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PURIFICATION AND SUBSTRATE KINETICS OF PLANT LACTATE DEHYDROGENASE

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Key Word Index—*Brassica rapa*; Cruciferae; turnip; *Lactuca sativa*; Compositae; lettuce; lactate dehydrogenase affinity purification.

Abstract—Lactate dehydrogenase (LDH) from turnip (*Brassica rapa*; Cruciferae), purified to electrophoretic homogeneity using affinity chromatography, has a native M_r of 157×10^3 and a subunit M_r of 38×10^3. The LDH from turnip shows the same relative effectiveness (relative V_{max} and K_m values) as the mammalian H_4 and M_4 isoenzymes with pyruvate, lactate and glyoxylate (oxoacetate and dihydroxyacetate) as substrates. All three LDH types show no activity with glycolate (hydroxyacetate). The affinities for these and a range of competitive inhibitory analogues shows a consistent pattern of highest affinity for the H_4 mammalian isoenzyme, medium affinity for the M_4 form and lowest affinity for the plant enzyme, in a ratio of about 10:3:1, respectively. The catalytic mechanism of the plant enzyme is very similar to that of the mammalian forms. The major physiological activity of the plant LDH is considered to be pyruvate reduction, rather than the disproportionation of glyoxylate that has been proposed as a plant cell pH-stat. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The classic work of Poerio and Davies [1] showed considerable similarities between mammalian lactate dehydrogenase (LDH) (EC 1.1.1.27) and that from potato tuber, although Rothe [2] disagreed on a number of points. The kinetic and molecular studies on the enzyme from lettuce leaf, barley and soybean seedlings [3–6] have supported Davies' conclusions. Here we present a kinetic-mechanistic study of the oxo/hydroxyacid substrates (Fig. 1) of the enzyme from turnip (*Brassica rapa*) together with some parallel comparative studies of the mammalian isoenzymes. Our results support the view that the plant enzyme is kinetically and mechanistically similar to the vertebrate isoenzymes.

RESULTS AND DISCUSSION

Affinity purification of plant lactate dehydrogenase

LDH occurs at low levels in plants [7] and therefore it can be difficult to obtain sufficient quantities of pure enzyme. An affinity system for L-LDH has been developed by O'Carra and Barry [8–10] and employs an oxamate derivative immobilised using a C_6-spacer (Fig. 2). This system depends upon the specificity of LDH for NADH and oxamate, and also on the kinetic mechanism of the enzyme. LDH from higher plants behaves in a similar manner to L-lactate specific LDH from vertebrates, invertebrates and bacteria, however, the plant LDH tends to leak from the affinity matrix in the presence of NADH (e.g. Fig. 2). This is due to a lower affinity of the plant enzyme for pyruvate/oxamate. Nevertheless, it was found that LDH from a number of plant sources (including turnip, leek, spring onion and lettuce) could be purified in a single affinity chromatographic step after ammonium sulphate fractionation. With leek crude extracts about 90% of the LDH could be recovered in a purified form, however, with other plant extracts lower recoveries (*ca* 75%) were usual owing to leakage. LDH preparations from these sources, after the affinity chromatographic purification, were found to be homogenous by the usual electrophoretic criteria [e.g. Fig. 3(A)]. The rapidity of this purification procedure may serve to reduce the possibility of artifactual alteration of the enzyme during isolation. This is particularly important in view of the interesting non-linear kinetics of the enzyme [11]. (Such phenomena with other enzymes have sometimes been ascribed

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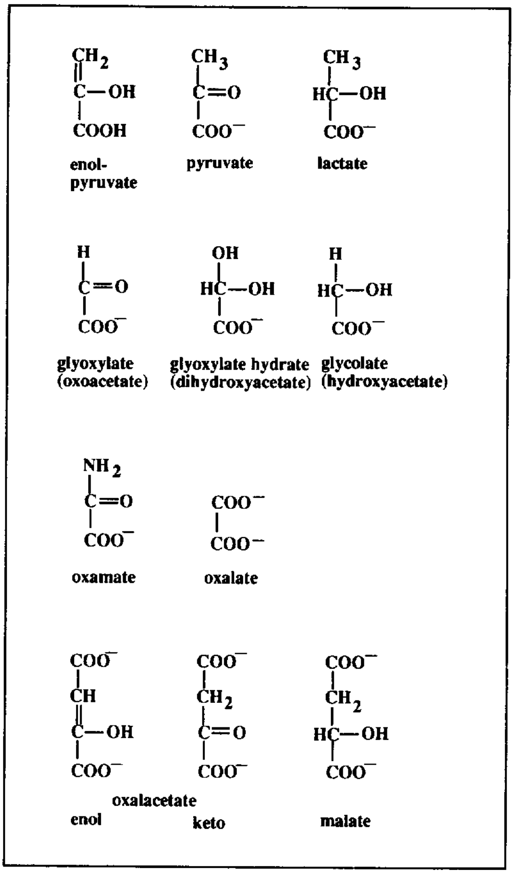

Fig. 1. Structures of the oxo/hydroxyacid substrates of lactate dehydrogenase and their inhibitory analogues.

