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PLANT LACTATE DEHYDROGENASE: NADH KINETICS AND INHIBITION BY ATP

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Key Word Index—*Brassica rapa*; Cruciferae; wild turnip; *Allium porrum*; Liliaceae; cultivated leek; lactate dehydrogenase; kinetics.

Abstract—Lactate dehydrogenase (LDH), isolated from either turnip (*Brassica rapa*, Cruciferae) or from leek (*Allium porrum*, Liliaceae) shows normal Michaelis–Menten kinetics with NAD^+ in the lactate dehydrogenase direction, but non-hyperbolic kinetics with NADH in the pyruvate reductase direction. These kinetics are not 'sigmoidal' and appear consistently as two intersecting straight lines in reciprocal plots, representing a sharp decline in the effectiveness of NADH as a substrate in its lower concentration range. The overall apparent affinity for NADH decreases with decreasing pH. Competitive inhibition by ATP, which is much stronger than that characteristic of mammalian LDH, also displays kinetics that seem to be biphasic, but the K_i value does not vary significantly with pH in the physiological range. The counter-inhibitory effect of Mg^{2+} ions is shown to be due to the formation of non-inhibitory Mg–ATP complexes. The dissociation of this is strongly pH dependent and this results in most of the ATP being complexed, and therefore non-inhibitory at pH 7.4 (the normal pH in plant cytosol) but largely uncomplexed and inhibitory at pH 6.4 (probably a lower cytosolic pH limit). These factors, and particularly the last, combine to inhibit the enzyme more strongly as the pH drops (as a result of lactate-terminating anaerobic glycolysis) and may act as an effective negative feed-back mechanism in plant cells, allowing a pH-activated switchover to ethanol-terminating glycolysis while preventing 'overshoot' into harmful acidosis. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Davies and Davies [1], Davies *et al.* [2] and Betsche [3] found that lactate dehydrogenase (LDH) of potato and lettuce displays 'non-linear' kinetics with NADH. These workers also noted the strong inhibition of the plant enzyme by ATP. Doubt was cast on any possibility of a physiological role by the effect of Mg^{2+} in relieving the inhibition [3]. Using LDH isolated from turnip, and with comparative data for the enzyme from leek and lettuce, our conclusions differ from those of previous workers and point to significant metabolic roles for all of these phenomena in controlling the pH-switch mechanism in anaerobic glycolysis.

RESULTS AND DISCUSSION

Non-hyperbolic kinetics

'Non-linear' kinetics has been reported for LDH from potato tuber and lettuce leaf [1, 3], though at unphysiologically low pH values. These kinetic deviations were either treated as sigmoidal, e.g. by the use of Hill plots, or were subjected to other empirical methods of processing that appeared to 'linearize' the data (e.g. reciprocal plots of the square of the substrate concentration). The LDH preparations we have isolated from turnip, leek and lettuce display normal Michaelis-Menten-type kinetics with pyruvate, lactate and their analogues [4] and also with NAD^+ [Fig. 1(A)] in the pH range 6.4–7.4. However, NADH, especially in the presence of ATP, displays 'non-hyperbolic' kinetics, that appear as biphasic patterns in reciprocal plots, rather than the parabolic ones characteristic of 'sigmoidal' kinetics. The reciprocal kinetics at the higher velocities are quite linear, and they are also linear at lower velocities but with a different slope, suggesting a lower intrinsic effectiveness of NADH as a substrate in its lower concentration range. This biphasic pattern is apparent under varied conditions that change the overall affinity of the enzyme for NADH considerably, e.g. when the pH is varied (see below) or in the presence of competitive inhibitors such as ATP [Fig. 1(B)]. Other methods of reciprocal plotting of these data, such as Hanes plots [5, 6], also yield biphasic patterns.

