Lactate dehydrogenase in *Phycomyces blakesleeanus*

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1. An NAD-specific L(+)-lactate dehydrogenase (EC 1.1.1.27) from the mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (–) was purified approximately 700-fold. The enzyme has a molecular weight of 135 000–140 000. The purified enzyme gave a single, catalytically active, protein band after polyacrylamide-gel electrophoresis. It shows optimum activity between pH 6.7 and 7.5. 2. The *Phycomyces blakesleeanus* lactate dehydrogenase exhibits homotropic interactions with its substrate, pyruvate, and its coenzyme, NADH, at pH 7.5, indicating the existence of multiple binding sites in the enzyme for these ligands. 3. At pH 6.0, the enzyme shows high substrate inhibition by pyruvate. 3-Hydroxypyruvate and 2-oxovalerate exhibit an analogous effect, whereas glyoxylate does not, when tested as substrates at the same pH. 4. At pH 7.5, ATP, which inhibits the enzyme, acts competitively with NADH and pyruvate, whereas at pH 6.0 and low concentrations of ATP it behaves in an allosteric manner as inhibitor with respect to NADH. GTP, however, has no effect under the same experimental conditions. 5. Partially purified enzyme from sporangiophores behaves in an entirely similar kinetic manner as the one exhibited by the enzyme from mycelium.

Phycomyces blakesleeanus is a unicellular Zygomycete fungus (Bergman *et al.*, 1969). Lactate dehydrogenases specific for D(–)-lactate or L(+)-lactate have been described in almost all animal tissues, in micro-organisms and also in plants. Gleason *et al.* (1966) and LéJohn (1971) showed that D(–)-lactate dehydrogenases are prevalent among several subclasses of Phycomycetes. Nevertheless the occurrence of L(+)-lactate dehydrogenases has been described in other fungi (Trinchant & Rigaud, 1974; Brown *et al.*, 1975; Allsopp & Matthews, 1975).

Lactate dehydrogenase is one of the most thoroughly investigated NAD-linked dehydrogenases. The enzyme plays an important role in the regulation of anaerobic glycolysis by providing NAD⁺ for glyceraldehyde phosphate dehydrogenase, thus maintaining ATP production. The lactate dehydrogenases from a large number of sources have been purified to homogeneity, and their physical, chemical and catalytic properties have been investigated in great detail in many laboratories (Everse & Kaplan, 1973; Holbrook & Gutfreund, 1973; Stinson & Holbrook, 1973; Holbrook & Stinson, 1973; Holbrook *et al.*, 1975).

With regard to regulation of lactate dehydrogenase activity, ATP has been reported as an allosteric effector for L(+)-lactate dehydrogenase from potato tubers (Davies & Davies, 1972) and from *Actinomyces viscosus* (Brown *et al.*, 1975) and as a competitive inhibitor of nicotinamide nucleotides for D(–)-lactate dehydrogenases in several subclasses of Phycomycetes (LéJohn, 1971). Likewise, GTP has been proposed as an allosteric inhibitor of D(–)-lactate dehydrogenases in fungi belonging to the Oomycetes and Hypochytridiomycetes (LéJohn, 1971). NADH appears to play a regulatory role in lobster tail L(+)-lactate dehydrogenase (Kaloustian & Kaplan, 1969).

In the present work, a partially purified lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (–) was used for the characterization of the enzyme activity in a preliminary study of the kinetic properties of the reduction of pyruvate associated with NADH oxidation. In addition, ATP and GTP were tested for their possible regulatory effects on lactate dehydrogenase activity.