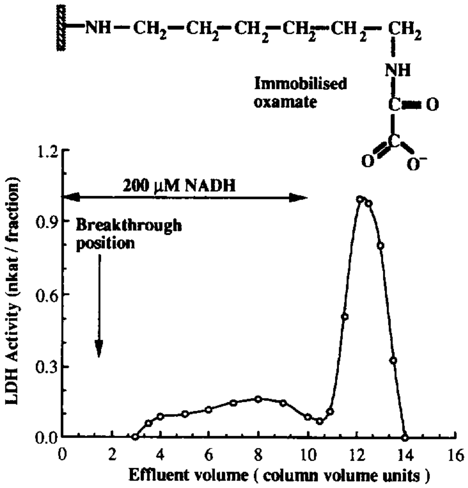

Fig. 2. Affinity chromatography of LDH from turnip (*Brassica rapa*), the 30–70% saturated ammonium sulphate cut of turnip crude extract, using the immobilised oxamate affinity system. The irrigant was 100 mM K-Pi buffer pH 7.0 containing 0.5 M KCl and 1 mM EDTA. 200 μM NADH was added to this buffer to promote adsorption of the LDH (see text) and its discontinuation (as indicated by the horizontal arrow) resulted in elution of the LDH. The chromatography was performed at 4°.

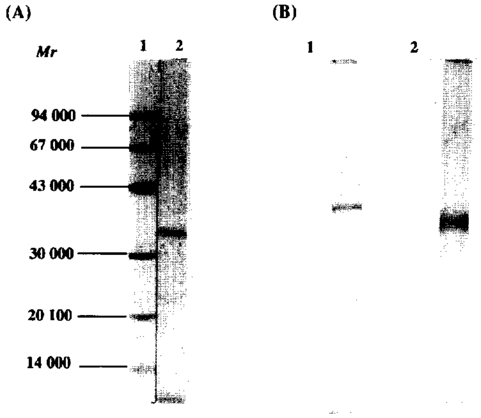

Fig. 3. Polyacrylamide gel electropherograms of plant LDH. (A) SDS-PAGE gels: lane 1, protein standards; lane 2, LDH from the turnip, *Brassica rapa*, purified in a single affinity chromatographic step. Gels were stained for protein using Coomassie Blue. (B) Non-denaturing PAGE: gel 1, LDH from the turnip, *Brassica rapa*; gel 2, LDH from the lettuce, *Lactuca sativa*. Gels were stained for LDH activity as described in the Experimental.

to alteration during lengthy purification procedures—see ref. 12.)

Electrophoretic properties and multiple forms

Betsche *et al.* [13] found only a single electrophoretic form in leaves of lettuce and Capsella, while Asker and Davies [14] reported a single form in potato leaves, but at least four LDH forms in potato tuber. The pattern of LDH isoenzymes in potato tubers reported by Asker and Davies was confirmed in the present work, as opposed to the patterns described by Rothe [2] and by Mayr *et al.* [15]. Furthermore, a single form of LDH was found in lettuce leaves [Fig. 3(B)] and this pattern applies to the entire plant and not just to the leaves. Electrophoretic examination of a range of tissues of leek, onion, turnip and swede also showed only a single resolvable LDH in all tissues [e.g. Fig. 3(B)]. This absence of isoenzymes argues against any functional importance for multiple forms. While Asker and Davies [14] have made a case for differential functions of the isoenzymes in potato, the kinetic differences on which their argument was based seem to be too slight to be of any great physiological consequence. In the roots of barley seedlings, the isoenzyme pattern has recently been shown to vary from strain to strain of this species, one strain having only a single isoenzyme [16]. Such isoenzymic differentiation, and that in potato, may be functionally neutral.

Subunit structure

The LDH from potato tubers and barley roots are tetrameric with variants based on two different subunit types [1, 4], but the enzyme from soybean seedlings is a homotetramer [5].

The M_r of native LDH from turnip and leek was investigated by gel chromatography on a calibrated G-200 column and these L-lactate dehydrogenase activities eluted as single symmetrical peaks with a M_r of *ca* 157×10^3, similar to the results obtained for potato [1] and barley [4] LDH (150×10^3 and 157×10^3, respectively).