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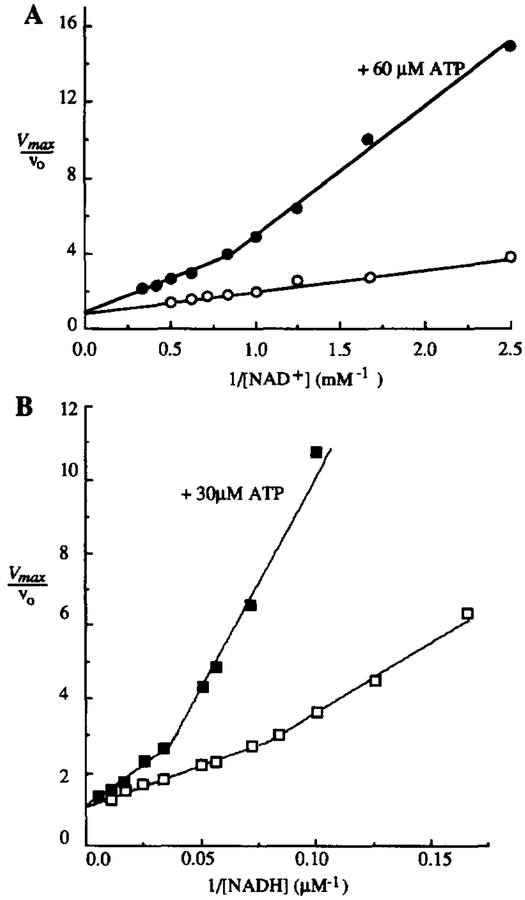

Fig. 1. Double reciprocal plots of the kinetics of ATP inhibition of LDH from turnip with: **A**, NAD$^+$ (substrate) in the lactate dehydrogenase direction and **B**, NADH (substrate) in the pyruvate reductase direction. Assays were performed in triplicate at 30° in 100 mM K-Pi buffer, pH 7, with 100 mM L-lactate for lactate oxidation and 100 mM K–Pi buffer pH 7.4 with 0.5 mM pyruvate for pyruvate reduction.

At lower pH values outside the physiologically relevant range (e.g. pH 6.4) the intersecting pattern is considerably more pronounced. Statistical evaluation of the individual sets of data (e.g. those shown in Fig. 2) yield excellent correlation coefficients ($R^2 = 1$ or nearly 1) for the data analysed as intersecting straight lines, although it is also possible to fit the data to arbitrary, complex and entirely empirical equations containing higher power terms of the substrate or inhibitor concentrations.

Dixon plots of competitive inhibition by ATP, ADP (and also by GTP) also show biphasic patterns [Fig. 2(A) and (B)], but competitive inhibition by AMP does not [Fig. 2(C)]. It might be concluded that the biphasic patterns with ATP are simply a reflection of the underlying kinetic pattern with NADH. However, ATP is also a competitive inhibitor with respect to NAD$^+$ (in the lactate dehydrogenase direction), and it converts the 'normal' monophasic kinetics with NAD$^+$ to a biphasic pattern [Fig. 1(A)], suggesting that the binding kinetics of ATP are independently biphasic.