Materials

Biochemical reagents

Reduced 3-acetylpyridine–adenine dinucleotide, NAD⁺, NADP⁺, NADH, NADPH, GTP, D(–)-

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lactate, L(+)-lactate, 3-hydroxypyruvic acid, 2-oxo-valeric acid, glyoxylic acid, L-asparagine monohydrate, thiamin hydrochloride, rabbit muscle pyruvate kinase, bovine serum albumin, carbonic anhydrase, phenazine methosulphate and Nitro-tetrazolium Blue dye were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. ATP was from Aldrich-Europe, Beerse, Belgium, Bacto-casitone was from Difco, Detroit, MI, U.S.A., agar no. 3 and yeast extract (Lab Lemco powder) were from Oxoid Ltd., London, U.K., Sephadex G-200 was from Pharmacia, Uppsala, Sweden, insulin (Insulin LEO) was from Nordisk Insulinlaboratorium, Copenhagen, Denmark, acrylamide, NN'-methylenebisacrylamide, NNN'-tetramethylethylenediamine, Bromophenol Blue, Coomassie Brilliant Blue, sodium dodecyl sulphate and molecular-weight marker mixture (range 14 300–71 500) were from Shandon Southern Products Ltd., Cheshire, U.K., and sodium pyruvate and all other chemicals were obtained from Merck, Darmstadt, West Germany.

Buffers

All chemicals used for the preparation of buffers were obtained from Merck. Tris was dissolved in water at 20°C and adjusted to the required pH with HCl. All buffers were prepared with glass-distilled water.

Calcium phosphate gel

This was prepared by the method of Davies & Davies (1972), modified by dissolving CaCl_2 (29.4 g) in 250 ml of water and mixing with 250 ml of a solution containing K_2HPO_4 (273.6 g/l). The gelatinous precipitate was filtered with suction and washed with 500 ml of water and 150 ml of 70% (v/v) ethanol. The dry gel was stored in the dark at 4°C.

Methods

Cell growth

The *Phycomyces blakesleeanus* wild-type strain N.R.R.L. 1555 (–) was used. *Phycomyces* was grown in a solid minimal medium, as described by Sutter (1975), containing, per litre of culture medium, D-glucose (20 g), L-asparagine monohydrate (2 g), KH_2PO_4 (5 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g), CaCl_2 (28 mg), thiamin hydrochloride (2 mg), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (1 mg), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (50 µg), $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (1.5 mg), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.3 mg) and agar (15 g). The medium was supplemented with yeast extract (1 g/l) and Bacto-casitone (1 g/l).

Phycomyces spores were heat-shocked by incubating 10 ml of an aqueous suspension containing 1.5×10^4 spores/ml for 15 min at 48°C. Four Petri dishes of 20 cm diameter containing 250 ml of solid medium were inoculated with 1.5 ml of the spore

suspension. The culture was kept at room temperature (18–22°C).

Protein measurement

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma Chemical Co.) as standard.

Enzyme assay

Lactate dehydrogenase was assayed at 30°C in 20 mM-sodium phosphate buffer, pH 7.5, by measuring the decrease in A_{340} associated with NADH oxidation in a Beckman model 25 spectrophotometer equipped with a recorder and a temperature-control unit. The assay mixture contained NADH (0.250 mM) in a final volume of 3.0 ml. The reaction was started by addition of 2 ml of the enzyme preparation suitably diluted, and its rate was found to be linearly related to enzyme concentration and reaction time.

Enzyme purification

The source of the lactate dehydrogenase was mycelium of *Phycomyces blakesleeanus* 4 days old. All enzyme fractionation steps were performed at 4°C.

The mycelium was cut into pieces and suspended in 300 ml of 20 mM-sodium phosphate buffer, pH 7.5, containing 0.01% (v/v) 2-mercaptoethanol and homogenized in a Sorvall homogenizer for 20 min. The homogenized mycelium was centrifuged at 20 000 g for 25 min and the pellet was discarded. The clear extract was stirred while solid $(\text{NH}_4)_2\text{SO}_4$ was added to adjust the suspension to a concentration of 30% saturation. After being stirred for 15 min the extract was centrifuged at 20 000 g for 15 min. The precipitate, devoid of lactate dehydrogenase activity, was discarded, and the supernatant was treated with solid $(\text{NH}_4)_2\text{SO}_4$ to increase the concentration to 60% saturation. The second precipitate, collected by centrifuging at 20 000 g for 15 min, was resuspended in 30 ml of 20 mM-Tris/HCl buffer, pH 7.4, containing 0.01% (v/v) 2-mercaptoethanol.

