NADH]/(K_{pyruvate}[NADH] + K_{NADH \cdot pyruvate})\}$, the magnitude of the complex kinetic constant $K_{NADH \cdot pyruvate}$ is infinitesimal compared with that of the product of the NADH concentration and the Michaelis constant for pyruvate, whereupon the term containing the kinetic parameters is effectively unity. The values shown as the final two entries for $\bar{k}_{E \cdot I}$ in Table 1 suggest that phosphate has no discernible effect on the magnitude of the intrinsic association

Table 1. Evaluation of binding constants by competitive inhibition of the enzymic reduction of pyruvate by rabbit muscle lactate dehydrogenase at pH 7.4, $I0.15$

The apparent Michaelis constants, K_m^1 and K_m , were determined in the presence and in the absence respectively of a concentration $[I]$ of inhibitor, $\bar{k}_{E \cdot I}$ being the reciprocal of the classical competitive-inhibition constant obtained therefrom. Inhibition by NAD^+ was tested with reaction mixtures containing 2.77 mM-pyruvate, 0.28 nM-enzyme and 10–110 μM -NADH, and that by oxamate with reaction mixtures containing 0.275 mM-NADH, 0.45 nM-enzyme and 0.42–5.32 mM-pyruvate.

Inhibitor	[I] (mM)	[P] (mM)	Apparent Michaelis constant (M)		$\bar{k}_{E \cdot I} (M^{-1})$
			K_m	K_m^1	
NAD ⁺	0.77	0	1.13×10^{-5}	2.67×10^{-5}	1800 (± 400)
	0.77	50	6.67×10^{-6}	2.53×10^{-5}	3600 (± 800)
Oxamate	0.48	0	2.22×10^{-4}	1.77×10^{-3}	14 600 (± 3200)
	0.48	50	2.12×10^{-4}	1.79×10^{-3}	15 500 (± 3000)

constant for the interaction of oxamate with the binary enzyme-NADH complex.

Further confirmation of the magnitude of the association equilibrium constant for the formation of ternary enzyme-NADH-oxamate complex was obtained from measurements of the quenching of NADH fluorescence by the presence of oxamate in mixtures of lactate dehydrogenase and NADH (Winer & Schwert, 1959). Since the inclusion of a concentration of NADH sufficient to saturate the coenzyme-binding sites of rabbit muscle lactate dehydrogenase under the present conditions (pH 7.4, 10.15) led to non-linearity in the concentration-dependence of fluorescence emission at 450nm, reaction mixtures were made up with a sub-saturating concentration ($5.5\mu\text{M}$) of reduced coenzyme. It has therefore been necessary to consider the distribution of enzyme and NADH between free, enzyme-NADH complex and ternary-complex forms, a complication that precludes adoption of the standard linearization procedures (Winer *et al.*, 1957; Winer & Schwert, 1959; Stinson & Holbrook, 1973) for evaluating the association constant for ternary-complex formation. Results obtained from fluorescence titrations of mixtures of rabbit muscle lactate dehydrogenase and NADH with oxamate in three phosphate-containing buffers (pH 7.4, 10.15) are shown in Fig. 2, together with the theoretical relationships predicted on the basis of eqn. (1) with $k_{\text{E}\cdot\text{P}} = 10\text{M}^{-1}$ and $k_{\text{EP}\cdot\text{NADH}} = 4k_{\text{E}\cdot\text{NADH}} = 6.0 \times 10^5\text{M}^{-1}$, and the formation of ternary complex from enzyme-NADH described by an association constant of 15000M^{-1} , the value deduced from the inhibition studies (Table 1). The good agreement between the theoretical and observed dependences of fluorescence emission on oxamate concentration in all three experiments clearly provides support for the adequacy of the same equilibrium constant to describe ternary-complex formation in buffers with different phosphate concentration. The binding of phosphate to rabbit muscle lactate dehydrogenase is therefore considered to have no effect on the interaction between enzyme-NADH complex and oxamate, and by inference to have no effect on the corresponding interaction involving pyruvate in place of its substrate analogue.

