MODIFICATION OF ARGININE RESIDUES OF PEA LACTATE DEHYDROGENASE BY PHENYLGLYOXAL

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Electrophoretically homogeneous lactate dehydrogenase was isolated from germinating pea seedlings by chromatography on AMP-Sepharose 4B. The amino acid composition of the enzyme was determined as well as the values of the Michaelis constants for four substrates and of the dissociation constants for the binary enzyme–coenzyme complexes. The treatment of the enzyme with phenylglyoxal resulted in the modification of nine arginine residues in its subunit. The modification was paralleled by a complete inactivation of the enzyme. The role of the arginine residues in the active center probably involves the binding of substrates, lactate and pyruvate, to the apoenzyme by an ionic bond.

Lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase EC 1.1.1.27) plays an important role in the metabolism of germinating seeds and in overcoming stress situations, such as, e.g. temporary oxygen insufficiency[1]. Whereas the enzyme from animal species has been explored in detail[2,3] our information on the enzyme from plants is meager. The data available so far characterize lactate dehydrogenase from potato tubers[4–6] and from germinating soybean seeds[7–9].

This paper describes our studies on the enzyme from pea seedlings. It is known that this enzyme has a relative molecular weight of 145 000 (ref.[10]) and that its structure is that of a tetramer composed of subunits seemingly identical on gel electrophoresis in sodium dodecyl sulfate[11]. A free SH group of cysteine is essential for the activity of the enzyme[12]. The aim of this work was to prepare the enzyme in such a quantity and in such a quality which would permit the amino acid analysis of native and modified lactate dehydrogenase to be performed and the role of the arginine residues in the catalytic mechanism to be studied.

EXPERIMENTAL

Material

The following chemicals were used in this study: NAD^+, $NADH\text{-}Na_2$ (Reanal, Budapest), sodium lactate, phenylglyoxal, Coomassie Brilliant Blue G-250, methylene-bis-acrylamide (Serva, Heidelberg), phenazine methosulfate (Aldrich, Milwaukee), AMP-Sepharose 4B, Sephadex

G-25 (Pharmacia, Uppsala), Sevatest-LDH, LDH-UV-test (Imuna, Šarišské Michaľany), tris-(hydroxymethyl)-aminomethane (Tris), sodium pyruvate, acrylamide, nitroblue tetrazolium chloride, glycine (Lachema, Brno). The remaining chemicals were of analytical purity grade.

Methods

Isolation of lactate dehydrogenase: The enzyme was extracted from pea seedlings (*Pisum sativum* L.), which were allowed to germinate 48 h at 25°C in water, with 10 mM Tris-acetate buffer at pH 7·4 containing 1mM 2-mercaptoethanol. The crude extract was used for the preparation of proteins precipitated at 30 and 40% saturation with ammonium sulfate. The latter was removed by dialysis and the desalted fraction was separated by affinity chromatography on a column of AMP-Sepharose using the procedure applied earlier for the isolation of the same enzyme from soybeans[8]. The active enzyme was eluted from the column by 5mM NAD^+ in the original buffer. The active fractions were pooled, desalted and concentrated by ultrafiltration. The activity of lactate dehydrogenase with respect to lactate oxidation was determined from the change in pyruvate concentration with time (Sevatest-LDH) and with respect to pyruvate reduction from the change in concentration of NADH with time (LDH-UV test, ref.[9]). Protein concentration was determined by the method of Bradford[13].

Polyacrylamide gel electrophoresis was carried out according to Davis[14]. The proteins were stained in 1% solution of Amido Black 10B in 7% acetic acid and the activity of the enzyme was determined according to King[7]. The electrophoresis in dodecyl sulfate has been described before[9].

Amino acid analysis was done on 0·3 mg samples of pure enzyme or, alternatively, on 0·3 mg samples of enzyme which had been incubated 1 h with 9·1 mg of phenylglyoxal at 25°C and then separated from low molecular weight contaminants on a 1 × 10 cm column of Sephadex G-25 equilibrated with 10 mM Tris-acetate buffer at pH 7·4. The pooled protein fractions were concentrated by lyophilization. The proteins were hydrolyzed 20 h in 6M-HCl at 110°C. The amino acid composition was determined in Durrum D-500 Amino Acid Analyzer. Norleucine was used as an internal standard.

Modification of enzyme by phenylglyoxal[15]. The enzyme was allowed to react at 25°C with various concentrations of phenylglyoxal in 0·2 M phosphate buffer at pH 8·0. Samples were removed from the reaction mixture at predetermined time intervals and the activity of lactate dehydrogenase was determined. Analogous reactions were performed with the enzyme which had been preincubated with NAD^+, NADH, lactate or pyruvate at various concentration.

