Influence of pH on the allosteric properties of lactate dehydrogenase activity of *Phycomyces blakesleeanus*

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1. Lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* showed positive homotropic interactions with NADH at all pH values studied (pH 5.0–7.7). The calculated values for the first and last intrinsic association constants remained unaltered with pH, in contrast with the Hill coefficient value, which varied significantly, reaching its maximum values at pH 6.0 and 7.7. This suggests the hypothesis that pH regulates these homotropic effects by changes in the value of the intermediate intrinsic association constants. 2. From pH 7.2 to 7.7 lactate dehydrogenase exhibited, likewise, positive homotropic interactions with pyruvate. There were practically no changes in the first and last intrinsic association constants and in Hill coefficient values with pH. At pH values below 7.2 (pH 5.0–6.8) the enzyme showed high substrate inhibition, which was highly dependent on pH, NADH concentration and temperature. By way of substrate inhibition pH regulates, primarily, lactate dehydrogenase activity towards pyruvate, since the homotropic effects appear not to be dependent on pH. 3. Fructose 1,6-bisphosphate is a true allosteric effector of lactate dehydrogenase of *Phycomyces blakesleeanus*. It decreases positive co-operativity with NADH, and on the other hand pyruvate co-operativity turns into mixed co-operativity. In addition, the effector decreases the inhibitory effect caused by pyruvate.

In the preceding paper (Soler *et al.*, 1982) some characteristics of the kinetic response of the lactate dehydrogenase from the mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (–) were described. Lactate dehydrogenase showed positive homotropic interactions with pyruvate and NADH, and a change of pH produced a change in the kinetic response for both ligands. The present paper emphasizes the details of the effect of pH on the homotropic interactions with pyruvate and NADH of the enzyme from mycelium of *Phycomyces blakesleeanus*. In addition, we include the effect produced by fructose 1,6-bisphosphate, which behaves as an allosteric effector of this lactate dehydrogenase.

Wolin (1964) demonstrated that lactate dehydrogenases from certain streptococci have a specific requirement for fructose 1,6-bisphosphate for catalytic activity. Similar lactate dehydrogenase activities have been found in other streptococci (Wittenberger & Angelo, 1970; Brown & Witten-

berger, 1972), bifidobacteria (de Vries & Stouthamer, 1968) and *Lactobacillus casei* (Holland & Pritchard, 1975). Regulation of lactate dehydrogenase activity by fructose 1,6-bisphosphate has been shown in *Acholeplasma laidlawii* (Neimark & Tung, 1973), *Rhotia dentocariosa* (Eisenberg *et al.*, 1976), *Butyrivibrio fibrisolvens* (Van Gylswyk, 1977) and *Actinomyces viscosus* (Brown *et al.*, 1975). Fructose 1,6-bisphosphate is a glycolytic intermediate that may provide a useful system for regulation of the normal pathway of glucose catabolism, and it is possible that it may be more widespread than previously recognized.

Materials

Biochemical reagents

NADH and fructose 1,6-bisphosphate were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were obtained as described in the preceding paper (Soler *et al.*, 1982).

Buffers

All chemicals used for the preparation of buffers were obtained from Merck, Darmstadt, Germany.

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All buffers were prepared with glass-distilled water as described by Gomori (1955).

Methods

Enzyme preparation

Lactate dehydrogenase purified from the mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (—) was used. *Phycomyces* was grown and harvested and lactate dehydrogenase was purified as described in the preceding paper (Soler *et al.*, 1982).

Enzyme assay

The rates of NADH oxidation were determined by using a Beckman model 25 spectrophotometer equipped with recorder and a temperature-control unit. The reactions were performed at 30°C in 20mM-sodium phosphate buffer for all pH values studied, except that at pH 5.0 it was in 20mM-sodium citrate buffer. The final volume of the reaction mixture was 3.0ml. The reaction was started by addition of 2ml of the enzyme preparation suitably diluted, and the initial rates of reactions were measured by following the decreases in A_{340} associated with NADH oxidation.

