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SUBUNIT COMPOSITION AND SUBSTRATE BINDING REGION OF POTATO L-LACTATE DEHYDROGENASE

ULRICH MAYR, REINHARD HENSEL and OTTO KANDLER

Botanisches Institut der Universität München, Menzinger Strasse 67, D-8000 München 19, West Germany

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Abstract—Four of the six electrophoretically distinguishable isoenzymes of the L-lactate dehydrogenase (EC 1.1.1.27) from potato tubers were purified from crude extracts. The isoenzymes are tetrameric and exhibit MWs around 145000. They are composed of mixtures of different subunits. Two of the isoenzymes together contain at least three, the other two together contain six different subunits indicating that the actual number of isoenzymes may be even greater than the number of electrophoretically detectable isoenzymes. Since the isoenzymes agree largely with respect to their enzymatic properties and to their primary structure as suggested from fingerprinting and amino acid analysis, it is suggested that the variation of the subunits is caused by proteolytic processing *in vivo* rather than by different genetic coding. The amino acid sequence of the substrate-binding region (Arg_6 peptide) shows a high homology to that of the L-lactate dehydrogenases of animals and bacteria indicating a common origin of plant, animal and bacterial enzymes.

INTRODUCTION

Whereas NAD-dependent L-lactate dehydrogenases from animals and bacteria have been studied in great detail including primary, tertiary and quaternary structure [cf. 1–9], investigations of the plant L-lactate dehydrogenases were restricted to kinetics and to determinations of the MW [10–17]. Because of the lack of data on the amino acid sequence the phylogenetic relation of the plant enzymes to the bacterial and animal enzymes is obscure as yet. This paper reports on the subunit composition of the potato L-lactate dehydrogenase isoenzymes and the amino acid sequence of the so-called Arg_6 peptide, a dodecapeptide of the substrate-binding region of L-lactate dehydrogenase (cf. [1]).

RESULTS

Electrophoretic separation of isoenzymes

Since the L-lactate dehydrogenase activity in the crude extract of potato tubers was very weak (Table 1) the enzyme was concentrated previous to gel electrophoresis by precipitation with 14% (v/v) polyethylene glycol (PEG) and dissolving the precipitate in a small volume of 20 mM imidazole buffer, pH 8.0, containing mercaptoethanol and EDTA. The recovery of activity after this concentration step was 100%.

Gel electrophoresis of the concentrated crude enzyme yielded six bands exhibiting L-lactate dehydrogenase activity. The bands, characterized by $R_{\text{bromophenol blue}}$ values of 0.41, 0.43, 0.48, 0.52, 0.57 and 0.62 were denoted as isoenzymes I–VI. Isoenzyme III was resolved into two bands in some of the electrophoretograms; however, the separation was not reproducible. Neither the presence of the protease inhibitor phenylmethanesulfonylfluoride (PMSF) nor the absence of mercaptoethanol during the preparation of the extract caused an alteration of the isoenzyme pattern. Also storage of the extracts at 7° for 5 hr in the presence of mercaptoethanol but without PMSF prior to electrophoresis had no influence on the pattern. However, the number of isoenzymes varied slightly in preparations obtained from different batches of potatoes. Whereas isoenzymes III–VI were present in all preparations, isoenzymes I and II were absent in some cases.

Enzyme purification and molecular weights

The sequence of purification steps and the purification data are summarized in Table 1. The purified enzyme contained the isoenzymes III–VI only. Isoenzyme III was present in a much smaller amount than the other isoenzymes. No other protein

Table 1. Purification of potato L-lactate dehydrogenases

Purification step	Sp. act. (kat/kg protein)	Enrichment	Recovery (%)
1. Crude extract	0.0046	—	100
2. PEG 6000 fraction (4–14%) and heat treatment	0.007	1.5	95
3. DEAE-cellulose and Sepharose–aminohexyl oxamate	0.058	12.5	56
4. Sepharose–blue dextran and 5′-AMP Sepharose	6.83	1464	25

bands than those exhibiting L-lactate dehydrogenase activity could be detected indicating that the isoenzymes were virtually purified to homogeneity.