RESULTS

Using a combination of ammonium sulfate fractionation and affinity chromatography on a column AMP-Sepharose 4B we isolated from pea seedlings lactate dehydrogenase of specific activity 552 U/mg which is eighty times higher than the activity of the crude extract. The course of the chromatography is shown in Fig. 1, the efficiency of the individual isolation steps in Table I. The enzyme preparation behaved as a homogeneous product on electrophoresis in polyacrylamide gel at alkaline pH. The activity zone coincided with the protein zone. When subjected to chromatography on a column of Sephadex G-150 the enzyme emerged as a uniform symmetrical peak corresponding to a molecular weight of 145 000. The enzyme dissociated in sodium

dodecyl sulfate to subunits of the same electrophoretical mobility and a relative molecular weight of 35 000.

The values of K_m for four substrates of lactate dehydrogenase and the values of dissociation constants of binary apoenzyme–coenzyme complexes, i.e. LDH–NAD^+ and LDH–NADH (Table II), were determined from the plot of reaction rate versus concentration of the individual substrates.

The enzyme activity of pea lactate dehydrogenase was decreased in the presence of phenylglyoxal. The inactivation was dependent on the concentration of the modifying

TABLE I

Isolation of lactate dehydrogenase from pea seedlings. The values in the table are expressed per 100 g of fresh weight

Fraction	Protein mg	Activity		Degree of purification
		total U	specific U/mg	
Crude extract	160	1 109	6·9	1
Sulfate fraction 30—40% saturation	99	1 453	14·7	2·1
Chromatography on AMP-Sepharose	0·63	348	552	80

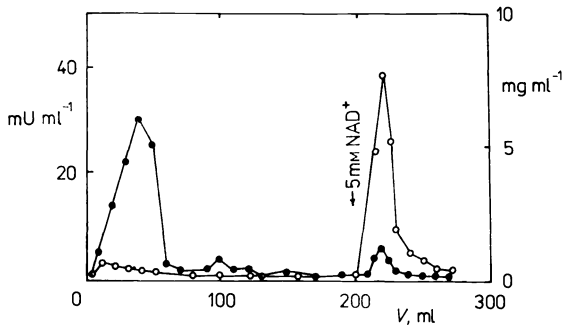

FIG. 1

Chromatography of ammonium sulfate fraction of pea lactate dehydrogenase on column of AMP-Sepharose 4B. mU ml^{-1} lactate dehydrogenase activity (○); mg ml^{-1} protein concentration (●)

TABLE II

Values of kinetic constants for lactate dehydrogenase from pea seedlings

Constant	Value mol l^{-1}	Constant	Value mol l^{-1}
K_m for lactate	$6{\cdot}6 \cdot 10^{-3}$	K_m for NADH	$3{\cdot}3 \cdot 10^{-4}$
K_m for pyruvate	$8{\cdot}3 \cdot 10^{-4}$	K_d LDH–NAD^+	$1{\cdot}5 \cdot 10^{-3}$
K_m for NAD^+	$1{\cdot}8 \cdot 10^{-4}$	K_d LDH–NADH	$4{\cdot}0 \cdot 10^{-5}$

TABLE III

Amino acid composition of pea lactate dehydrogenase. The inactivation was effected in 20 mM phenylglyoxal for 1 h. The number of amino acid residues is expressed per enzyme subunit of molecular weight 35 000

Amino acid	Number of residues in enzyme subunit	
	native enzyme[a]	enzyme modified by phenylglyoxal[b]
Aspartic acid	23·4	23·3
Threonine	13·3	14·1
Serine	15·8	16·0
Glutamic acid	24·7	24·3
Proline	11·7	12·0
Glycine	27·9	28·0
Alanine	22·8	22·5
Valine	17·4	18·0
Methionine	3·4	3·2
Isoleucine	13·3	13·3
Leucine	21·6	21·1
Tyrosine	6·3	6·2
Phenylalanine	9·1	9·1
Histidine	5·7	5·8
Lysine	15·0	14·7
Arginine	10·4	0·9
Half-cystine	5·9	5·6
Tryptophan	n.d.	n.d.

[a] 100% of activity; [b] less than 10% of activity.

reagent and on time. The rate constant of inactivation was directly proportional to phenylglyoxal concentration (Fig. 2). The inhibition of lactate dehydrogenase could not be reversed by the removal of the excess reagent. The treatment of the enzyme with 10 mM phenylglyoxal for 1 h decreased the activity to 10% of the original value. After the low molecular weight compounds had been removed from the reaction mixture the activity of the enzyme remained unchanged for at least 48 h.

The number of arginine residues labeled with phenylglyoxal was determined from the comparison of the amino acid composition of native and modified lactate dehydrogenase. The results are summarized in Table III. The subunit of the enzyme which was almost completely inactivated by phenylglyoxal contained 9 labeled arginine residues out of 10. The correlation between the number of modified residues and the activity of the enzyme was not made.