Calibrated SDS-PAGE [Fig. 3(A)] indicated a subunit M_r of *ca* 38×10^3 for turnip LDH. This compares with reports of 37.5×10^3 [1] and 40×10^3 [4], respectively, for potato tuber and barley LDH. There is no indication of polypeptide size-heterogeneity by the SDS-PAGE technique. Thus, the experimental data for turnip LDH is consistent with a tetrameric holoenzyme consisting of similar, or identical subunits. SDS-PAGE of LDH from leek resulted in two protein bands with M_rs of about 45×10^3 and 62×10^3, although the 62×10^3 band was sometimes absent. Initial experiments with the turnip enzyme gave a similar result which seems to be artefactual. The protein is prepared for this electrophoretic method by heating in the presence of SDS and mercaptoethanol. If the turnip enzyme, so treated, is electrophoresed immediately in SDS, the result described above and illustrated in Fig. 3(a) is obtained consistently. However, if electrophoresis of the treated material is delayed, additional bands of M_r 45×10^3 and 62×10^3 appear and eventually become dominant (e.g. after overnight storage). This result indicates that the two higher M_r proteins are artefacts of the true LDH subunits. With the leek enzyme, the apparently anomalous result was obtained even when the preparations were electrophoresed immediately. The cause of this is unknown, but a similar result was found for potato LDH [17].

Other workers have had difficulties when attempting to determine the subunit composition of some plant LDHs, notably potato LDH: Rothe *et al.* [17] reported three LDH isoenzymes in potato tubers with M_rs of 84.5×10^3; 106×10^3; and 115×10^3 (by gel filtration) and two different subunits of M_r 41×10^3 and 43×10^3 (by SDS-PAGE); Mayr *et al.* [15] reported six LDH isoenzymes in potato tubers and claims that each electrophoretically distinguishable isoenzyme is a mixture of different tetramers (composed of combinations from up to six different subunit types).

These results have been variously attributed to modification of thiol groups or to proteolysis [1] during extraction and purification. For this reason Davies advocated the inclusion of thiol-protecting and proteolysis-inhibiting reagents in extraction media. In the present work this has not been found necessary: the enzyme prepared rapidly from turnip, leek and lettuce without the use of such reagents being apparently homogeneous, as described above.

Substrates and analogues

LDH from turnip and leek shows normal Michaelis–Menten kinetics with the oxo/hydroxyacid substrates listed in Table 1 and with NAD$^+$, the only departures being the inhibition by high substrate concentrations discussed below. The kinetics with NADH [11] are however, complexly non-linear, as reported previously for LDH from potato and lettuce. The pattern of double reciprocal plots with varied substrate and cofactor concentrations is typical of a sequential (single displacement) mechanism, as already indicated by Davies and Davies [18] for the potato enzyme. Furthermore, the affinity chromatographic behaviour (see above) shows that the turnip and leek enzyme preparations have a compulsory sequential order of ligand binding, with NADH binding before oxamate/pyruvate, as found for the mammalian LDH isoenzymes.

The kinetic constants for the turnip enzyme with the oxo/hydroxyacid substrates are summarised in Table 1. The kinetic constants are generally similar to, though not identical with, those reported for the enzyme from potato tuber [14], lettuce leaf [3] and barley seedlings [4]. Comparison with the kinetic constants for the bovine H_4 and M_4 LDH isoenzymes, determined under identical conditions, shows that the K_m values for the various substrates and the K_i values for the competitive inhibitors stand in the same ratio to one another for each of the three LDH preparations, including those for the competitive inhibitors listed in Table 1. However, the *absolute* K_m and K_i values are about 10-fold higher for the plant enzyme than for the H_4 mammalian isoenzyme, while the M_4 isoenzyme has intermediate values—about three-fold higher than those for the H_4 isoenzyme. The data presented in Table 2 serves to illustrate this for pyruvate. Thus, the plant LDH shows considerably lower affinity for its substrates than the mammalian LDHs.

As shown by Duncan and Tipton [19] and Warren [20], glyoxylate is a substrate for LDH in two ways. As the keto form, oxoacetate, it undergoes reduction (with NADH as cofactor), and as the hydrated, dihydroxyacetate form which predominates in aqueous solution, it is dehydrogenated (with NAD$^+$ as cofactor) to oxalate. This dual activity with glyoxylate has also been reported for LDH from lettuce leaves [21], and we find similar dual activity with the turnip enzyme. However, the dehydrogenase activity, even at its pH optimum (pH 8.5–9.0) is very slow, and requires a higher NAD$^+$ concentration than is required for lactate oxidation (K_m for NAD$^+$ at pH 8.5: 1.56 mM with glyoxylate; 0.91 mM with lactate). We have been unable to detect any activity in the dehydrogenase mode at pH 7 (Table 1), and in the physiologically important pH range, 7.4–7.6, we have also been unable to detect any significant reproducible dehydrogenase activity with glyoxylate (see below under Physiological Importance).