The MWs of the native isoenzymes III–VI were investigated by gel chromatography of the purified mixture on a Sephadex G-200 column (1 m length, 0.9 cm diameter) using cytochrome *c*, albumin from hen egg, aldolase and catalase as markers and 20 mM Tris–HCl buffer, pH 8.5, containing 0.2 M NaCl, 2 mM EDTA and 10 mM mercaptoethanol as rinsing buffer. The L-lactate dehydrogenase activity was eluted in a single symmetrical peak without any indication of a separation of the isoenzymes. The apparent MW of the mixture was *ca* 145000.

Electrophoresis of the purified mixture of isoenzymes III–VI on a dodecylsulfate polyacrylamide gradient (5–25%, logarithmic) slab gel with DNA-dependent RNA polymerase, bovine serum albumin and trypsin inhibitor from soya beans as markers, yielded five protein bands with MWs of *ca* 38000, 37500, 37000, 35500 and 35000, thus indicating a tetrameric structure of the native isoenzymes of *ca* 145000 as already suggested by Poerio and Davies [11]. The multiplicity of subunits further indicates that the isoenzymes are composed of different subunits. As already mentioned in the case of the isoenzyme pattern, the presence of PMSF during purification also has no influence on the number and relative concentration of the subunits.

Subunit composition of individual isoenzymes

Isoenzymes III–VI were isolated from each other by preparative gel electrophoresis. Recovery of the total activity of the separated enzymes was 60–70% of that of the mixture of isoenzymes applied to gel electrophoresis. The separated isoenzymes were concentrated by dialysis in 15 mM imidazole–HCl buffer, pH 7.5, containing 30% PEG 20000, 20 mM mercaptoethanol and 2 mM EDTA and were then subjected to electrophoresis in polyacrylamide urea gels and in dodecylsulfate polyacrylamide gradient slab gels. The subunit composition of the isoenzymes obtained by urea gel electrophoresis is shown in Table 2.

Each of the isoenzymes yielded several different subunits. Isoenzyme III gave rise to essentially three bands. The same bands are also present in isoenzyme IV, which also yielded three additional bands. A similar pattern was found in isoenzyme V, with the three slower migrating bands being present at a lower, and the faster migrating three bands at a higher amount than in isoenzyme IV. Isoenzyme VI yielded virtually the faster migrating three bands only.

The use of dodecylsulfate polyacrylamide gradient slab gels led to similar results. Isoenzyme III yielded one band corresponding to a MW 38000. Isoenzyme IV showed three bands corresponding to the MWs of 38000, 37500, and 37000. In isoenzyme V significantly less of the 38000 form than of the two other forms was present. Isoenzyme VI consisted mainly of the 37500 and 37000 forms. In isoenzymes IV–VI bands corresponding to a MW of 35500 and 35000 were observed in trace amounts.

Comparing the results of the two gel systems one may conclude that the band corresponding to a MW of 38000 is resolved in the urea gel into the three slower migrating bands, whereas the bands characterized by the MWs of 37500 and 37000 correspond to the faster migrating three bands seen in the urea gel. The protein content of the bands representing MWs of 35500 and 35000 was too low to be detected in the urea gel.

In conclusion, several different subunits are present in each of the isoenzymes, at least six in isoenzymes IV and V and three to four in isoenzymes III and VI, hence indicating that the electrophoretically distinguishable 'isoenzymes' each are mixtures of different tetramers.

Enzymatic properties of the L-lactate dehydrogenases

The isoenzymes showed a very similar pH dependence with an optimum around 7.0. They are different from each other, however, with respect to their affinity to pyruvate. The Michaelis constant, determined at pH 7.0 (80 mM imidazole–HCl buffer), increases from isoenzyme III to VI (K_m = 0.5, 1.1, 1.8, 2.1 mM) indicating a decreasing affinity to the substrate with increasing content of subunits of lower MW.

Isolation and characterization of the Arg_6 peptide

The purified mixture of isoenzymes was digested with trypsin (EC 3.4.21.4). Staining of the fingerprints with fluorescamine yielded about 25 peptide spots corresponding to the average number of lysine and arginine residues present per subunit (Table 3). The peptide spots located at a position similar to the Arg_6 peptides of the previously investigated bacterial enzymes [6] were scraped off, hydrolysed and analysed. One of these peptides showed an amino acid composition (Table 3) very similar to the Arg_6 peptides of the bacterial L-lactate dehydrogenases.