Interaction of oxalate with the binary enzyme-NAD⁺ complex

The final interaction to be examined experi-

Fig. 2. Effect of oxamate on the fluorescence of mixtures of NADH and rabbit muscle lactate dehydrogenase. Mixtures of enzyme ($0.71\mu\text{M}$), NADH ($5.5\mu\text{M}$) and oxamate ($5\mu\text{M}$ – 3mM) in buffers (pH 7.4, 10.15) with phosphate concentrations of 5 mM (○), 20 mM (□) and 50 mM (■) were subjected to an excitation wavelength of 340nm and the emission intensities were measured at 450nm. The curves are theoretical relationships based on predetermined values of the equilibrium constant for the enzyme-NADH interaction and an intrinsic association constant of 15000M^{-1} for ternary-complex formation. For further details see the text.

mentally is the formation of the ternary complex between enzyme-NAD⁺ and oxalate, a non-metabolized analogue of the natural substrate, lactate. The method adopted is based on the difference-spectroscopy procedure outlined by Schmid *et al.* (1976a), in which the difference in absorbance at 280nm and 302nm, ΔA , is expressed as a fraction of the maximal difference, ΔA_{max} , and the resulting ratio is considered to represent the fraction of enzyme active sites occupied by oxalate. On this basis the following expression may be deduced for a binding function r' , defined as the mol of oxalate bound per mol of enzyme subunits (sites):

$$r' = \frac{\Delta A}{\Delta A_{\text{max}}} = \frac{\bar{k}_{\text{E}\cdot\text{NAD}^+} \bar{k}_{\text{ENAD}^+\cdot\text{O}} [\text{NAD}^+] [\text{O}]}{1 + \bar{k}_{\text{E}\cdot\text{NAD}^+} [\text{NAD}^+] + \bar{k}_{\text{E}\cdot\text{NAD}^+} \bar{k}_{\text{ENAD}^+\cdot\text{O}} [\text{NAD}^+] [\text{O}]} \quad (2)$$

mentally is the formation of the ternary complex between enzyme-NAD⁺ and oxalate, a non-metabolized analogue of the natural substrate,

$\bar{k}_{\text{E}\cdot\text{NAD}^+}$ denotes the mean association constant for the interaction between oxidized coenzyme and an enzyme subunit, and $\bar{k}_{\text{ENAD}^+\cdot\text{O}}$ the corresponding

equilibrium constant for the binding of oxalate, O, to the binary complex. The weak binding of NAD^+ to rabbit muscle lactate dehydrogenase makes it necessary to consider results in terms of this expression rather than the simplified form that pertains (Schmid *et al.*, 1976a,b) when the denominator is dominated by the last two terms.

For a system with a total concentration of enzymic sites $[\bar{\text{E}}]$, the concentration of free oxalate, $[\text{O}]$, is given by the difference between $[\bar{\text{O}}]$, the total oxalate concentration, and $r'[\bar{\text{E}}]$, the concentration of oxalate bound to binary enzyme– NAD^+ complex. Since $[\text{O}] \gg [\text{E}]$ and $r' \leq 1$, $[\text{O}]$ provides an acceptable estimate of $[\bar{\text{O}}]$ for use in eqn. (2). Furthermore, consideration of the distribution of coenzyme between its various forms shows that the total ($[\bar{\text{NAD}}^+]$) and free ($[\text{NAD}^+]$) concentrations of nicotinamide nucleotide are related by the expression:

$$[\bar{\text{NAD}}^+] - r'[\bar{\text{E}}] = [\text{NAD}^+](1 + \bar{k}_{\text{E} \cdot \text{NAD}^+}[\text{E}])$$

where $\bar{k}_{\text{E} \cdot \text{NAD}^+}$ is $1800\text{--}3600\text{M}^{-1}$ (Table 1) and $[\text{E}]$ is in the micromolar range ($[\text{E}] < [\bar{\text{E}}] = 7.6\text{ }\mu\text{M}$). The difference between the total coenzyme concentration and the concentration of bound oxalate thus approximates very closely to the free concentration of NAD^+ , the quantity required for application of eqn. (2). Indeed, provided that the magnitude of $\bar{k}_{\text{E} \cdot \text{NAD}^+}$ is predetermined, the value of the equilibrium constant for ternary-complex formation may be obtained as the only remaining parameter in eqn. (2) whose magnitude is still to be determined.