By contrast, the reductase activity of the plant enzyme with glyoxylate has a V_{max} slightly better than that with pyruvate. The high apparent K_m value relative to that with pyruvate is attributable to the low proportion of the reducible, oxo form of glyoxalate in

Table 1. Kinetic constants of LDH from *Brassica rapa* for pyruvate, lactate and analogues. Assays were carried out in 100 mM K-Pi buffer at the pH values indicated with 0.5 mM pyruvate, 100 mM lactate or 50 mM glyoxylate and NADH (200 μM for pyruvate or glyoxylate reduction) or NAD^+ (3 mM for lactate oxidation or 10 mM for glyoxylate oxidation). All assays were performed at 30° in duplicate

Substrate	Co-substrate	K_m (mM)	Relative* V_{max}
At pH 7.0:			
Pyruvate	NADH	0.4	1.0
Lactate	NAD^+	25.0	0.2
Glyoxylate	NADH	21.0	1.1
(as oxoacetate)†		(1.1)	
Glyoxylate	NAD^+	No detectable activity	
Glycolate	NADH	No detectable activity	
At pH 8.5:			
Lactate	NAD^+	38.5	0.30
Glyoxylate	NAD^+	22.2	0.05
(as dihydroxy-acetate)†		(21.1)	
Glycolate	NADH	No detectable activity	
NAD^+	Lactate	0.9	
NAD^+	Glyoxylate	1.6	
Inhibitor (at pH 7.0)	Competitive against:	K_i (mM)	
Oxamate	Pyruvate	0.3	
Oxalate	Lactate	0.1	
Oxalacetate	Lactate	1.0	
Malate	Lactate	36.8	

* All V_{max} values were extrapolated to saturation with both the oxo acid and NAD(H) and expressed relative to V_{max} with pyruvate and NADH at pH 7.0.

† At these pH values, glyoxylate is 95% hydrated to the dihydroxy-acetate oxidizable form, the remaining 5% being the reducible oxoacetate form [23]. The K_m values in parentheses are corrected for these proportions.

aqueous solution owing to extensive hydration to the dihydroxy form. The equilibrium constant of the hydration reaction indicates that only 5–6.2% of glyoxylate exists as the unhydrated oxo form at neutral pH [22, 23]. We used the former value to calculate the true K_m values for the oxo and dihydroxy forms given in Table 1.

We were unable to detect any dehydrogenase activity with glycolate (hydroxyacetate), the strictly equivalent lactate analogue (Fig. 1). However, this was also characteristic of the mammalian LDH forms, a fact not previously recorded.

Oxalate and oxamate are classic inhibitors of vertebrate LDH, [24] and they similarly inhibit turnip LDH in the classic competitive fashion, oxalate being competitive against lactate and non-competitive against pyruvate, and oxamate being strictly competitive against pyruvate (Fig. 1 and Table 1). The K_i values show consistent ratios with the K_m values discussed above, being about 10-fold higher than the equivalent values for the mammalian H_4 isoenzyme.

Oxalacetate and malate (4-carbon, dicarboxylate analogues of pyruvate and lactate respectively—see Fig. 1) also inhibit turnip LDH. While malate shows the expected competitive pattern against lactate, oxalacetate is unexpectedly competitive against lactate and noncompetitive against its apparent analogue, pyruvate (Table 1). We have also found this same unexpected pattern with mammalian LDH. Oxalacetate exists to the extent of 7–10% in the enol form in solution [25] and as such it may act as an analogue of lactate. However, malate, which seems a much closer analogue of lactate (Fig. 1), is a much weaker inhibitor (Table 1), a point of possible physiological significance as discussed below. It is possible that the enol form of oxalacetate is a transition state analogue, although this is not immediately obvious in terms of current views of the mechanism of catalysis by LDH.

Substrate inhibition

Substrate inhibition of the H form of mammalian LDH by high concentrations of pyruvate has been advocated as a major functional property of this isoenzymic form [26]. However, the M form is also subject to substrate inhibition by pyruvate (Table 2). While this inhibition is not as strong as that of the H form, $I_{0.5}$ (the pyruvate concentration resulting in half

Table 2. Comparison of the pyruvate kinetics of turnip LDH and the bovine H_4 and M_4 isoenzymic forms. All determined with 200 μM NADH at 30° in 50 mM K-Pi buffer, pH 7.0

LDH type	K_m (mM)	$I_{0.5}$* (mM)	$I_{0.5}/K_m$
Turnip	0.46	44.0	96
Bovine H_4	0.05	4.8	100
Bovine M_4	0.18	18.0	99

* $I_{0.5}$ is the inhibitory pyruvate concentration that causes a decrease of v_o back to half V_{max} on the inhibitory side of plots of v_o against [pyruvate]. V_{max} is not identical with the 'optimal velocity' on such plots and must be determined by reciprocal plots at the lower, 'non-inhibitory' pyruvate concentrations.

V_{max} on the inhibition side) bears almost the same ratio to K_m as for the H form (Table 2). The plant enzyme is also inhibited by high concentrations of pyruvate, the ratio of $I_{0.5}$ to K_m being very similar to that for the mammalian H_4 isoenzyme (Table 2). Thus, this substrate inhibition is common to all three LDH types, differing only in the same relative way as the affinities for the oxo/hydroxyacid substrates and their analogues.