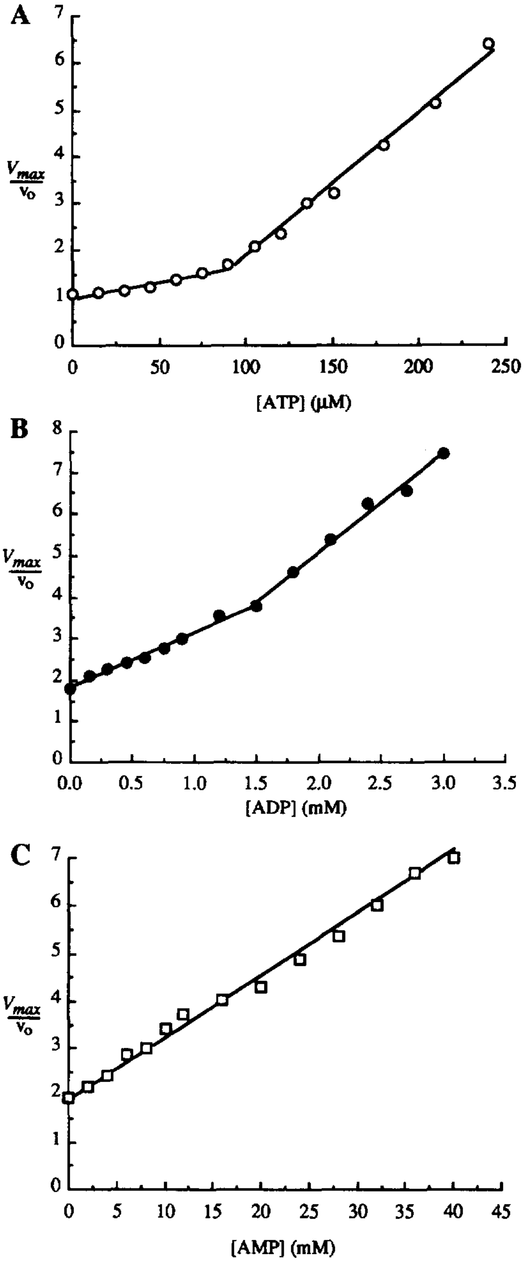

Fig. 2. Dixon plots of inhibition by adenosine phosphates of LDH from turnip in the pyruvate reductase direction. Dixon plots of the inhibition by: **A**, ATP; **B**, ADP; **C**, AMP. All assays were performed in triplicate at 30° in 100 mM K-Pi buffer pH 7.4 with 0.5 mM pyruvate and 200 μM NADH (for plot **A**) or 40 μM NADH (plots **B** and **C**).

The kinetics with NADH indicate co-operative binding, but something unusual is suggested by the sharp discontinuity of the kinetic pattern. Heterogeneity of enzyme preparations cannot be invoked as an explanation since the purified turnip LDH preparation consistently appears as a single very sharp band on polyacrylamide gel electopherograms under a variety of conditions [4]. Furthermore, like the mammalian H_4 and M_4 LDH isoenzymes, the turnip enzyme is a tetramer of identical subunits [4].

Table 1. Inhibition of plant (turnip) and mammalian LDH by ATP and other adenosine phosphates at pH 7.4. All assays were carried out at 30° with 0.5 mM pyruvate and 40 μM NADH (except in the case of ATP inhibition, where 200 μM NADH was used). The buffer used was 100 mM K–Pi buffer

Inhibitor	Turnip LDH*	Mammalian LDH† M_4	H_4
	K_i values (μM)		
NAD^+	5500	470	270
AMP	7800	820	1100
ADP	690	820	950
ATP	21	1200	2000

* The K_i values for the turnip enzyme were determined from Dixon plots. With ATP, ADP and NAD^+ only the line corresponding to higher activities was extrapolated (see text).

† Constants listed for the mammalian isoenzymes are those determined by Geyer [11, 12] for the human enzymes. In the present study, determination of some comparable parameters for the bovine and porcine enzymes, under similar conditions, agreed well with Geyer's values.

Table 2. Inhibition of turnip LDH by ATP: effect of pH on K_i, degree of inhibition, and the counter-inhibitory effect of Mg^{2+}. Assay conditions: 200 μM NADH, 0.5 mM pyruvate, 100 mM ammonium acetate buffer

	pH 7.4	7.0	6.4
1. K_i ATP (μM)	21.0	18.0	22.4
2. K_m NADH (μM)	25	42	105
3. % inhibition* by: 100 μM ATP	49	81	97
4. 100 μM ATP + 1 mM Mg^{2+}	27.4	61.3	94.9
5. 100 μM ATP + 6 mM Mg^{2+}	0.05	6.5	49.5
6. $[ATP^{4-}]/[ATP^{3-}]$	2.7	1.1	0.27

* Note that these % inhibitions are not predictable or interpretable solely on the basis of the kinetic constants given above and the pH variation in K_{diss} for Mg · ATP. The apparent discrepancies are due largely to the complex nonhyperbolic kinetics with NADH and with ATP, the quoted kinetic constants being valid only in the upper velocity range (see text). The kinetics with pyruvate, while themselves hyperbolic are another source of variation of velocity with pH [4] and, thereby, cause further complex pH-dependent modulation of the nonhyperbolic kinetics with NADH.

However, the former have hyperbolic kinetics with both NADH and NAD^+. The most directly comparable kinetic phenomenon seems to be the discontinuous 'multiphasic' kinetic pattern of glutamate dehydrogenase with NAD^+ [7], but extensive studies have failed to provide a clear explanation of the molecular mechanism for the sharp kinetic discontinuities with this enzyme (e.g. [8–10]).