Results

Influence of pH on the homotropic interactions with NADH

At all pH values studied (5.0, 6.0, 6.8, 7.5 and 7.7) sigmoidal kinetics were observed, indicating the existence of positive co-operativity for NADH. The $[S]_{0.5v}$ values (Atkinson, 1966; Stadtman, 1966) for

NADH were between 0.065mM at pH 5.0 and 0.130mM at pH 7.7 at a fixed pyruvate concentration (20mM) and increased markedly as the pH of the reaction mixture was raised. In addition, for each pH value starting at pH 6.0, the $[S]_{0.5v}$ value for NADH increased as the pyruvate concentration decreased (Figs. 1a and 1b).

The Hill coefficient (h) values (Cornish-Bowden & Koshland, 1975; Cadenas, 1978) obtained from a Hill plot were between 1.5 and 3.5 as the pH varied, indicating more than two NADH-binding sites in the enzyme. The values obtained for h remained unchanged when pyruvate concentration varied. When only co-operative binding was considered to occur, a Hill plot provided the values of first (1k_s) and last (sk_s) intrinsic association constants, as defined in Cadenas (1978) (Table 1). At all pH values studied (sk_s) and (1k_s) remained unchanged as pyruvate concentration was increased. At the same time the (1k_s) value increased roughly 10-fold as the pH changed. As a consequence the site-site interaction energy appears as a function of pH.

Influence of pH on the homotropic interactions with pyruvate

When experiments were performed at varied pyruvate concentration and fixed NADH concentration in a range of pH values (5.0, 6.0, 6.8, 7.5 and 7.7), the existence of sigmoidal kinetics was only observed at pH 7.2, 7.5 and 7.7. The $[S]_{0.5v}$ values for pyruvate increased from 1.5mM at pH 7.2 up to 12mM at pH 7.7 at fixed NADH concentration (0.250mM) as the pH value was raised. On the other

Fig. 1. Hill plot of the data obtained for the kinetic response of lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (—) to NADH

The assays were performed as described in the Methods section. The incubation mixture contained 39 μ g of protein. The V_{max} values were obtained from Scatchard plots. (a) pH 6.8: $\bullet$, 4 mM-pyruvate; $\blacktriangle$, 20 mM-pyruvate. (b) pH 7.7: $\blacktriangle$, 7.5 mM-pyruvate; $\bullet$, 20 mM-pyruvate.

Table 1. Influence of pH on binding co-operativity of NADH to lactate dehydrogenase from mycelium of *Phycomyces blakesleeianus* N.R.R.L. 1555 (—)

The experiments were performed at varied NADH concentrations (0.01–0.3 mM) as described in the Methods section. The data were obtained from Hill plots.

pH	[Pyruvate] (mM)	<i>h</i>	¹ <i>k_s</i> (M ^{−1})	^s <i>k_s</i> (M ^{−1})	Δ <i>G</i> ⁰ (kJ/mol)
5.0	0.25	1.5	7.7 × 10 ³	1.6 × 10 ⁴	1.9
	20.00	1.5	9.5 × 10 ³	2.1 × 10 ⁴	2.0
6.0	0.25	2.9	8.9 × 10 ²	2.8 × 10 ⁴	8.7
	20.00	3.5		2.8 × 10 ⁴	
6.8	4.00	2.3	3.2 × 10 ³	2.4 × 10 ⁴	5.3
	20.00	1.9	9.5 × 10 ³	2.4 × 10 ⁴	2.5
7.5	10.00	2.2		1.5 × 10 ⁴	
	20.00	2.3		1.7 × 10 ⁴	
7.7	7.50	2.9	8.5 × 10 ²	1.3 × 10 ⁴	7.0
	20.00	2.5	5.2 × 10 ²	2.0 × 10 ⁴	9.2

hand, for each pH value the [S]_{0.5*v*} value for pyruvate increased as NADH concentration decreased.