It has been widely accepted that the substrate inhibition is caused by the small proportion of pyruvate present as the enol form (Fig. 1) in solution. But, we find that the glyoxylate reductase activity of turnip LDH is also subject to substrate inhibition at high glyoxylate concentrations. High concentrations of lactate also cause substrate inhibition of all three LDH forms in the dehydrogenase direction. Glyoxylate (oxoacetate) cannot form an enol derivative, although perhaps the proportion of glyoxylate present in the hydrated form contributes to the inhibition. However, there are no alternative forms of lactate analogous to the enol and hydrated forms discussed above and therefore lactate inhibition can only be ascribed to unaltered lactate binding to the 'wrong' enzyme-cofactor complex (i.e. E.NADH) on the product side of the catalytic turnover. It therefore seems likely that the introduction of enol-pyruvate to explain substrate inhibition is probably an unnecessary complication and that (keto) pyruvate binds to the enzyme–NAD^+ complex to form the abortive complex on the product side of the reductase catalytic turnover.

Effects of pH and their mechanistic implications

Analysis of pH profiles has played an important role in the formulation of the catalytic mechanism of vertebrate LDH [27]. We have carried out an analogous study on turnip LDH (summarised in Fig. 4), showing that the decline in activity on either side of the pH optimum at pH 6.5 results from an increasing K_m for pyruvate on the alkaline side and increasing K_m for NADH on the acid side, V_{max} remaining essentially constant throughout this pH range. The variation in apparent affinity for pyruvate suggests that its binding requires protonation of an active site group with a pK_a of about 7. In the case of the vertebrate enzyme, this has been identified as the imidazolyl side chain of an active-site histidine residue [28, 29]. Besides its role in the binding process, this same proton has been implicated in the catalytic process and it is less easy to understand on this basis why the V_{max} remains unaffected over the same pH range. Presumably the proton necessary for binding of pyruvate is so 'locked-in' by the binding step that the subsequent catalytic proton transfer remains unaffected by the external pH.

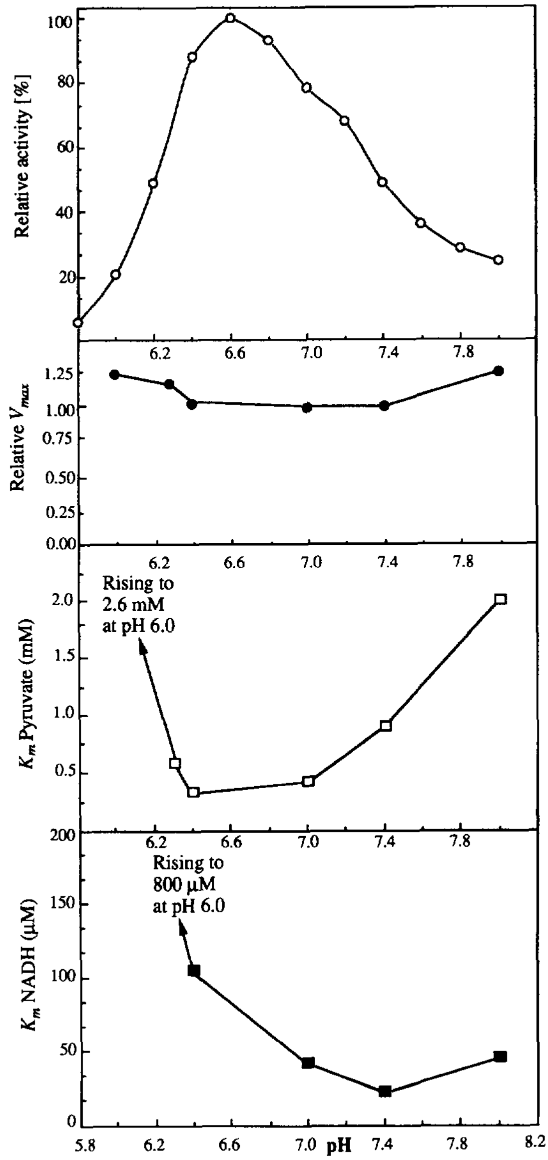

Fig. 4. Analysis of pH activity-profiles of lactate dehydrogenase from turnip (*Brassica rapa*): v_o values were determined with 0.5 mM pyruvate and 200 μM NADH; V_{max}values were plotted relative to a value of 1 for V_{max} at pH 7.0; V_{max} values were all extrapolated to infinite concentrations of both pyruvate and NADH.

The pH profile of the turnip enzyme in the direction of lactate dehydrogenation again shows similarity with the vertebrate enzymes. The effect of pH is opposite to that on the pyruvate side, the apparent affinity for lactate increasing between pH 6.5 and 7.0 (as indicated by a decrease of K_m without change of $V_{\max}$ and then remaining relatively constant as the pH is raised to higher values. Again a group with a pK_a of about 7 is indicated, this being identified in the vertebrate enzymes as the same imidazolyl group involved in the binding of pyruvate, but required to be in the unprotonated state for the binding and dehydrogenation of lactate.