Fractions of this clear extract were each diluted to 100 ml with a content of 1.4 mg of protein/ml and stirred while calcium phosphate gel (0.9 g) was added. After being stirred for 10 min the gels were collected by centrifugation at 7000 g for 5 min and discarded. Each supernatant was treated with further calcium phosphate gel (0.9 g), which was similarly collected and discarded. The supernatants were then again treated with calcium phosphate gel (1.8 g), and the gels were collected as described above but this time retained. Each gel was resuspended in 30 ml of 20 mM-potassium phosphate buffer, pH 7.8, and after standing for 10 min the gels were collected by centrifuging at 7000 g for 5 min and the supernatants discarded. The gels were each treated with

30 ml of 0.1 M-potassium phosphate buffer, pH 7.8, and after standing for 10 min the calcium phosphate gels were removed by centrifugation at 7000g for 5 min and the supernatants containing lactate dehydrogenase activity collected.

Fractions (2.5 ml) of this last extract were applied to a column (2.5 cm × 35 cm) of Sephadex G-200 that had been equilibrated with 20 mM-sodium phosphate buffer, pH 7.5. The column was calibrated with Blue Dextran (M_r 2×10^6), rabbit muscle pyruvate kinase (M_r 230 000), bovine serum albumin (M_r 67 000), carbonic anhydrase (M_r 30 000) and insulin (M_r 5700). Enzyme activity was diluted for the column with the equilibrating buffer. The enzyme emerged in a volume of eluate corresponding to a molecular weight of 135 000–140 000, estimated by a plot of log (molecular weight) against K_{av} . Those tubes that contained significant enzyme activity were pooled and stored at -20°C after being adjusted to 0.01% (v/v) 2-mercaptoethanol.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was used as a means of evaluating the purity of the enzyme. It was performed in 5% acrylamide gels, pH 8.3, by a technique similar to that of Davis (1964). After electrophoresis one set of gels was stained with Coomassie Brilliant Blue for total protein and another set for lactate dehydrogenase activity by the tetrazolium method (LéJohn, 1971).

For polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, gels containing 10% acrylamide and 1% sodium dodecyl sulphate were prepared and run essentially as described by Weber *et al.* (1972). After electrophoresis gels were stained with Coomassie Brilliant Blue.

Results

Lactate dehydrogenase activity in crude preparations

The amounts of lactate dehydrogenase activity from mycelium and sporangiophores of *Phycomyces blakesleeanus* N.R.R.L. 1555 (–) were

determined. Lactate dehydrogenase activity per mg of protein in crude preparations from mycelium was approximately 5-fold greater than that obtained from 4-day-old sporangiophores.

We have partially purified lactate dehydrogenase activity from mycelium and sporangiophores by following the same method. A summary of the purification scheme is shown in Table 1. The above procedure resulted in a 700-fold increase in the lactate dehydrogenase activity with approx. 25% recovery.

Polyacrylamide-gel electrophoresis revealed a single cathodic protein band with a coincidence in the relative migration value of lactate dehydrogenase activity and protein. In the presence of sodium dodecyl sulphate, the migration was detected of one component with an estimated molecular weight of 35 000. This is in agreement with the estimated molecular weight obtained from Sephadex G-200 chromatography, assuming that the one component obtained in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis corresponds to the monomeric form of the enzyme.