All the kinetics and kinetic constants discussed below, unless otherwise indicated, refer to the linear reciprocal kinetics determined in the higher initial velocity range (lower reciprocal range).

Inhibition by ATP and other adenosine phosphates

Some plant LDH preparations are much more strongly inhibited by ATP than mammalian LDH [1, 3]. We find strong inhibition also of the enzyme from turnip and leek with K_i values in the range 20–25 μM (Table 1). Even lower K_i values have been reported, e.g. 0.2 μM for LDH from lettuce leaf lamina [3], but we have been unable to confirm this result and find much the same kinetics for the enzyme from this source.

AMP can be regarded as half of the NAD molecule and is a relatively weak inhibitor of all the LDH species, weakest in the case of the plant enzyme (Table 1). In the case of both mammalian isoenzymes, addition of the terminal phosphate in ADP and the further phosphate in ATP does not result in significant change in the strength of inhibition of the mammalian isoenzymes (Table 1). With the plant enzyme, however, ATP is far more inhibitory against NADH than is the complete NAD^+ molecule (Table 1). Addition of a further terminal phosphate, as in adenosine tetraphosphate (K_i: 110 μM), decreases the inhibition; while altering the base, as in GTP (K_i: 90 μM), also decreases the inhibition.

Counter-inhibitory effect of Mg^{2+}

Mg^{2+} abolishes the allosteric effects of ATP and GTP on glutamate dehydrogenase at least in part by complexing of the nucleotides by Mg^{2+} [13]. Morrison [14] has suggested that such complexing might have important implications for the physiological relevance of many *in vitro* studies of such nucleotide 'effectors'. The ATP inhibition of the turnip enzyme is also relieved by Mg^{2+} ions and this counter-inhibitory effect is pH dependent (Table 2). We have now analysed this Mg^{2+} effect kinetically. From the change in inhibition by ATP in the presence of varying concentrations of Mg^{2+} and the kinetic constants for NADH and free ATP, we calculated the variation of $[ATP]_{free}$ with $[Mg^{2+}]$ and the apparent dissociation constant for the Mg·ATP complex. These calculations yield a value of 70 ± 5 μM, in agreement with the value of 73 μM determined directly in ref. [13]. This indicates that no other mechanism need be invoked to explain the counter-inhibitory effect of Mg^{2+}. An alternative route to the same conclusion involves using the latter dissociation constant to calculate the concentration of free ATP at various (total) concentrations of ATP used to inhibit LDH in the presence of various concentrations of Mg^{2+}. When such $[ATP]_{free}$ values were used in a Dixon plot, they all fitted on the (biphasic) lines, indicating that only free ATP (and not Mg·ATP or Mg^{2+}) has a direct effect on the activity of the enzyme.

The much weaker inhibition of the plant LDH by ADP is relieved by much higher concentrations of Mg^{2+}, and similar analysis of such data is fully consistent with the much higher dissociation constant of the Mg·ADP complex (cf. footnote to Table 1 of ref. [14]). The inhibition of mammalian LDH by ATP is also relieved by Mg^{2+} and can be attributed entirely to the formation of a non-inhibitory Mg·ATP complex.

Physiological relevance: effect of pH

In Table 2, showing results on the effect of pH on the kinetic parameters, the pH range is chosen for its physiological relevance: from pH 7.4, representing normal cytoplasmic pH, to pH 6.4, which is acidotic and below the level normally tolerated by plant cells [15].

Davies *et al.* [2] proposed that anoxia in plant cells results first in lactate-terminating glycolysis. The initial accumulation of lactate lowers the pH, which activates pyruvate decarboxylase and switches glycolysis to the non-acidifying ethanol-terminating variant which plant cells are also capable of carrying out. The operation of such a mechanism in living plant cells has since been supported by NMR studies [16, 18] and indirectly by other studies (e.g. [18]).

Davies also proposed that this LDH-activated pH-switch should be self regulating to avoid harmful acidosis, but the mechanism proposed, involving pH variation in the K_i for ATP, seemed unconvincing. This is particularly true when, as shown in Table 2 (top row), the turnip enzyme shows no significant pH variation in its K_i with ATP. This is also the case for the enzyme from leek.

