ISOLATION OF PLANT LACTATE DEHYDROGENASE BY AFFINITY CHROMATOGRAPHY AND ROLE OF HISTIDINE IN THE MOLECULE OF THE ENZYME

Jana BARTHOVÁ, Pavla PLACHÁ and Sylva LEBLOVÁ

Department of Biochemistry,
Charles University, 128 40 Prague 2

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Lactate dehydrogenase (EC 1.1.1.27) was isolated from soybean seedlings (*Glycine max.* L.) by affinity chromatography on an AMP-Sepharose 4B column. The enzyme obtained was inactivated by treatment with diethyl pyrocarbonate; the inactivation rate was proportional to the molar ratio of the enzyme to the reagent. The plot of the inactivation rate *versus* pH shows that of all the functional groups of the protein the imidazole groups of histidine only were modified by diethyl pyrocarbonate. By this procedure 20 histidine residues were ethoxyformylated in the molecule of soybean lactate dehydrogenase yet 8 only, *i.e.* two in every subunit were essential for the activity of the enzyme. A comparison of the effect of diethyl pyrocarbonate on the lactate dehydrogenase apoenzyme with its effect on the binary complexes of the enzyme with coenzymes or on ternary complexes with its both substrates permits the conclusion that histidine is involved not only in the proton transfer during the redox reaction but also in the coenzyme-binding site.

The germination of seeds starts with a shorter or longer anaerobiosis period during which the organism must satisfy large energetic needs by anaerobic degradation of the substrates. It is during this initial stage where lactate dehydrogenase plays an important role[1-4]. We decided to examine the function of this enzyme, besides others, also by studying the structure of the isolated enzyme[5] and especially of its active center. Kinetic assays of the effect of pH and temperature on the activity of the enzyme have shown that the groups involved in the active site of lactate dehydrogenase are most likely the sulfhydryl group of cysteine, the hydroxy group of tyrosine, and the imidazole ring of histidine[6]. It has been demonstrated in modification experiments that the enzyme contains 24 sulfhydryl groups in one molecule of which 4, *i.e.* one in every subunit, are localized in the neighborhood of the coenzyme-binding site[5-7], and that the hydroxy group of tyrosine directly participates in the binding of the coenzyme[8].

The aim of the present study was to examine the role of histidine in the molecule and particularly in the active site of soybean lactate dehydrogenase.

EXPERIMENTAL

Material

The chemicals used in this study were tris-(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid (EDTA), sodium pyruvate (Lachema, Brno), Sevac-test-LDH (Imuna, Šarišské Michalany), NAD, sodium L-lactate (Serva, Heidelberg, FRG), NADH (Merck, Darmstadt, FRG), AMP-Sepharose 4B (Pharmacia, Uppsala, Sweden), and diethyl pyrocarbonate (Fluka, Buchs, FRG). The remaining chemicals used were of analytical purity unless stated otherwise.

Methods

Soybean seedlings 32 h old served as a source of the enzyme. The enzyme was isolated by affinity chromatography on a column of AMP-Sepharose 4B essentially by the method of Mosbach[9] which was partially modified for the purpose of isolation from plant material. The fraction from ammonium sulfate fractionation (30—40% saturation, about 80 mg of proteins) was desalted and applied to a 2 × 4·5 cm AMP-Sepharose 4B column. The latter was equilibrated with 0·1M Tris-acetate buffer, pH 8·6, containing 1 mM mercaptoethanol. The enzyme was eluted from the column by 5 mM-NAD solution in the same buffer. Active fractions were pooled, desalted, and lyophilized.

The activity of the enzyme was examined by measurement of pyruvate formation which gives a colored hydrazone with 2,4-dinitrophenylhydrazine in alkaline media. Sevac-test-LDH was employed for the determination.

The concentration of proteins was determined by the method of Lowry and coworkers[10].