Substrate and coenzyme specificity of lactate dehydrogenase

Lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (–) catalyses the NADH-dependent conversion of pyruvate into L(+)-lactate. The reduction of pyruvate was tested with either NADH or NADPH. Although oxidation of NADPH was observed when equimolar concentrations were substituted for NADH in the standard reaction mixture, the rate of reaction was less than 0.5% of that obtained when assayed by oxidation of NADH (Table 2). The rate of pyruvate-dependent NADH oxidation was maximal between pH 6.7 and 7.5. The rate of the reverse reaction was approx. 45-fold lower and showed marked specificity for L(+)-lactate and NAD⁺ (Table 2) at pH 7.5. Therefore lactate dehydrogenase appeared to catalyse preferentially the pyruvate reduction associated with NADH oxidation.

Table 1. Summary of a purification of lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (–)

The fractionation and assay procedures are described in the Methods section. A unit of enzyme activity is defined as that amount of enzyme to catalyse the oxidation of 1 μmol of NADH/min at 30°C .

Fraction	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)	Purification (fold)
Extract	422.5	1.3	100	1.0
(NH ₄) ₂ SO ₄ fractionation	355.3	2.2	85	1.7
Calcium phosphate gel eluate	211.3	13.2	50	9.5
Sephadex G-200 peak	106.0	924.0	25	695.5

Homotropic interactions of lactate dehydrogenase with pyruvate

Lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* showed sigmoidal kinetics with

Table 2. Substrate- and coenzyme-specificity of lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* N.R.R.L. 1555 (—)

Experiments were performed in 20 mM-sodium phosphate buffer, pH 7.5, at 30°C, and the L(+) and D(—)-lactate-dependent reduction of NAD⁺ and pyruvate-dependent oxidation of NADH catalysed by lactate dehydrogenase were assayed by monitoring the reduction of NAD⁺ or oxidation of NADH at 340 nm respectively. For the experiments with lactate as substrate the reaction mixtures contained L(+) or D(—)-lactate (20 mM), NAD⁺ (3 mM) and 510 µg of protein in a reaction volume of 3.0 ml. Equimolar amounts of NADP⁺ were substituted for NAD⁺ in the standard reaction mixture where indicated. For the experiments with pyruvate as substrate the incubation mixture contained 510 µg of protein, pyruvate (20 mM) and NADH (0.250 mM) in a reaction volume of 3.0 ml. Equimolar amounts of NADPH were substituted for NADH in the standard assay where indicated.

Substrate	Lactate dehydrogenase activity (µmol of product/min)
L(+)-Lactate + NAD ⁺ + NADP ⁺	0.078 ≤ 0.003
D(—)-Lactate + NAD ⁺ + NADP ⁺	≤ 0.003 ≤ 0.003
Pyruvate + NADH + NADPH	3.670 0.011

respect to pyruvate concentration at pH 7.5 (Fig. 1a), indicating positive co-operativity. This view was supported by a Hill plot (Fig. 1b), which gave a line with a maximal slope (Hill coefficient, *h*) close to 2, indicating more than two pyruvate-binding sites in the enzyme (Stadtman, 1966; Cornish-Bowden & Koshland, 1975; Cadenas, 1978). On the assumption that there is only binding co-operativity, the Hill plot allowed an estimation of the values for the first (¹*k_s*) and last (^s*k_s*) intrinsic association constants. [¹*k_s* represents each of the intrinsic association constants, where *s* indicates the number of binding sites in the enzyme for the ligand and *i* indicates the number of binding sites that are occupied by ligand (Cadenas, 1978)]. The ^s*k_s* value obtained for pyruvate remained unchanged when the coenzyme concentration varied.

Lactate dehydrogenase showed marked high pyruvate inhibition at pH 6.0 (Fig. 2), which decreases as coenzyme concentration in the reaction mixture is decreased.

The experiments conducted at both pH values with lactate dehydrogenase from sporangiophores of *Phycomyces blakesleeanus* exhibited an analogous kinetic pattern (Table 3).