However, other parameters can produce the desired pH-dependent inhibition of LDH. Firstly, the K_m for NADH increases significantly with decreasing pH (Table 2, row 2) so that ATP effectively becomes more inhibitory against it. This inhibition is intensified by the biphasic kinetics which result in an abrupt drop in activity once it is below a certain level. Consequently, ATP appears far more inhibitory than might otherwise be expected at the lower pH (Table 2, row 3).

The influence of pH on the Mg^{2+} effect is probably more important. At levels of Mg^{2+} that might be expected in the cell, the inhibition by ATP is practically abolished at pH 7.4, but only partly effective at pH 6.4. This is because ATP has a proton dissociation constant of 6.97 and at pH 7.4 exists mostly in the ATP^{4-} form (Table 2, bottom row) which has a high affinity for Mg^{2+} (K_{diss}: 14.3 μM). But at pH 6.4 the situation is reversed, ATP now being predominantly in the ATP^{3-} form which has a much lower affinity for Mg^{2+} (K_{diss}: 1.44 mM). (For relevant dissociation constants, see footnote to Table II of ref. [14].) All these parameters combine to inhibit LDH progressively more strongly with decreasing pH, as shown in rows 4 and 5 of Table 2, and could act as the feedback control mechanism. Such a mechanism envisages LDH on 'active standby' (i.e. uninhibited) under normal aerobic conditions with the cytosolic pH at about 7.4. The biphasic kinetics with NADH may serve to minimise pyruvate reduction under such conditions when the cytosolic NADH concentration would be low. In anoxic conditions, the rise in NADH concentrations above a critical level would result in a disproportionate increase in the rate of reduction of pyruvate to lactate. The resulting drop in pH, which activates the alternative alcohol-producing glycolysis, would simultaneously release ATP from its Mg-complexed state causing inhibition of pyruvate reduction and limiting further decrease in pH.

Proposed control of plant LDH as a 'pH switch' in anaerobic glycolysis

The ideas discussed in this and in ref. [4] are outlined in Fig. 3. It is envisaged that: under aerobic conditions (Phase 1) the enzyme is inactive because of the low levels of NADH and pyruvate, the high pH (7.4), and concerted inhibition by high levels of NAD^+ and oxalacetate (OAA). Hypoxia (Phase 2) results in relief of the inhibition as OAA is converted to the much less inhibitory malate, and NADH consequently accumulates while NAD^+ decreases (thus releasing the concerted inhibition). Owing to the non-hyperbolic kinetics of plant LDH with NADH, even a relatively small increase in the concentration of NADH can result in a disproportionate increase in the pyruvate reductase activity of LDH. LDH now converts pyruvate to lactate with a resulting decrease in cytoplasmic pH (Phase 3). This causes activation of ethanol-terminating glycolysis, largely because pyruvate decarboxylase only becomes active below pH 7.0 [2]. Simultaneously, the lower pH releases ATP from the ATP–magnesium complex and this free ATP strongly inhibits LDH thereby preventing further decreases of pH and shunting pyruvate into the ethanol-terminating sequence. As suggested in ref. [4] if any cytoplasmic component of the system responsible for regeneration of NAD^+ from NADH (e.g. the malate shuttle) were inhibited by rising pH, Phase 2 and, partly, Phase 3 of this scheme could effectively come into play, resulting in a readjustment of the pH downward, thus providing a pH-stat keeping the cytoplasmic pH constant under normal aerobic conditions.

EXPERIMENTAL

Chemicals. All of the general materials used in this study were obtained from Sigma.