In every case the *h* values obtained from Hill plots (Fig. 2) were close to 2, indicating more than two pyruvate-binding sites in the enzyme. These *h* values appear to be independent of both pH and coenzyme concentration.

When feasible, the evaluation of the first and last intrinsic association constants (¹*k_s* ≈ 10 M^{−1}; ^s*k_s* ≈ 10² M^{−1}) indicated also their independence of both pH and NADH concentration. The values obtained are shown in Table 2.

Substrate inhibition

Experiments performed with pyruvate at various substrate concentrations in the pH range 5.0–6.8 showed high substrate inhibition for concentrations above 1.5 mM. At pH values above 6.8 this was not observed, not even at 60 mM-pyruvate concentration (Fig. 3).

The pH value appears to determine first the minimum pyruvate concentration at which substrate inhibition can be detected, and secondly the percentage inhibition as determined by the activity obtained with 20 mM-pyruvate as a function of the activity obtained with pyruvate concentration that produces the maximum activity.

At pH 6.0 and at 30 °C at a fixed NADH concentration of 0.125 mM there was 39% inhibition by 1.5 mM-pyruvate. In addition, percentage inhibition for a given pH value appears to depend on coenzyme concentration. At pH 6.0 and at 30 °C the percentage inhibition was increased and the minimum pyruvate concentration at which inhibition could be detected decreased as coenzyme concentration increased (Fig. 4).

A Dixon-like plot of 1/*v* versus pyruvate concentration in the inhibition range for various fixed NADH concentrations gave a family of parallel

Fig. 2. Hill plot of the data obtained for the kinetic response of lactate dehydrogenase from mycelium of *Phycomyces blakesleeianus* N.R.R.L. 1555 (—) to pyruvate at pH 7.7

The experiments were performed as described in the Methods section. The incubation mixture contained 39 μg of protein. The *V_{max}* value were obtained from Scatchard plots. ●, 0.250 mM-NADH; ▲, 0.125 mM-NADH.

Table 2. Influence of pH on binding co-operativity of pyruvate to lactate dehydrogenase from mycelium of *Phycomyces blakesleeianus* N.R.R.L. 1555 (—)

The experiments were performed at varied pyruvate concentrations (0.1–20.0 mM) as described in the Methods section. The data were obtained from Hill plots.

pH	[NADH] (mM)	<i>h</i>	¹ <i>k_s</i> (M ^{−1})	^s <i>k_s</i> (M ^{−1})
7.2	0.125	1.5	1.3 × 10 ²	5.5 × 10 ²
	0.250	1.5	3.3 × 10 ²	1.0 × 10 ³
7.5	0.125	1.7		1.3 × 10 ²
	0.250	2.3		1.9 × 10 ²
7.7	0.125	1.8	1.3 × 10	
	0.250	2.1	3.2 × 10	1.2 × 10 ²

Fig. 3. Kinetic response of lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (—) to pyruvate at various pH values

The experiments were performed as described in the Methods section. The incubation mixture contained 39 μg of protein. The NADH concentration was held constant at 0.125 mM. ▲, pH 5.0; △, pH 6.0; ●, pH 6.8; ○, pH 7.7.

Fig. 5. Effect of temperature on the substrate inhibition of lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (—) at pH 6.0

The experiments were performed as described in the Methods section. The incubation mixture contained 39 μg of protein. The NADH concentration was 0.250 mM. ●, 30°C; ○, 37°C; ▲, 23°C.

Fig. 4. Influence of NADH concentration on the substrate inhibition of lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (—) at pH 6.0 and 30°C

The experiments were performed as described in the Methods section. The incubation mixture contained 39 μg of protein. ○, 0.125 mM-NADH; △, 0.0625 mM-NADH; ▲, 0.03125 mM-NADH.

lines, indicating uncompetitive inhibition. Experiments performed at pH 6.0 and at various temperatures for a fixed NADH concentration suggested that high substrate inhibition was less marked as the temperature was raised (Fig. 5).