Kinetic response of lactate dehydrogenase with other 2-oxo acids

Evidence of high substrate inhibition for lactate dehydrogenase of *Phycomyces blakesleeanus* with 2-oxo acids analogous to pyruvate at pH 6.0 and fixed NADH concentration (0.250 mM) was obtained. Experiments performed with 3-hydroxypyruvate, 2-oxovalerate and glyoxylate showed high substrate inhibition for 3-hydroxypyruvate and 2-oxovalerate in the concentration range studied (0.1–50 mM). However, the 2-oxo acid concen-

Fig. 1. Kinetic response of lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* to pyruvate at pH 7.5 (a) The standard activity was assayed as described in the Methods section. The pyruvate concentration was varied as shown. The incubation mixture contained 5 µg of protein. O, 0.125 mM-NADH; ●, 0.250 mM-NADH. (b) Hill plot of the data shown in (a). The V_{max} values were obtained from a Scatchard plot.

Table 3. Summary of the data obtained from Hill plots of the pyruvate-dependent oxidation of NADH catalysed by lactate dehydrogenase from mycelium and sporangiophores of *Phycomyces blakesleeanus* N.R.R.L. 1555 (—) Experiments were performed in 20 mM-sodium phosphate buffer at the pH values indicated at 30°C. The V_{max} values for the Hill plots were obtained from the Scatchard plots. The incubation mixture contained (a) 39 μ g of lactate dehydrogenase from mycelium or (b) 5 μ g of lactate dehydrogenase from sporangiophores of *Phycomyces blakesleeanus*.

		pH 6.0			pH 7.5		
		<i>h</i>	1k_s (M ⁻¹)	sk_s (M ⁻¹)	<i>h</i>	1k_s (M ⁻¹)	sk_s (M ⁻¹)
Variable NADH concentrations (0.01–0.3 mM) and fixed pyruvate concentration (20 mM)	(a)	2.9	8.9×10^2	2.8×10^4	2.3		1.7×10^4
	(b)	2.6		2.8×10^4	2.5	2.2×10^2	7.7×10^3
Variable pyruvate concentrations (1–20 mM) and fixed NADH concentration (0.250 mM)	(a)	High substrate inhibition			2.2		1.9×10^2
	(b)				1.7	3.5×10	1.5×10^2

Fig. 2. Kinetic response of lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* to pyruvate at pH 6.0

The standard activity assay as described in the Methods section was used, except that the pH value and pyruvate concentration were those shown. The incubation mixture contained 39 μ g of protein. O, 0.0625 mM-NADH; ●, 0.125 mM-NADH.

trations from which the inhibitory effect was noticeable were 4 mM and 20 mM for 3-hydroxypyruvate and 2-oxovalerate respectively, in contrast with pyruvate, which showed this effect at 1.5 mM under the same experimental conditions. Apparent K_m values, obtained from double-reciprocal plots, were in the region of 10^{-4} mM for pyruvate and 3-hydroxypyruvate, whereas that for 2-oxovalerate was more than 10-fold greater. Apparent V_{max} values were similar for pyruvate and 3-hydroxypyruvate, whereas that for 2-oxovalerate was over 15–20-fold smaller. Furthermore, glyoxylate exhibits no inhibition in the range of concentration studied (1–300 mM) and shows hyperbolic kinetics. The apparent K_m value, from a double-reciprocal plot, obtained for glyoxylate was more than 100-fold that for pyruvate. The values obtained are shown in Table 4.

Table 4. Apparent kinetic constants of four substrates of lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus*

Each incubation mixture contained NADH (0.025 mM), varied 2-oxo acid concentrations in 20 mM-sodium phosphate buffer, pH 6.0, and 9 μ g of protein in a reaction volume of 3.0 ml. The assays were performed at 30°C.