The turnip and vertebrate enzymes diverge in the nature of their response below pH 6.3. The steep fall in pyruvate reductase activity of turnip LDH in this range (Fig. 4) is due to a steep increase in the K_m values for both pyruvate and NADH (which rise to 2.6 mM and 800 μM, respectively, at pH 6.0). These changes are not seen with mammalian LDH and seem too great and abrupt to be attributable to a localised active-site effect. They are more reminiscent of a larger pH-induced conformational change. The fact that the activity still reaches the same $V_{\max}$ at saturation levels of pyruvate and NADH (Fig. 4) suggests that the conformational change is reversed by binding of these substrates at high concentrations.

As discussed above, the commonly accepted mechanism of substrate inhibition by pyruvate postulates enol-pyruvate as the inhibitory species (Fig. 1). This is considered to be structurally more closely analogous to lactate than to pyruvate. It is proposed that it binds (on the product side of the mechanism) to the enzyme–NAD$^+$ complex inhibiting the dissociation of NAD$^+$ and slowing the catalytic turnover. This proposed 'abortive' binding is therefore analogous to the 'productive' binding of lactate and is commonly assumed to show kinetic characteristics similar to those with lactate. That this is not so with the turnip enzyme can be concluded from the results summarised in Table 3, which shows that there is no significant variation in the apparent inhibition with pH. If the productive and abortive binding of pyruvate were to require different protonation states of the active-site imidazolyl group, the binding affinities in the two modes should diverge with pH causing much stronger relative inhibition at higher pH values. Any slight variation we observe is in the opposite direction. This data strongly suggests that abortive and productive binding vary in parallel resulting in no overall change in the degree of 'conflict' between the two binding modes, and hence no significant change in apparent inhibition index. Reinvestigation of the H and M bovine isoenzymic forms showed exactly the same pattern (unpublished data).

Table 3. Turnip LDH: effect of pH on pyruvate kinetics. All constants were determined with 200 μM NADH at 30°C in 50 mM K-Pi buffer

pH	K_m (mM)	$I_{0.5}$* (mM)
6.4	0.36	42.0
7.0	0.46	44.0
7.4	0.89	40.0

* See Table 2.

Physiological aspects

The relatively high K_m of plant LDH for pyruvate, together with the non-linear kinetics with NADH discussed elsewhere [11], seem particularly well suited to minimising pyruvate reduction under normal aerobic conditions, while allowing a rapid and automatic increase in activity when the concentrations of both NADH and pyruvate rise under anoxic conditions. This could constitute an effective substrate level control mechanism. The substrate inhibition by pyruvate requires such high levels of pyruvate to become even marginally important with the turnip enzyme that it could not possibly be of physiological importance. It is tempting to speculate, however, that the relatively very strong inhibition by oxalacetate (Table 1) may be important, particularly in the presence of NAD$^+$ which potentiates the inhibitory action of oxalacetate (see Table 4). This might provide a further element of control by inhibiting LDH activity under aerobic conditions, when the levels of oxalacetate and NAD$^+$ would be high in the cytosol. LDH would be quickly released from such inhibition under anaerobic conditions as oxalacetate became reduced to the very much less inhibitory malate (see above and Table 4). These ideas regarding control of the activity of LDH are discussed in the accompanying paper [11].

Such ability to keep anaerobic glycolysis nearly inactive under normal aerobic conditions, but 'on standby' for immediate response to periods of anaerobiosis, is almost certainly important in roots, which may suffer periods of anoxia [30]. However, this seems

Table 4. Concerted inhibition by oxalacetate (OAA) and NAD$^+$ of the pyruvate reductase activity of LDH from turnip. Assays were performed with 0.5 mM pyruvate and 100 μM NADH in 100 mM K-Pi buffer, pH 7.0, at 30°

Addition	Relative v_o
None	1
OAA (4 mM)	0.99
NAD$^+$ (3 mM)	0.91
OAA (4 mM) with NAD$^+$ (3 mM)	0.54
NAD$^+$ (6 mM)	0.78
OAA (4 mM) with NAD$^+$ (6 mM)	0.41
OAA (8 mM)	0.98
OAA (8 mM) with NAD$^+$ (6 mM)	0.23

unlikely to be directly important in other parts of plants—particularly in leaves, but also in stems, the bases of which seem to be a major focus of 'standby' LDH activity under normal conditions [7].