Table 2. Relative amount of the subunits observed after polyacrylamide urea gel electrophoresis of isoenzymes III–VI

Isoenzyme	R_f = 0.14	R_f = 0.16	R_f = 0.18	R_f = 0.23	R_f = 0.25	R_f = 0.26
III	+++	+++	++	(+)	—	—
IV	+++	+++	++	+	+	+
V	+	++	+	++	++	++
VI	+	—	—	+++	+++	+++

The protein bands are characterized by $R_{\text{bromophenol blue}}$ values. The number of crosses indicates the staining intensity.

Table 3. Amino acid composition of the mixture of isoenzymes III–VI and of the Arg_6 peptide

Amino acid	Mol amino acid/mol of Isoenzyme subunit*	Mol amino acid/mol of Arg_6 peptide
Cysteic acid	3.4	
Aspartic acid	33.7	1.9(2)
Methionine sulfone	7.3	
Threonine	14.0	0.9(1)
Serine	28.7	2.6(3)
Glutamic acid	32.0	
Proline	12.0	
Glycine	26.5	2.1(2)
Alanine	23.9	
Valine	36.8	1.1†(1)
Isoleucine	26.7	0.9†(1)
Leucine	38.5	1.0(1)
Tyrosine	7.7	
Phenylalanine	10.3	
Lysine	13.1	
Histidine	8.5	
Arginine	14.0	0.95(1)
Tryptophane	4.0	

*The values were calculated using an average MW of the isoenzyme subunits of 37500. Valine and isoleucine were calculated from 150 hr hydrolysis; serine was corrected for partial destruction during hydrolysis.

†After 72 hr hydrolysis.

The peptide does not contain cysteine as shown by the analysis of the oxidized peptide. As in the case of bacterial Arg_6 peptides [6], prolonged hydrolysis yielded an increase of the amount of valine and isoleucine indicating that the sequence Val–Ile is a dipeptide which is very resistant to hydrolysis. The amino acid sequence of the Arg_6 peptide obtained from three different fingerprints is shown in Fig. 1.

DISCUSSION

The existence of L-lactate dehydrogenase isoenzymes in potato tubers has been reported by several authors [10–13, 17]. Rothe and Cörper [18] suggested that the number of electrophoretically distinguishable isoenzymes may depend on the addition of thiol reagents, e.g. mercaptoethanol, whereas Poerio and Davies [11] assume that the presence of the protease inhibitor PMSF during preparation of the extracts diminishes the number of isoenzymes. These findings suggest that the potato L-lactate dehydrogenase isoenzymes, or at least some of them, may originate during the preparation of the extracts. However, we could not confirm the above-mentioned influence of PMSF or mercaptoethanol. Moreover, the pattern of electrophoretically distinguishable isoenzymes did not alter in extracts being stored for several hours without PMSF previous to electrophoresis. This indicates that the six isoenzymes found in our preparations already existed in the uninjured potato tubers although they may have arisen by modifications rather than by different genetical coding.

Since each of the tetrameric isoenzymes of MW *ca* 145000 is composed of at least three different types of subunits with two of the isoenzymes containing six different subunits, one may assume that the isoenzyme bands contain a mixture of different tetramers composed of different mixtures of subunits. Thus, the actual number of isoenzymes may be far greater than the number of electrophoretically distinguishable bands.

Possibly, the multiplicity of only slightly different subunits results from proteolytic activities in the potato tubers removing small peptides not important for the catalytic activity. An essentially identical amino acid sequence of the thus modified subunits is indicated by the agreement of the numbers of peptides in the fingerprints and the arginine and lysine residues in the mixture of isoenzymes.