Substrate	K_m (M)	$10^3 \times V_{max}$ (μ mol of NADH oxidized/min)
Pyruvate	2.0×10^{-4}	203.5
3-Hydroxypyruvate	3.4×10^{-4}	204.8
2-Oxovalerate	5.2×10^{-3}	12.0
Glyoxylate	7.2×10^{-2}	263.0

Homotropic interactions of lactate dehydrogenase with NADH

Lactate dehydrogenase showed a sigmoidal kinetic response to NADH at pH 6.0 and 7.5 (Figs. 3a and 3b). Hill plots of the data obtained from plots of v versus [NADH] gave lines with a slope (Figs. 4a and 4b) (h) greater than 2. This value remained constant when pyruvate concentration varied. The findings support the existence of various NADH-binding sites in the enzyme. The value obtained for the last (sk_s) intrinsic association constant was in the region of 10^4 M⁻¹. The value of the first (1k_s) intrinsic association constant was found to be in the region of 10^2 M⁻¹ when its evaluation was feasible. The results obtained for lactate dehydrogenase of sporangiophores (Table 3) led to identical conclusions. The experiments performed with a derivative of the coenzyme, reduced acetylpyridine–adenine dinucleotide, also showed sigmoidal kinetics at pH 7.5 and fixed pyruvate concentration (20 mM). The values obtained for the Hill coefficient (h) and the last intrinsic association constant were similar to the ones obtained with NADH.

Effects of ATP and GTP on lactate dehydrogenase

The effects of ATP and GTP on L-(+)-lactate

Fig. 3. Kinetic response of lactate dehydrogenase from mycelium of *Phycomyces blakesleeanus* to NADH at (a) pH 6.0 and at (b) pH 7.5

(a) The standard activity was assayed as described in the Methods section. The NADH concentration was varied as indicated. The incubation mixture contained 39 μ g of protein. $\blacktriangle$, 0.25 mM-Pyruvate; $\bullet$, 20 mM-pyruvate. (b) The standard activity was assayed as described in the Methods section. The NADH concentration was varied as indicated. The incubation mixture contained 5 μ g of protein. $\blacktriangle$, 10 mM-Pyruvate; $\bullet$, 20 mM-pyruvate.

Fig. 4. Hill plots of the data shown in (a) Fig. 3(a) and (b) Fig. 3(b)

(a) The $V_{\max}$ values were obtained from Scatchard plots. $\blacktriangle$, 0.25 mM-Pyruvate; $\bullet$, 20 mM-pyruvate. (b) The $V_{\max}$ values were obtained from Scatchard plots. $\bullet$, 10 mM-Pyruvate; $\circ$, 20 mM-pyruvate.

dehydrogenase from *Phycomyces blakesleeanus* were tested at pH 6.0 and 7.5. The nucleotides were added to the reaction mixture at different concentrations, and pyruvate reduction associated with NADH oxidation was measured as described in the Methods section.

Lactate dehydrogenase showed inhibition by ATP at pH 6.0 and 7.5, the effect being less pronounced at pH 7.5 (Fig. 5a). The nucleotide appeared to exert a slight competitive inhibition with respect to both pyruvate and NADH at pH 7.5, whereas at pH 6.0

ATP showed non-competitive inhibition (Fig. 6) with respect to pyruvate and only could exhibit competitive inhibition with respect to NADH at relatively high (5 mM) ATP concentration. A Hill plot of the data shown in Fig. 5(a) gave two lines with different slopes at pH 6.0 and 7.5, indicating that the pH value affects the degree of binding co-operativity for ATP (Fig. 5b). In turn, low concentrations of ATP appeared to affect the degree of co-operativity for NADH binding, since it lowers the corresponding h values (Table 5).

Fig. 5. Kinetic response of lactate dehydrogenase from *Phycomyces blakesleeanus* to ATP at two pH values (a) The standard lactate dehydrogenase assay as described in the Methods section was used, except ATP was included in the reaction mixture at the concentrations indicated. Each reaction mixture contained 6.5 μ g of protein. O, pH 6.0; ●, pH 7.5. (b) Hill plots of the data shown in (a).