The histidine residues of the enzyme were modified by diethyl pyrocarbonate; a modification of the method of Wallis and Holbrook[11] was used. Lactate dehydrogenase dissolved in 0·1M-NaH_2PO_4, whose pH had been adjusted to 6 by 5M-NaOH, was mixed at an appropriate ratio with 1 mM solution of diethyl pyrocarbonate in absolute ethanol and the mixture was incubated at 25°C. Samples for activity determination were withdrawn from the reaction mixture at selected time intervals. A mixture in which the solution of diethyl pyrocarbonate had been replaced by a corresponding volume of ethanol served as a blank. The number of ethoxyformyl groups incorporated into the protein as ethoxyformylimidazole was determined from the difference in absorbance at 240 nm of the modified and the native enzyme and from the molar extinction coefficient of this compound[12]. The deacylation of the modified enzyme was effected by dilution of the samples with an equal volume of 1M hydroxylamine in 0·1M phosphate buffer, pH 7·5, containing 2 mM mercaptoethanol, and incubation at 4°C as described by Choong and coworkers[13].

RESULTS

Isolation of enzyme: The enzyme was purified more than 100 times by a procedure involving extraction, ammonium sulfate fractionation, and chromatography on a column of AMP-Sepharose. The efficiency of the individual isolation steps is documented in Table I.

The treatment of the enzyme with diethyl pyrocarbonate results in a rapid loss of activity. The inactivation rate is proportional to the concentration of the inactivating reagent and to the molar diethyl pyrocarbonate to lactate dehydrogenase ratio,

respectively. The plot of the inactivation rate expressed by a first order reaction constant *versus* diethyl pyrocarbonate concentration is linear.

Inactivation as function of pH: The inactivation of soybean lactate dehydrogenase by diethyl pyrocarbonate was examined in 0·1M phosphate buffers at pH 5·0 to 8·0 (Fig. 1). The dissociation constant (K_H) of the group modified can be calculated from this plot using the formula proposed by Ehrlich and Colman[14]

$$k_{obs} - k_{min} = k_{max} - k_{min}/1 + ([H^+]/K_H),$$

where k_{obs} is the inactivation rate constant at the given hydrogen ion concentration. When k_{max}, *i.e.* the inactivation rate constant of the unprotonated enzyme was substituted by 0·693 and k_{min}, *i.e.* the inactivation rate constant of the protonated enzyme by 0·124, then the pK_H for lactate dehydrogenase was 7·0. The pK-values for the modified group obtained in different experiments varied between 6·8 and 7·0.

Number of modified histidine residues in the molecule of soybean lactate dehydrogenase and their function: As a result of the action of diethyl pyrocarbonate on the enzyme solution its absorbance at 240 nm increases in proportion to the number of modified histidine residues. On condition that the molecular weight of soybean lactate dehydrogenase is 140 000 (ref.[5]) and that the molar extinction coefficient of the derivative formed is 3 500 l mol^{-1} cm^{-1}, as follows from experiments with the modification of free histidine by diethyl pyrocarbonate, 20 histidine residues of lactate dehydrogenase are labeled under our experimental conditions (Fig. 2).

TABLE I

Isolation of Lactate Dehydrogenase from Soybean Seedlings

The values in the Table are based on 50 g of fresh weight.

Step	Protein mg	Activity		Degree of purification
		total U . 10^{-3}	specific U/mg	
Crude extract	1 250	223·0	178	1
Sulfate fraction 30—40% saturation	185	75·8	410	2·5
Chromatography on AMP-Sepharose 4B	3·5	62·5	18 800	105

If we correlate the number of modified histidine residues with the decrease of the activity of the enzyme (Fig. 2) it can be seen that the enzyme is completely inactivated after eight residues in the molecule, *i.e.* two in every subunit have been labeled.

Reactivation experiments: The treatment of lactate dehydrogenase modified by diethyl pyrocarbonate with hydroxylamine leads gradually to complete deacylation of the histidine residues, as shown in Fig. 3. The activity of the enzyme was restored only after hydroxylamine had been dialyzed off; this is due to the fact that hydroxylamine inhibits also native lactate dehydrogenase itself. After 48-h dialysis against the buffer the activity of modified and originally completely inactive lactate dehydrogenase was restored to 86% compared to the control (the enzyme mixed with hydroxylamine at equal ratios).