Hence, the origin of isoenzymes may differ in

		160			165			170	
Chicken M_4, Pig H_4, M_4, Rabbit H_4, Beef H_4	[2-4]	Arg - Val - Ile -	Gly - Ser - Gly	-	Cys	- Asn -	Leu - Asp	- Ser - Ala -	Arg
Chicken H_4	[4]	Arg - Val - Ile -	Gly - Ser - Gly	-	Cys	- Asn -	Leu - Asp	- Thr - Ala -	Arg
Dogfish M_4	[2]	Arg - Ile - Ile -	Gly - Ser - Gly	-	Cys	- Asn -	Leu - Asp	- Ser - Ala -	Arg
Lobster	[2]	Arg - Val - Ile -	Gly - Ser - Gly	-	Thr	- Asx -	Leu - Asx	- Ser - Ser -	Arg
Potato		Val - Ile -	Gly - Ser - Gly	-	Thr	- Asx -	Leu - Asx	- Ser - Ser -	Arg
Lactobacillus curvatus	[6]	Arg - Val - Ile -	Gly - Ser - Gly	-	Thr	- Ser -	Leu - Asp	- Ser - Ala -	Arg
L. xylosus	[6]	Val - Val -	Gly - Ser - Gly	-	Thr	- Ser -	Leu - Asx	- Thr - Ala -	Arg
L. casei	[6]	(Val, Val,	Gly, Ser, Gly	,	Thr	, Ser ,	Leu, Asx	, Thr, Ala,	Arg)
L. plantarum	[6]	(Val, Ile,	Gly, Ser, Gly	,	Thr	, Ser ,	Leu , Asx	, Ser, Ser,	Arg)
L. acidophilus	[6]	(Val, Ile,	Gly, Ser, Gly	,	Thr	, Ser ,	Leu , Asx	, Thr, Gly,	Arg)
Bacillus stearothermophilus	[6]	Val - Ile -	Gly - Ser - Gly	-	Thr	- Ile -	Leu - Asx	- Thr - Ala -	Arg

Fig. 1. Amino acid sequence of a dodecapeptide from the substrate-binding region of several L-lactate dehydrogenases.

animals and plants. Whereas the five animal isoenzymes (A_4, A_3B, A_2B_2, AB_3, B_4) result from combining two different genetically determined types of subunits, the potato isoenzymes may have derived from only one polypeptide by proteolytic processing.

With respect to the phylogenetic relationship among L-lactate dehydrogenases of plants, animals and bacteria, the close homology of the Arg_6 peptide of various members of these groups (Fig. 1) indicates a common origin of all L-lactate dehydrogenases. It also shows the very pronounced conservative character of this substrate-binding region—pyruvate is bound to Arg_{171} (cf. [1])—with six positions (162, 163, 164, 167, 168, 171) being constant and six others exhibiting only very limited variations. The changes in two of these positions namely 165 and 166 are characteristic of particular groups of organisms and may mark phylogenetically important events.

So, asparagine at position 166 marks the eucaryotic line of evolution whereas serine is characteristic of procaryotes. An exception is the replacement of serine by isoleucine in the case of the *Bacillus stearothermophilus* enzyme. Since this enzyme operates in the thermophilic range, the replacement of the hydrophilic serine by the hydrophobic isoleucine may be of selective advantage. Another significant replacement is the change from threonine to cysteine at position 165. It may mark the branching point of the vertebrates since cysteine is restricted to representatives of the various groups of the vertebrates, whereas threonine is found in all the other organisms, bacteria, plants (potato) and invertebrates (lobster).

EXPERIMENTAL

Plant material. Potato tubers (*Solanum tuberosum* cv Sieglinde) were purchased from the market.

Affinity chromatography resins. Sepharose-aminohexyl oxamate [19–21] and Sepharose-blue dextran [22] were prepared as recently described. 5'-AMP Sepharose was purchased from Pharmacia.

Polyacrylamide gel electrophoresis. Electrophoretic separation was performed with the gel system No. 1 of Maurer [23] in 10 cm tubes (0.6 cm diam.) using, however, 5 or 6% polyacrylamide instead of 7.5%. To isolate the isoenzymes the polyacrylamide gels were incubated in imidazole–HCl buffer (0.1 M), pH 7.0, containing NADH and pyruvate. The bands visible in UV light were cut out. Subsequently the enzymes were extracted from the gel slices electrophoretically using two glass tubes (0.6 cm diameter) connected by a Si tube. The upper glass tube (3 cm long) contained 0.3 cm^3 5% polyacrylamide gel on which the gel slice was applied, the lower glass tube (1.5 cm long) was filled with running buffer and closed with a dialysis membrane at the bottom. After 1 hr of electrophoresis (180 V and *ca* 2 mA/tube) the enzymes had migrated out of the applied gel slice through the 5% polyacrylamide gel into the running buffer of the lower glass tube.