Fig. 6. Non-competitive inhibition by ATP at pH 6.0 with respect to pyruvate at different ligand concentrations. The concentration of protein used was 1 μ g. NADH concentration was 0.250 mM. ATP concentrations were as specified in the Figure.

No significant activation or inhibition was observed with GTP at concentrations of 0.5 and 1.0 mM at both pH values.

Discussion

Lactate dehydrogenase activity from mycelium and sporangiohores of *Phycomyces blakesleeanus* N.R.R.L. 1555 (—) was identified. The amount of lactate dehydrogenase activity per mg of protein from 4-day-old mycelium was approximately 5-fold greater than that obtained from sporangiohores. Mycelium was therefore used as routine source of lactate dehydrogenase activity. Gleason *et al.* (1966) reported that many fungi are capable of growth in anaerobic or microaerobic conditions. Lactic fermentation could be a means to obtain the energy, and therefore one would expect that lactate dehydrogenase activity could play an important regulatory role.

The kinetic properties of the preparation reported in the present paper and obtained from mycelium of *Phycomyces blakesleeanus* are consistent with its designation as NAD⁺-specific L(+)-lactate dehydrogenase; the enzyme preparation is active with L(+)-lactate but not with D(–)-lactate, and at pH 7.5 the ratio apparent $V_{\max}$ (lactate oxidation)/apparent $V_{\max}$ (pyruvate reduction) is 0.02. Likewise the enzyme preparation catalyses preferably the pyruvate reduction associated with NADH oxidation, since the ratio between apparent $V_{\max}$ obtained with NADH and NADPH is 32.5 at pH 7.5. Gleason *et al.* (1966) and LJohn (1971) described D(–)-lactate dehydrogenases in several Subclasses of Phycomycetes (Oomycetes, Hypochytridiomycetes and

Table 5. Effect of ATP on NADH binding to lactate dehydrogenase at pH 6.0

Lactate dehydrogenase activity was assayed as described in the Methods section except that the reaction mixture contained the ATP concentrations listed. NADH concentrations were varied from 0.010 mM to 0.250 mM. Each reaction mixture contained 1 μ g of protein. The h values from NADH were estimated from Hill plots.

ATP (mM)	[NADH] _{0.5} (mM)	h
0	0.03	3.0
0.5	0.05	1.8
2.0	0.09	2.1
5.0	34.00	2.9

Chytridiomycetes). *Phycomyces* belongs to the lower fungi. This group, which used to have the status of a 'Class' (Phycomycetes), is now recognized as a taxonomic artifact. The lower fungi have accordingly been re-assigned to a group of Classes. In this scheme, then, *Phycomyces* belongs to the Class Zygomycetes (Order Mucorales) (Bergman *et al.*, 1969). L(+)-Lactate dehydrogenases have been also described in *Actinomyces viscosus* (Brown *et al.*, 1975), *Rhizobium meliloti* (Trinchant & Rigaud, 1974) and ten strains of *Mycoplasma mycoides* (Allsopp & Matthews, 1975). In addition, *Phycomyces blakesleeanus* L(+)-lactate dehydrogenase has a molecular weight of 135 000–140 000 and appears to consist of four subunits, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, which is in good agreement with the results obtained for L(+)-lactate dehydrogenases from several sources (Eichner & Kaplan, 1978; Dixon & Webb, 1979).