Fig. 3 presents, in a format suggested by eqn. (2), the results obtained with mixtures of rabbit muscle lactate dehydrogenase, oxidized coenzyme and oxalate in buffer (pH 7.4, $I=0.15$) in which the phosphate concentration was zero (●) and 50 mM (■), the values of $\bar{k}_{\text{E} \cdot \text{NAD}^+}$ having been taken from Table 1. The two sets of results may clearly be described in terms of a single linear relationship, which, on the basis of least-squares calculations, corresponds to an intrinsic association constant of $3.0(\pm 0.2) \times 10^4\text{M}^{-1}$ for the interaction of oxalate with the binary enzyme– NAD^+ complex. We therefore conclude that phosphate is also without effect on ternary-complex formation between enzyme– NAD^+ complex and oxalate, the inference again being that this finding mimics the situation where lactate replaces the substrate analogue.

Phosphate activation of the enzymic reaction

The enzymic conversion of pyruvate (Py) into lactate (L) is generally considered (Takenaka & Schwert, 1955) in terms of the following five equilibrium reactions, which are governed by the appropriate association equilibrium constants, k :

The first point to note from the present investigation is the confirmation of the earlier conclusions (Lovell & Winzor, 1974; Ward & Winzor, 1982) that phosphate affects the binding of both the oxidized and the reduced nicotinamide nucleotides to rabbit muscle lactate dehydrogenase in the formation of the binary enzyme–coenzyme complex. Secondly, within the limits of experimental accuracy, the two

Fig. 3. Interaction of oxalate with the binary complex between NAD^+ and rabbit muscle lactate dehydrogenase. Difference spectroscopy (Schmid *et al.*, 1976a) was used to study ternary-complex formation in mixtures containing enzyme ($1.9\text{ }\mu\text{M}$), NAD^+ ($83\text{ }\mu\text{M}$) and oxamate ($0.09\text{--}0.75\text{ mM}$) in buffers (pH 7.4, $I=0.15$) containing zero (●) and 50 mM (■) phosphate. The results are plotted in accordance with the reciprocal form of eqn. (2) with $Z = \bar{k}_{\text{E} \cdot \text{NAD}^+}[\text{NAD}^+]$. For further details see the text.

interactions are influenced to the same relative extents in that the presence of 50 mM-phosphate causes a doubling of $k_{E \cdot NADH}$ (Fig. 1) and $k_{E \cdot NAD^+}$ (Table 1). Thirdly, with the proviso that the formation of ternary complexes with the two substrate analogues reflects the kinetic situation, the observed insensitivity to phosphate concentration of the equilibrium constants for the formation of ternary complexes (Table 1 and Figs. 2 and 3) implies that k_1 and k_3 in the above scheme are not affected by phosphate. The only reaction on which comment remains to be made is the isomerization equilibrium between the two forms of ternary complex.

If the enzyme-catalysed conversion of pyruvate into lactate is allowed to proceed to equilibrium, the final concentrations of products and reactants are related by the expression:

$$K_{eq}^{app.} = K_{eq} [H^+] = [NAD^+]_{eq.} [L]_{eq.} / [NADH]_{eq.} [Py]_{eq.} \quad (3a)$$

From the above reaction scheme the apparent equilibrium constant, $K_{eq}^{app.}$, may also be written as:

$$K_{eq}^{app.} = k_2(k_1/k_3)(k_{E \cdot NADH}/k_{E \cdot NAD^+}) \quad (3b)$$

At the fixed pH and ionic strength of the present study the same $K_{eq}^{app.}$ should apply in the presence and in the absence of phosphate (Winer & Schwert, 1958). Furthermore, we have already noted the constancy of each of the equilibrium-constant ratios within parentheses in eqn. (3b) when phosphate concentration is varied. It follows that k_2 , the equilibrium constant governing isomerization of the ternary complexes, is also unaffected by the interaction of phosphate with rabbit muscle lactate dehydrogenase. We therefore conclude that enhanced coenzyme binding is the source of phosphate activation of the rabbit muscle lactate dehydrogenase system. In this regard, X-ray-crystallographic analysis (Adams *et al.*, 1973) has revealed the existence of two sites for the binding of bivalent anions, one being coincident with the active site for pyruvate and the other in the region of subunit-subunit contact. Although either of these sites would meet the requirement that coenzyme and phosphate be accommodated simultaneously by the enzyme (Lovell & Winzor, 1974), the lack of an effect of phosphate on parameters governing ternary-complex formation suggests that the activation reflects an interaction with anionic sites at

the interfaces between the subunits of tetrameric lactate dehydrogenase.

The partial support of this investigation by the Australian Research Grants Scheme is gratefully acknowledged, as is the Commonwealth Postgraduate Award to L. D. W.

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