Influence of fructose 1,6-bisphosphate on the kinetic response of lactate dehydrogenase

Experiments performed with 30 mM-fructose 1,6-bisphosphate at fixed pyruvate concentration (20 mM) and varied NADH concentration (0.01–0.3 mM) at 30°C and at pH 6.0 and 7.7 showed the existence of positive co-operativity for the co-enzyme, which is analogous to that obtained without effector. At pH 6.0 activation by fructose 1,6-bisphosphate was observed and the $[S]_{0.5v}$ remained practically unchanged (0.07 mM without effector and 0.12 mM with it). However, at pH 7.7 no activation was observed, whereas the $[S]_{0.5v}$ increased markedly (6–7-fold), which indicates that the affinity for the coenzyme decreased (Fig. 6). At both pH values the presence of fructose 1,6-bisphosphate caused a decrease in the h value of more than 1 unit (Fig. 7). The intrinsic association constants 1k_s and 5k_s at pH 6.0 remained unchanged in the presence of effector. However, at pH 7.7 the 5k_s value decreased more than 10-fold in the presence of the effector, whereas 1k_s remained unchanged (Table 3).

On the other hand, experiments performed at 30°C at pH 6.0 and 7.7 with 30 mM-fructose 1,6-bisphosphate at fixed NADH concentration (0.250 mM) and varied pyruvate concentration (0.025–50 mM) showed that the high substrate inhibition that was apparent at pH 6.0 did not disappear in the presence of the effector. Instead, the pyruvate concentration at which inhibition could be detected was increased from 1.5 mM to 10 mM by the

Fig. 6. Effect of fructose 1,6-bisphosphate on the kinetic response to NADH of lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (—) at various pH values
The experiments were performed as described in the Methods section. The incubation mixture contained 10 μg of protein. The pyruvate concentration was held constant at 20 mM. (a) pH 6.0: ●, 30 mM-fructose 1,6-bisphosphate; ▲, without fructose 1,6-bisphosphate. (b) pH 7.7: ●, 30 mM-fructose 1,6-bisphosphate; ▲, without fructose 1,6-bisphosphate.

Fig. 7. Hill plot of the data shown in Fig. 6
(a) pH 6.0: ●, 30 mM-fructose 1,6-bisphosphate; ▲, without fructose 1,6-bisphosphate. (b) pH 7.7: ●, 30 mM-fructose 1,6-bisphosphate; ▲, without fructose 1,6-bisphosphate.

Table 3. Effect of fructose 1,6-bisphosphate on the homotropic interactions of lactate dehydrogenases from mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (—) with NADH
The experiments were performed at varied NADH concentrations (0.01–0.3 mM) and fixed pyruvate concentration (20 mM) as described in the Methods section. The data were obtained from Hill plots.

pH	[Fructose 1,6-bisphosphate] (mM)	<i>h</i>	¹ <i>k_s</i> (M ⁻¹)	¹³ <i>k_s</i> (M ⁻¹)	Δ <i>G</i> ⁰ (kJ/mol)
6.0	0	3.7	1.0 × 10 ³	1.7 × 10 ⁴	7.0
	30	2.3	2.7 × 10 ³	4.6 × 10 ⁴	7.2
7.7	0	2.9	7.4 × 10 ²	2.5 × 10 ⁴	8.9
	30	1.4	4.7 × 10 ²	9.4 × 10 ²	1.7

effector and percentage inhibition decreased from 42% to 9% (Fig. 8). The presence of 30 mM-fructose 1,6-bisphosphate did not appear to influence the $V_{\max}$ obtained from a double-reciprocal plot of $1/v$ versus $1/[\text{pyruvate}]$. The apparent K_m value obtained from the same plot was increased 10-fold in the presence of the effector. The manner in which high substrate inhibition depends on the fixed coenzyme concentration was not altered with 30 mM-fructose 1,6-bisphosphate.

Pyruvate was shown to be a competitive inhibitor by means of a Dixon-like plot of $1/v$ versus pyruvate concentration, in the inhibitory range, at fixed concentration of NADH in the presence of 30 mM-fructose 1,6-bisphosphate (Fig. 9).