The pH optimum for the pyruvate-dependent oxidation of NADH was between pH 6.8 and 7.5. Kinetic studies conducted at pH 7.5 showed that reaction rate exhibited a sigmoidal dependence on pyruvate concentration as well as on NADH concentration. The existence of positive co-operativity in the binding of these ligands, substrate and coenzyme, in the absence of an allosteric effector could be a possible explanation of this kinetic pattern. Nevertheless, a sigmoid dependence between velocity and substrate concentration can arise for several purely kinetic reasons in the absence of any co-operative binding phenomenon. The preferred order for a two-substrates enzyme mechanism suggested by Ferdinand (1966) that provides such dependence cannot explain our results, since we show sigmoidal curves with either substrate at pH 7.5, at lower and higher fixed concentration of the other substrate. At pH 6.0 there is sigmoidal dependence with NADH, whereas pyruvate exhibits substrate inhibition. Models based on isomerization reactions between two or more enzymic forms (Rabin, 1967; Ricard *et al.*, 1974) also could give a behaviour that resembles positive co-operativity. On the other hand, sigmoidal kinetics for pyruvate have been reported for lactate dehydrogenases from *Streptococcus* mutants (Brown & Wittenberger, 1972), *Acholeplasma laidlawii* (Neimark & Tung, 1973) and *Escherichia coli* (Tarny & Kaplan, 1968), whereas lactate dehydrogenase from *Actinomyces viscosus* (Brown *et al.*, 1975) exhibited homotropic interactions with pyruvate and NADH. From the results obtained in the present work and the fact that the enzyme from *Phycomyces blakesleeanus* and other sources is in a tetrameric form, we assume that NADH and pyruvate bind in a positive co-operative manner. In addition, experiments not presented here showed that the observed co-operativity was not

dependent on protein concentration (protein concentration range between 3.0 and 28 $\mu\text{g/ml}$), and hence the association–dissociation model suggested by Frieden (1967) and Nichol *et al.* (1967) is excluded as a possible explanation.

The existence of these positive homotropic interactions at pH 7.5 becomes more evident when Hill plots are used to analyse the kinetic data. The h values of 2.0 and 2.3 for pyruvate and NADH respectively suggest the existence of more than two binding sites for substrate as well as for coenzyme. It is possible that the number of binding sites for both ligands could be four. Our results indicate that binding sites are not independent but that there is some degree of interaction between them. From Hill plots, and on the assumption that there is only binding co-operativity, were determined the first and last intrinsic association constants when their evaluation was feasible. The last intrinsic association constant (*k_s) for pyruvate was in the region of 10^2 M^{-1} whereas that for NADH was more than 100-fold greater.

At pH 6.0 sigmoidal kinetics with NADH were quite marked, showing h values close to 3. The last intrinsic association constant value was unchanged, the first intrinsic association constant being in the region of 10^2 M^{-1} in this case. However, at this pH value lactate dehydrogenase showed a marked inhibitory effect by high concentrations of pyruvate. At 30°C and 0.125 mM-NADH, for 1.5 mM-pyruvate substrate inhibition was observed. By using 2-oxo acids analogous to pyruvate it was shown that at this pH value 3-hydroxypyruvate and 2-oxovalerate showed substrate inhibition, though a high substrate concentration was needed for the inhibitory effect to be noticeable. However, lactate dehydrogenase exhibited a normal dependence on substrate concentration when glyoxylate was used. Several authors (Burgner *et al.*, 1978; Burgner & Ray, 1978; Wilton, 1979) have proposed the formation of a ternary complex between enzyme–NAD⁺ and pyruvate (enol) to explain high substrate inhibition. Our results are consistent with this interpretation, and in support of the same view is the fact that glyoxylate, which is not able to undergo keto–enol tautomerism, is the only assayed substrate that fails to show high substrate inhibition.

ATP binds co-operatively to the lactate dehydrogenase from *Phycomyces blakesleeanus*, indicating that there are several binding sites on the enzyme for ATP at pH 7.5 and 6.0. In addition, ATP inhibits the enzyme at both pH values. Under slightly acid conditions ATP was a more potent inhibitor than at neutral ones, showing that ATP³⁻ is effective rather than ATP⁴⁻. ATP competes for the NADH-binding sites, and in addition at pH 6.0 the inhibition appears to be allosteric and therefore there is an allosteric site for ATP. Lactate dehydro-

genase, however, was not influenced by GTP at either pH value.

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