Modification of soybean lactate dehydrogenase in the presence of substrates and coenzymes: The rate of inactivation of soy bean lactate dehydrogenase by diethyl pyrocarbonate was not essentially affected by any of the substrates if its concentration was at the K_m-level of the corresponding substrate or even higher by one order (Table II). NAD only at concentrations close to a hundred-fold excess of the K_m-value slowed down the inactivation of the enzyme any markedly.

TABLE II

Inactivation of Soybean Lactate Dehydrogenase by Diethyl Pyrocarbonate in Presence of Substrates

Ligand added to reaction medium	K_m M	Concentration M	Inactivation rate k, min^{-1}
—	—	—	0·144
Lactate	$4 . 10^{-2}$	10^{-2}	0·131
		10^{-1}	0·144
Pyruvate	$1 . 10^{-4}$	10^{-4}	0·151
		10^{-3}	0·139
NADH	$4 . 10^{-5}$	10^{-5}	0·126
		10^{-4}	0·134
NAD	$5 . 10^{-4}$	10^{-4}	0·118
		10^{-3}	0·108
		$2 . 10^{-2}$	0·029
NAD + lactate		$10^{-4} + 10^{-2}$	0·053
NADH + pyruvate		$10^{-5} + 10^{-4}$	0·866

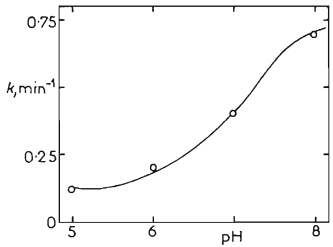

FIG. 1

Plot of Rate of Lactate Dehydrogenase Inactivation by Diethyl Pyrocarbonate *versus* Hydrogen Ion Concentration of Medium

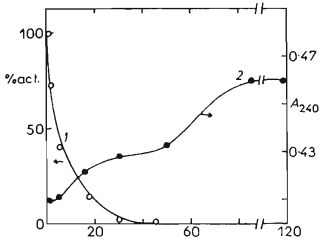

FIG. 2

Effect of Diethyl Pyrocarbonate on Soybean Lactate Dehydrogenase

1 Enzyme activity in per cent of original activity, *2* quantity of modified histidine residues expressed as absorbance change at 240 nm.

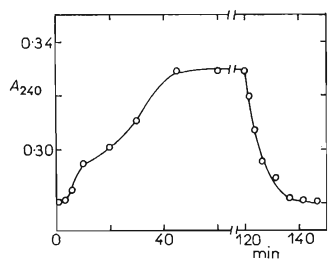

FIG. 3

Modification of Histidine Residues of Soybean Lactate Dehydrogenase by Diethyl Pyrocarbonate with Subsequent Deacylation by 1M Hydroxylamine

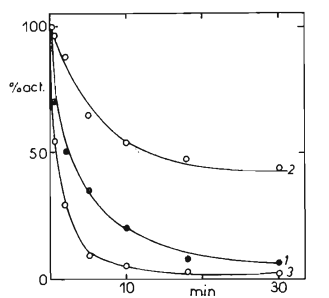

FIG. 4

Inactivation of Ternary Complexes of Soybean Lactate Dehydrogenase (LDH) by Diethyl Pyrocarbonate

% of original activity of *1* apoenzyme, *2* LDH–NAD–lactate complex, *3* LDH–NADH–pyruvate complex.

Finally, we investigated whether the histidine residues of the enzyme are protected against modification in ternary complexes of the enzyme. As documented by Table II and Fig. 4, the inactivation is considerably slowed down in the enzyme–NAD–lactate complex whereas its rate is considerably higher in the enzyme–NADH–pyruvate complex.