Purification of plant LDH. Affinity chromatographic purification of LDH from *Brassica rapa* was carried out by a rapid method based on the immobilised oxamate system of refs [4, 19, 20], the enzyme being purified to homogeneity within *ca* 4 hr

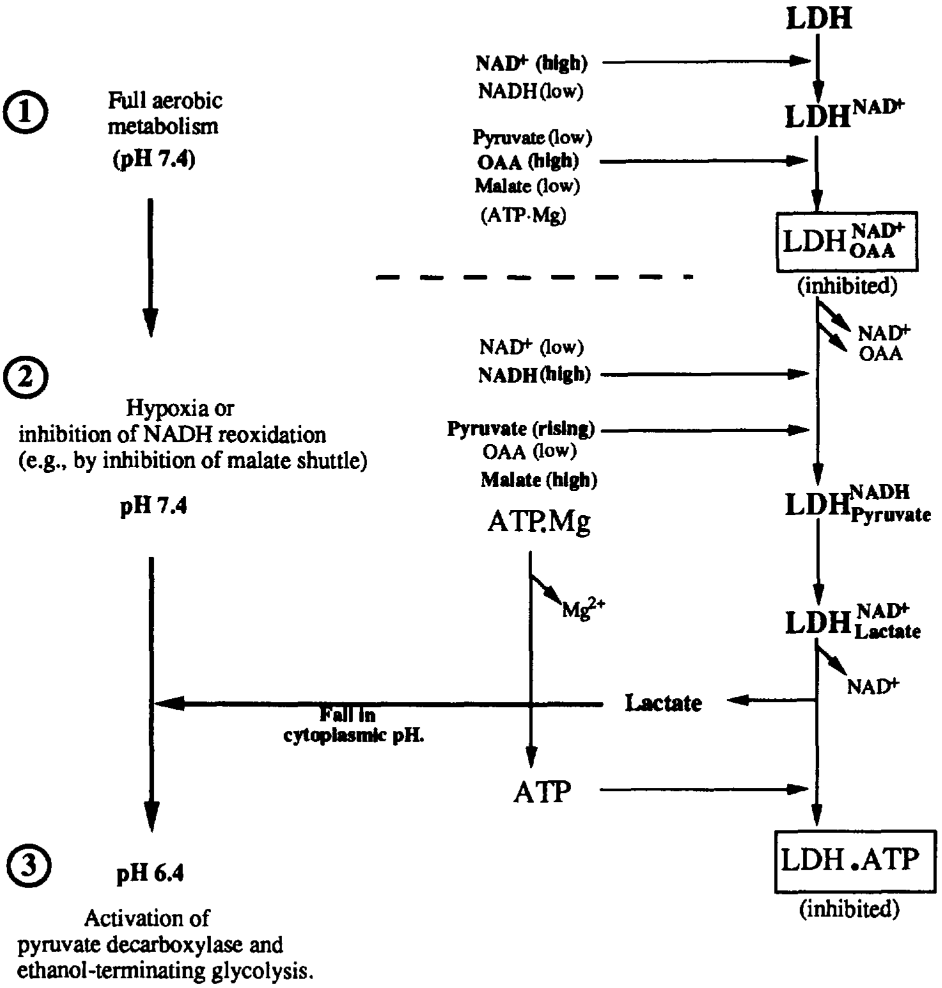

Fig. 3. Outline of suggested mechanism for control of the action of LDH as a 'pH switch' in anaerobic glycolysis. (OAA: oxalacetate).

of extraction. LDH-containing frs were passed through a Sephadex G-25 gel filtration column prior to kinetic analysis in order to remove the contaminating NADH.

Since Mg^{2+} complexes with phosphate, it was necessary to avoid Pi buffer when studying the counter-inhibitory effect of Mg^{2+}. Alternative buffers proved unsatisfactory for other reasons (Tris and imidazole caused inhibition and/or instability of the enzyme). Therefore, NH_4OAc buffers were used instead, the LDH preparation being equilibrated into such buffers using a Sephadex G-25 gel-filtration column. Since NH_4OAc has a poor buffering capacity, pH values were carefully monitored, and if necessary adjusted.

Enzyme assay and evaluation of kinetic analysis. LDH was assayed in triplicate at 30° by measuring the decrease or increase of A of NADH at 340 nm. All sets of kinetic data were fitted to straight lines by linear regression analysis using a Macintosh Cricket Graph program. The resulting fits and kinetic constants were concurrently evaluated statistically, R^2 being better than 0.99, and usually 1.0, for all the data presented. In connection with the biphasic kinetics, this latter point only applies to the individual straight line sections of the plots.

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