Davies [21, 31] proposed that the function of LDH in leaves derives from its activity with glyoxylate which it can both reduce and dehydrogenate (see above). If these reactions occurred simultaneously, the result would be dismutation of glyoxylate to form glycolate and oxalate, with NAD^+/NADH cycling between the two reactions. The resulting oxalate production has been proposed [21, 31] as a downward adjuster of pH and as a pH-stat mechanism dependent on the overlap of the pH optima of the reductase and dehydrogenase activities. While Davies and Asker [21] demonstrated that LDH can promote such complex glyoxylate dismutation *in vitro*, their experiments were conducted at pH 8.5, very far removed from the pH range at which pH stasis would be appropriate in plants cells whose cytosol is maintained at pH 7.3–7.5 under normal aerobic conditions [30, 32].

However, we find both the glyoxylate reductase and dehydrogenase activities extremely slow at the glyoxylate concentrations one might reasonably expect in the cytosol. Compounding this is the fact that the pH optima of the two activities are far apart and the overlap of their pH profiles is poor. Indeed, with the turnip enzyme we have been unable to detect any convincing glyoxylate dehydrogenase activity in the physiologically appropriate 'overlap region' around pH 7.4. The strong inhibition of any such dehydrogenase activity by its product, oxalate, also militates against a role for such activity under physiological conditions.

It might be argued that even barely detectable glyoxylate dismutase activity might be sufficient to maintain something as nearly static as a pH-stat. However, we believe that even at the low concentration of pyruvate and NADH pertaining in the cytosol under aerobic conditions, the pyruvate reductase activity of LDH would far outstrip the glyoxylate dismutase activity. There is ample evidence that lactate is produced constantly in fully aerobic plant tissues such as leaves [33] and this would certainly tend to reduce the cytosolic pH if the NAD^+ so-produced were appropriately recycled. It is possible that such lactate production itself might constitute part of a pH-stat if coupled to some switch mechanism activated by rising pH and creating a transient approach to 'anaerobic' type metabolism. For example, such an effect might be produced if rising pH inhibited any component of the malate shuttle, so inhibiting transfer of NADH reducing equivalents into the mitochondrion.

EXPERIMENTAL

Chemicals. All of the biochemicals used in this study were the purest grade supplied by Sigma. Pyruvate and oxalacetate solns were freshly prepared and checked for purity. Oxalacetate undergoes a slow spontaneous decarboxylation [34], which not only decreases its conc but also yields pyruvate. Pyruvate in soln undergoes oligomerisation and polymerisation yielding 'parapyruvate' [34].

Purification of plant LDH. LDH from turnip (*Brassica rapa*) and leek (*Allium porrum*) was purified by a rapid affinity chromatographic method [8, 9], the enzyme being purified to electrophoretic homogeneity within 4 hr of extraction. Using a Waring blender, 800 g of washed plant material was homogenised in 800 ml of 50 mM K-Pi buffer pH 7.4 containing 1 mM EDTA and 64 g of insoluble PVP. The resulting extract was centrifuged at 10 000 *g* for 20 min and strained through muslin. $(NH_4)_2SO_4$ was added to a final concn of 30% satn and the prepn was centrifuged at 10 000 *g* for 20 min. The supernatant was then brought to 70% $(NH_4)_2SO_4$ satn and centrifuged again at 10 000 *g* for 20 min. The resulting pellet was dissolved in 35 ml of 0.1 M K-Pi buffer pH 7 containing 0.1 M EDTA and 0.5 M KCl. Aminohexyl oxamate was synthesised according to ref. [10]. 0.5 mM NADH was added to the plant extracts immediately prior to their application to the amino-hexyl oxamate column (10 ml bed vol.). At least eight column-vols of the irrigating buffer (0.1 M K-Pi buffer pH 7 containing 0.5 M KCl, 1 mM EDTA, and 0.2 mM NADH) was used to wash the column before eluting the enzyme by omitting NADH from the irrigating buffer.

Electrophoretic procedures. Non-denaturing polyacrylamide gel electrophoresis was according to ref. [35]. LDH activity was detected in gels with the staining cocktail of ref. [36]. SDS-PAGE was carried out according to ref. [37], using a separating gel of 13% acrylamide. M_r calibration of SDS gels was achieved using the following protein 'markers': α-lactalbumin (14×10^3), trypsin inhibitor (20.1×10^3), carbonic anhydrase (30×10^3), ovalbumin (43×10^3), BSA (67×10^3) and phosphorylase B (94×10^3). Staining for protein was carried out by first fixing the gels in 12% TCA for 30 min, followed by staining with Coomassie Blue (0.2% in 7% HOAc) for 3 hr. Stained gels were cleared of residual dye by leaching overnight in 7% v/v HOAc.

Native M_r *determination.* The native M_r of purified LDH was estimated by gel filtration on a calibrated G-200 column (bed dimensions: 2.5 × 53 cm). The proteins used for calibration (and their M_rs in parentheses) were: ferritin (400×10^3) catalase (232×10^3), aldolase (158×10^3), porcine heart LDH (142×10^3), bovine serum albumin (67×10^3) and ovalbumin (43×10^3). The irrigating buffer in all cases was 50 mM K-Pi buffer pH 7.4 containing 0.1 M KCl. The flow rate was maintained at 12 ml hr^{-1}.