At pH 7.7 with 30 mM-fructose 1,6-bisphosphate the apparent $V_{\max}$ showed an approx. 50% decrease. The $[S]_{0.5v}$ increased from 9 mM to 35 mM in the presence of fructose 1,6-bisphosphate. A Hill plot

Fig. 8. Effect of fructose 1,6-bisphosphate on the substrate inhibition of lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (—) at pH 6.0

The experiments were performed as described in the Methods section. The incubation mixture contained 10 μg of protein. The NADH concentration was held constant at 0.250 mM. ●, 30 mM-Fructose 1,6-bisphosphate; ▲, without fructose 1,6-bisphosphate.

Fig. 9. Dixon-like plot showing the inhibition class shown by lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (—) with pyruvate and with NADH

The experiments were performed as described in the Methods section. The incubation mixture contained 10 μg of protein. ○, 30 mM-Fructose 1,6-bisphosphate and 0.125 mM-NADH; ●, 30 mM-fructose 1,6-bisphosphate and 0.250 mM-NADH

Fig. 10. Hill plot of the data obtained for the kinetic response of lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (—) to pyruvate at pH 7.7 in the presence of 30 mM-fructose 1,6-bisphosphate

The experiments were performed as described in the Methods section. The incubation mixture contained 10 μg of protein. The NADH concentration was held constant at 0.250 mM. The $V_{\max}$ values were obtained from Scatchard plots.

Table 4. Effect of fructose 1,6-bisphosphate on the homotropic interactions of lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (—) with pyruvate

The experiments were performed at 30°C at pH 7.7 with varied pyruvate concentrations (0.025–50 mM) and fixed NADH concentration (0.250 mM) in the presence of 30 mM-fructose 1,6-bisphosphate as described in the Methods section. The data were obtained from Hill plots.

	h	1k_s (M^{-1})	4k_s (M^{-1})	5k_s (M^{-1})	ΔG^0 (kJ/mol)
Low pyruvate concentration	0.34	1.6×10^2	1.5×10	4.1×10	-3.4
High pyruvate concentration	1.86				

(Fig. 10) revealed mixed co-operativity. At low pyruvate concentrations negative co-operativity ($h = 0.34$) was observed, whereas at high pyruvate concentrations it became positive ($h = 1.86$). The results obtained are shown in Table 4.

Discussion

The conclusions reached in the preceding paper (Soler *et al.*, 1982) indicate that lactate dehydrogenase from the mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (–) exhibits at pH 7.5 positive homotropic interactions with its substrate, pyruvate, and its coenzyme, NADH. The h values obtained support the existence of more than two binding sites in the enzyme for each ligand, sites that are not independent. Furthermore, when a kinetic study was conducted at pH 6.0, a peculiar behaviour with pyruvate was revealed.

A detailed study of the change in lactate dehydrogenase activity with pH showed that, whereas the enzyme exhibits positive homotropic effects for NADH at all pH values studied (between pH 5.0 and 7.7), for pyruvate this effect is only apparent at neutral pH and above. The $[S]_{0.5}$ values obtained for both ligands indicate that the enzymic affinity for each decreases when pH is raised, the change being much more marked with pyruvate (6-fold). The h values obtained at varied pyruvate concentration remain unaffected by pH, whereas at varied NADH concentration there are two pH values (pH 6.0 and 7.7) at which the h value is at least 1 unit greater than those obtained at the other pH values. For both ligands the h value at each pH appears to be independent of the ligand the concentration of which remains fixed. The $^s k_s$ value calculated for pyruvate as well as that for NADH are independent of pH and the concentration of the ligand that remains fixed. With regard to $^i k_s$, which begins at a value of the same order as $^s k_s$ for pyruvate as well as that for NADH, a decrease of 10-fold with a change of pH was observed. Subsequent variations of pH failed to produce any significant alteration in the $^i k_s$ value, suggesting that it is at a certain pH value that the homotropic effects manifest themselves. In addition, at each pH value, $^i k_s$ is independent of the fixed ligand concentration. The fact that $^s k_s$, $^i k_s$ and h values are unchanged with the fixed ligand concentration seems to mean that the binding sites for NADH are independent from the sites for pyruvate.