Polyacrylamide–dodecylsulfate slab gels with an exponential polyacrylamide gradient from 5 to 25% and polyacrylamide–urea gels using 10 cm tubes (0.6 cm diameter) were prepared according to refs. [24–26]. Protein bands and enzyme activity were visualized as previously described [27].

Determination of protein. Protein was determined by a micro assay according to ref. [28].

Enzyme assay. The standard assay mixture for the determination of lactate dehydrogenase activity contained 80 mM imidazole–HCl buffer, pH 6.5; 0.5 mM NADH; and 17 mM pyruvate in a total vol. of 1 ml. The pH dependence of pyruvate reduction was tested with buffers containing 60 mM imidazole, 60 mM acetate and 60 mM Tris. L- and D-lactate dehydrogenase activities were determined in a mixture (1 ml) containing 80 mM Tris–HCl buffer, pH 8.0, 8.5 or 9.0; 45 mM NAD; and 0.2 M L- or D-lactate.

Enzyme purification. Potato tubers were peeled, cut into pieces and rinsed in ice-cold 10 mM acetate buffer, pH 5.4. The pieces (1 kg) and 50 ml 0.5 M acetate buffer, pH 5.4, containing 4% PEG 6000, 20 mM EDTA and 0.2 M mercaptoethanol were passed through a domestic AEG juice extractor. Subsequently, the extract was sucked through a filter and centrifuged at 27000 *g* for 15 min. For all further steps buffers containing 2 mM EDTA and 20 mM mercaptoethanol were used. Acetate buffer (50 mM), pH 5.4, containing 50% PEG 6000 was added to the clear crude extract (500 ml) while stirring, to give a final concn of 4% PEG. After centrifugation at 27000 *g* for 15 min additional PEG soln was added to the supernatant up to a final concn of 14% PEG. The ppt. was collected by centrifugation at 27000 *g* for 15 min, suspended in 20 mM imidazole–HCl buffer, pH 8.0, plus 10% glycerine and heated to 60° for 4 min. After removal of the ppt. by centrifugation, DEAE-cellulose equilibrated with 10 mM Tris–HCl buffer, pH 8.5, was added to the supernatant while stirring. The DEAE-cellulose was introduced into a column and rinsed with three bed vols of the same buffer containing 0.05 M NaCl. The elution of L-lactate dehydrogenase was performed with a linear gradient of 0.05–0.3 M NaCl. The eluted enzyme was concd by precipitation with $(NH_4)_2SO_4$ (0–60%) and dialysed in 15 mM Tris–HCl buffer, pH 8.5. After addition of 50 μM NADH the soln was loaded on a Sepharose–oxamate column, preequilibrated with 10 mM imidazole–HCl buffer, pH 6.5, containing 50 μM NADH. The column was rinsed with three bed vols of the same buffer plus 0.1 M NaCl and with two bed vols of buffer plus 0.15 M NaCl before the enzyme was eluted by omitting NADH and increasing the NaCl concn to 0.3 M. After dialysis in 15 mM imidazole–HCl buffer, pH 6.5, the eluted enzyme was loaded on a Sepharose–blue dextran column. The column was rinsed with four bed vols of the same buffer containing 0.08 M NaCl and the enzyme was desorbed by adding 50 μM NADH and increasing the NaCl concn to 0.2 M. Finally, the eluted and dialysed (the same buffer as before) extract was passed through a 5'-AMP–Sepharose column. After rinsing the column with four bed vols of 15 mM imidazole–HCl buffer, pH 6.5, plus 0.15 M NaCl the enzyme was eluted by adding 50 mM NADH and decreasing NaCl to 0.04 M.

Mapping of tryptic peptides, amino acid analysis and sequencing. Fingerprinting of the tryptic peptides on Si gel plates, isolation of the potato Arg_6 peptide from the gel plate, amino acid analysis and sequencing were performed as previously described [6].

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