Enzyme assay and evaluation of kinetic analysis. LDH was assayed in duplicate at 30° by measuring the decrease or increase in *A* of NADH at 340 nm. All sets of kinetic data were fitted to straight lines by linear regression analysis using a Mackintosh Cricket Graph programme. 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 here.

REFERENCES

1. Poerio, E. and Davies, D. D., *Biochemical Journal*, 1980, **191**, 341.
2. Rothe, G. M., *Archives of Biochemistry and Biophysics*, 1974, **162**, 17.
3. Betsche, T., *Biochemical Journal*, 1981, **195**, 615.
4. Hoffman, N. E. and Hanson A. D., *Plant Physiology*, 1986, **82**, 664.
5. Jervis, L., Robertson, E. R. and Schmidt, C. N. G., *Phytochemistry*, 1981, **20**, 2117.
6. Goodman, R. A. and Jervis, L., *Biochemistry Society Transactions*, 1985, **13**, 378.
7. O'Carra, P. and Mulcahy, P. *Phytochemistry*, 1996, **42**, 581.
8. O'Carra, P. and Barry, S., *FEBS Letters*, 1972, **21**, 281.
9. O'Carra, P. and Barry, S., *Methods in Enzymology*, vol. 34, ed. W. Jakoby and M. Wilckek 1974, p. 598.10. Mulcahy, P. and O'Carra, P., *Biochemistry Society Transactions*, 1989, **17**, 381.
11. O'Carra, P. and Mulcahy, P. *Phytochemistry*, 1997, **45**, 897.
12. Scheek, R. M. and Slater, E. C., *Biochimica et Biophysica Acta*, 1978, **526**, 13.
13. Betsche, T., Bosbach, K. and Gerhardt, B. *Planta*, 1979, **146**, 567.
14. Asker, H. and Davies, D. D., *Planta*, 1984, **161**, 272.
15. Mayr, U., Hensel, R. and Kandler, O., *Phytochemistry*, 1982, **21**, 627.
16. Hoffman, N. E., Hondred, D., Hanson, A. D. and Brown, A. H. D., *Journal of Heredity*, 1988, **79**, 110.
17. Rothe, G. M., Coerper, A. and Bacher, W., *Phytochemistry*, 1978, **17**, 1701.
18. Davies, D. D. and Davies, S., *Biochemical Journal*, 1972, **129**, 831.
19. Duncan, R. J. S. and Tipton, K. F., *European Journal of Biochemistry*, 1969, **11**, 58.
20. Warren, W. A., *Journal of Biological Chemistry*, 1970, **245**, 1675.
21. Davies, D. D. and Asker, H., *Plant Physiology*, 1983, **72**, 134.
22. Sorensen, P. E., Bruhn, K. and Lindelov, F. (1974) *Acta Chimica Scandanavica*, *A*, 1974, **28**, 162.
23. Cooper, A. J. L. and Redfield, A. G., *Journal of Biological Chemistry*, 1975, **250**, 527.
24. Novoa, W. B., Winer, A. D., Glaid, A. J. and Schwert, G. W., *Journal of Biological Chemistry*, 1959, **234**, 1143.
25. Kokesh, F. C., *Journal of Organic Chemistry*, 1976, **41**, 3593.
26. Everse, J. and Kaplan, N. O., in *Isozymes Vol II: Physiological Function*, ed. C. L. Markert. Academic Press, New York, 1975, p. 29.
27. Schwert, G. W., Miller, B. R. and Peanasky, R. J., *Journal of Biological Chemistry*, 1967, **242**, 3245.
28. Clarke, A. R., Atkinson, T. and Holbrook, J. J. *TIBS* 1989, **14**, 101.
29. Clarke, A. R., Atkinson, T. and Holbrook, J. J. *TIBS* 1989, **14**, 145.
30. Roberts, J. K. M., Callis, J., Jardetzky, O., Walbot, V. and Freeling, M. *Proceedings of the National Academy of Science, U.S.A.*, 1984, **81**, 6029.
31. Davies, D. D., in *Isozymes: Current Topics in Biological and Medical Research*, Vol. 16, ed. M. C. Rattazzi and A. R. Liss, New York, 1987, p. 193.
32. Wray, V., Schmiel, O., Berlin, J. and Witte L., *Archives of Biochemistry and Biophysics*, 1985, **236**, 731.
33. Betsche, T., *Phytochemistry*, 1983, **22**, 1341.
34. von Korff, R. W., *Analytical Biochemistry*, 1964, **8**, 1718.
35. Davis, B. J., *Annals of the New York Academy of Science*, 1964, **121**, 404.
36. Epstein, C. J., Kwok, L. and Smith, S., *FEBS Letters*, 1971, **13**, 45.
37. Laemmli, U. K., *Nature*, 1970, **227**, 680.