We have also examined the influence of the individual substrates of lactate dehydrogenase on its inactivation by phenylglyoxal. The enzyme was preincubated 10 min with the substrate and then treated with phenylglyoxal (Table IV). NAD^+ in a concentration corresponding to the K_m-value did not affect the interaction of the enzyme with the modifying reagent any substantially; inactivation was slowed down only after NAD^+ had been used at a concentration corresponding to a ten-fold

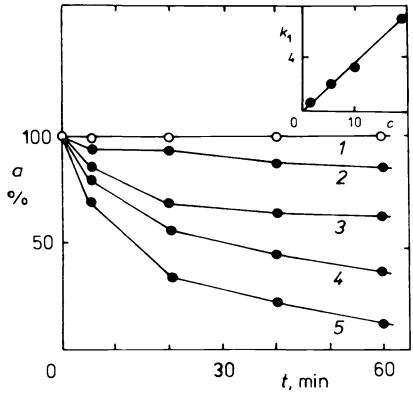

FIG. 2

Time dependence of lactate dehydrogenase (LDH) inactivation by phenylglyoxal (PGO), *a* activity of LDH in % of control. *1* control (LDH), *2* LDH + 1 mM PGO, *3* LDH + + 5 mM PGO, *4* LDH + 10 mM PGO, *5* LDH + 20 mM PGO. Insert: dependence of inactivation rate constant (k_1, min^{-1}) on phenylglyoxal concentration (c, mmol l^{-1})

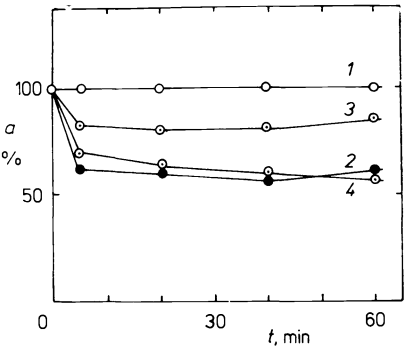

FIG. 3

Effect of NAD^+ on inactivation of pea lactate dehydrogenase (LDH) by phenylglyoxal (PGO). *1* control (LDH), *2* LDH + + 10 mM PGO, *3* LDH + 25 mM NAD^+ + + 10 mM PGO, *4* LDH + 2·5 mM NAD^+ + + 10 mM PGO

value of K_m (Fig. 3). The influence of the reduced enzyme was different. Lower NADH concentrations did not affect the inhibition, concentrations of NADH

TABLE IV

Effect of substrates on incativation of pea lactate dehydrogenase by 10 mM phenylglyoxal

Substrate	Concentration mol l^{-1}	k_1 min^{-1}
—	—	0·028
NAD^+	2·6	0·024
	26·4	0·006
NADH	0·3	0·026
	3·0	0·090
	30·0	0·105
Lactate	6·6	0·002
	66·0	0·0006
Pyruvate	0·5	0·002
	5·0	0·0004

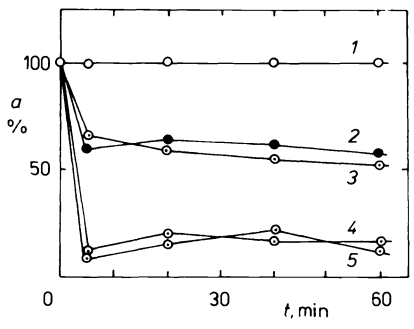

FIG. 4

Effect of NADH on inactivation of pea lactate dehydrogenase (LDH) by phenylglyoxal (PGO). *1* control (LDH), *2* LDH + + 10 mM PGO, *3* LDH + 0·3 mM NADH + + 10 mM PGO, *4* LDH + 3·0 mM NADH + + 10 mM PGO, *5* LDH + 30 mM NADH + + 10 mM PGO

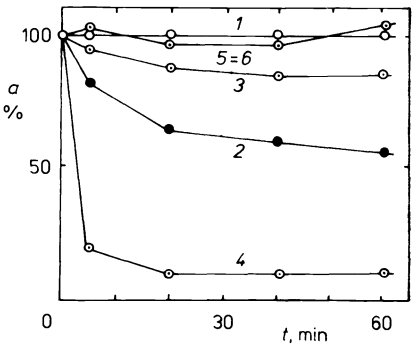

FIG. 5

Effect of substrates on inactivation of pea lactate dehydrogenase (LDH) by phenylglyoxal (PGO). *1* control (LDH), *2* LDH + + 10 mM PGO, *3* LDH + 25 mM NAD^+ + + 10 mM PGO, *4* LDH + 3 mM NADH + + 10 mM PGO, *5* LDH + 7 mM lactate + + 10 mM PGO, *6* LDH + 0·5 mM pyruvate + 10 mM PGO

corresponding to ten to hundred times the K_m-value enhanced the inactivation significantly (Fig. 4). In contrast, the substrates, lactate and pyruvate, were very efficient in preventing the enzyme against inactivation; even when applied at concentrations close to their K_m-values there was practically no inaticvation. The efficiency of the individual substrates in protecting pea lactate dehydrogenase against inactivation by phenylglyoxal is compared in Fig. 5.