The Hill coefficient for an enzyme with three or more binding sites for a ligand depends on the differences between all the association constants (Cadenas, 1978). Since $^s k_s$ and $^i k_s$ for NADH are independent of pH ($^i k_s$ from pH 6.0 onwards) in order to justify the pH-dependence of h , it is necessary to assume that the changes in pH

determine variations in the intermediate intrinsic association constants. On the other hand, since the first and last intrinsic association constants differ only 10-fold, and on the assumption that there are four binding sites for NADH (Holbrook & Gutfreund, 1973), the values of the second and third association constants must be very close. Even when the separation between both intrinsic association constants values is very slight (smaller than 10-fold), these values should differ to obtain h values equal to or greater than 3. Any assumption on the values of the intermediate intrinsic association constants that considers less than four different constants would always lead to h values smaller than 3. It therefore seems that pH alters the intermediate intrinsic association constants, and in this way controls lactate dehydrogenase activity through the percentage of intermediate saturation with NADH.

With regard to the homotropic effects of lactate dehydrogenase with pyruvate at different substrate concentrations, which begin to be noticeable at neutral pH, the h , $^s k_s$ and $^i k_s$ values were unchanged by pH. This indicates that the values of all intrinsic association constants, including the intermediate constants, suffered no alteration with pH, and therefore that the allosteric kinetics of the enzyme with pyruvate are not regulated by pH.

At pH values below neutral, lactate dehydrogenase activity appears to be regulated by high substrate inhibition by pyruvate. Our results indicate that the inhibitory effect of pyruvate is highly dependent on pH, and, as Cleland (1970) indicated, this effect can be considered to be uncompetitive. Results obtained recently by us (D. De Arriaga, J. Soler & E. Cadenas, unpublished work) are compatible with the proposal that an abortive ternary complex involving enzyme, NAD⁺ and pyruvate is responsible for this inhibition, in the same manner as proposed by several authors (Sugrobova *et al.*, 1975; Burgner *et al.*, 1978; Burgner & Ray, 1978; Wilton, 1979; Trommer *et al.*, 1979; Wenzel & Trommer, 1979). Moreover, we found that the inhibitory effect of pyruvate depends also on the temperature, a fact that has been reported for lactate dehydrogenase from several sources (Somero, 1973; Eichner & Kaplan, 1977; Kao & Farley, 1978), and also on NADH concentration in such a way that inhibition of lactate dehydrogenase should increase with an increased concentration of NADH. The possible physiological importance of these findings requires further investigation.

Our results indicate that fructose 1,6-bisphosphate is a true allosteric effector of lactate dehydrogenase of *Phycomyces blakesleeanus*. With regard to NADH at pH 6.0, the presence of fructose 1,6-bisphosphate causes the alteration of the intermediate intrinsic association constant value, since,

without a change in the 1k_s and 3k_s values, h decreased by more than 1 unit. At pH 7.7 the presence of effector causes a decrease of more than 10-fold in the 3k_s value, making it of the same order as 1k_s , which did not vary. In addition, the h value decreased by more than 1 unit, and the affinity for the coenzyme markedly decreased (6–7-fold). Consequently, positive co-operativity with NADH remained in the presence of fructose 1,6-bisphosphate, although its presence supposes decrease in positive co-operativity that similarly depends on pH.

As for pyruvate at pH 6.0, which shows high substrate inhibition, the presence of fructose 1,6-bisphosphate decreases this effect and enzyme affinity for pyruvate at the same time. The effect of fructose 1,6-bisphosphate at pH 7.7 is remarkable, since a mixed co-operativity (negative–positive) was observed with a change in the sign of net co-operativity from positive to negative, while producing a decrease in the affinity for pyruvate (approx. 4-fold).

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