DISCUSSION

The isolation of lactate dehydrogenase from plants is considerably more elaborate than from animal tissues. The enzyme has been isolated so far from soybean seeds[1,7-9], from potato tubers[4-6] and from barley[16] to a purity which permitted some structure studies to be performed. We used affinity chromatography on AMP-Sepharose 4B to prepare the enzyme from germinating pea seeds in a quality and quantity which enabled us to perform some kinetic and structure studies.

The values of the Michaelis constants as well as the values of the dissociation constants of binary apoenzyme–coenzyme complexes (Table II) point to a certain difference between the enzyme from pea and soybean[1] seeds. The pea enzyme shows a relatively strong affinity for NAD^+ and lactate, the K_m-values are at least by one order lower than those measured with the enzyme from soybean seeds. The value of the dissociation constant of the enzyme–NAD^+ complex is even by two orders lower and corresponds to the value of the constant obtained with the enzyme from potato tubers[17]. It is likely that the physiological role of lactate dehydrogenase in germinating pea seeds involves not only NADH oxidation during the period of anaerobiosis yet also the reversal oxidation of lactate to pyruvate after the restoration of aerobic conditions in plant tissues. Likewise, as regards its amino acid composition the pea enzyme resembles most the enzyme from potato tubers[6].

From the structure viewpoint pea lactate dehydrogenase is an enzyme of relative molecular weight 145 000 showing the structure of a tetramer. The M_r of the subunit is approximately 35 000. Essential for the activity of the enzyme is its sulfhydryl group[10,12]. Both in the enzyme from animal tissues[2,18] and in the enzyme from potato tubers[6] is arginine an important part of the catalytic domain of the active center. Studies on the role of this amino acid in the catalytic mechanism have made use of 1,2-dicarbonyl compounds which selectively interact with the guanidine group[19]. We have used phenylglyoxal in our studies on the role of arginine in the active center of pea lactate dehydrogenase. Phenylglyoxal inactivated the pea enzyme and the inactivation followed the kinetics of the first order; this seems to indicate that one or a few arginine groups only are essential for the activity of the enzyme. These groups are modified at the same rate under the conditions of our experiment. The dependence of the rate constant of the first order on inhibitor concentration is linear (Fig. 2); hence, there is no formation of a saturation complex in the beginning

of the reaction which is a true chemical modification of the protein. The modification rate constant of the second order is 3·1 l mol^{-1} min^{-1}, a value which exactly corresponds to the values recorded, e.g. for the inactivation of phospholipase A (ref.[20]) or ornithine transcarboxylase[15] by phenylglyoxal. A comparison of the amino acid composition of the native and phenylglyoxal-modified enzyme shows that no lysine or histidine residues yet exlusively the guanidine moiety of the arginine residues were modified under the conditions of our experiment. Neither were the sulfhydryl groups modified any significantly. The number of tryptophan residues was not determined and neither was assayed the sugar content (if any) of pea lactate dehydrogenase. The data published so far on the structure of lactate dehydrogenase of both animal[2,3] and plant[6,16] origin do not consider the possibility that the enzyme contains a sugar moiety in its molecule.

The amino acid analysis shows that almost all arginine residues of the enzyme molecule were modified. A correlation of the number of modified residues with the activity of the enzyme would require the use of radioactively labeled phenylglyoxal; such experiments were not possible in our laboratory.

The experiments in which the enzyme was preincubated first with one of its substrates and subsequently labeled with phenylglyoxal show that the arginine residue is located in the catalytic domain of the active center of pea lactate dehydrogenase; it participates in the binding of the substrate, i.e. lactate or pyruvate. The nature of this binding is obviously that of an ionic interaction between the positively charged guanidine group and the negatively charged carboxyl of the substrate. Likewise, the oxidized coenzyme (NAD^+) showed a certain protective effect which could be observed when NAD^+ was employed in a concentration which corresponded to a 100-fold of the K_m-value. The arginine residue is obviously located also in the coenzyme domain of the active site, similarly to the animal enzyme[2]. It participates in the binding of the coenzyme to the apoenzyme via interaction with its pyrophosphate moiety.

The binary apoenzyme–reduced coenzyme (NADH) complex was inactivated at a higher rate than the free enzyme. It is likely that the conformational change of the protein brought about by the binding of NADH (ref.[21]) makes the arginine necessary for the binding of the coenzyme or substrate more accessible for modification.

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