DISCUSSION

In the present study we isolated the enzyme by affinity chromatography on an AMP--Sepharose column. A 40-fold purification of the enzyme was achieved and the recovery was more than 80%. These results are more favorable than those obtained with a Blue Sepharose used before (16-fold purification, 38% recovery, ref.[6,7]). A certain disadvantage of the present method represents the consumption of NAD necessary for the elution of the enzyme from the column and the procedure is therefore a little more costly. The enzyme obtained was electrophoretically homogeneous and was used in all the subsequent experiments.

The investigation of the pH of the medium on the activity of soybean lactate dehydrogenase showed[6] that the group playing a role in the catalytic process itself has a pK of about 7 and we suspected therefore the imidazole group of histidine. We made an effort in this study to verify this assumption in a series of modification experiments.

It has been postulated long ago[15] that histidine forms a part of the active center of animal lactate dehydrogenase yet this postulate was confirmed much later after it had been shown that diethyl pyrocarbonate is suitable for selective histidine modification especially in weakly acidic media[16]. Holbrook and Ingram[17] using this reagent were able to determine the pK of the group playing a role both in the apo- and the holoenzyme of animal lactate dehydrogenase and thus to show that the imidazole ring of histidine is involved.

We found that plant lactate dehydrogenase is also inactivated by diethyl pyrocarbonate and that the inactivation rate is proportional to the molar ratio of the enzyme to the reagent. The plot of the inactivation rate *versus* pH over the entire investigated range has a shape indicating that one group of a pK between 6·8 and 7·0 is modified. This shows that the imidazole of histidine is labeled. Using this method a total number of 20 histidines were ethoxyformylated in one molecule of soybean lactate dehydrogenase. Amino acid analyses of lactate dehydrogenases from various animal and bacterial species show the presence of 7 to 12 histidines in one subunit[18]. No amino acid analysis of the plant enzyme has so far been reported. Our data show that the soybean lactate dehydrogenase subunit contains 5 histidines which can be modified by diethyl pyrocarbonate. However, not all of these histidines are essential for the activity of the enzyme. Even after the modification of eight histidines, *i.e.* of two per subunit, soy bean lactate dehydrogenase was completely inactivated (Fig. 2). In this respect interest deserves the ununiform and essentially repetitive

course (Fig. 2 and 3) of histidine inactivation as a function of time. Holbrook and Ingram[17] declare that the essential histidine reacts with diethyl pyrocarbonate up to ten times faster than the remaining histidines or than free histidine. Our observation could perhaps be interpreted in an analogous manner. The treatment of the ethoxyformylated and completely inactive lactate dehydrogenase with hydroxylamine decreased its 240 nm absorbance to the value characteristic of the native enzyme. This shows that all ethoxyformyl groups were removed. The removal was paralleled by an almost complete (86%) reactivation of the enzyme. This is another piece of evidence in favor of the importance of histidine groups for the activity of soybean lactate dehydrogenase. The activity was restored by the action of hydroxylamine during 15 min, *i.e.* during a very short time. From the data of Melchior and Fahrney[19] only the ethoxyformyl-histidine bonds are readily cleaved by hydroxylamine. The hydrolysis of bonds with other groups, *e.g.* with amino groups, requires much longer periods.

The results of experiments in which we endeavored to protect the enzyme against modification by preincubation with individual substrates and coenzymes show that histidine is obviously not localized at the site of attachment of the substrates, *i.e.* of lactate or pyruvate or that the binding of substrates to the enzyme cannot prevent it from chemical modification. Histidine, however, is most likely the part of the coenzyme domain and participates here in the binding of the oxidized coenzyme (NAD) since NAD at a concentration close to its binding constant ($4 . 10^{-1}$M, ref.[6]) decreased the inactivation rate constant five times. The reduced coenzyme, whose binding constant is $1 . 10^{-6}$M (ref.[6]) is lacking a similar effect even if applied at higher concentrations. Similarly, the results of experiments in which diethyl pyrocarbonate was allowed to react with the ternary complexes of the enzyme with both substrates seem to suggest that histidine is protected against modification in the complex of the enzyme with the oxidized coenzyme and lactate only. By contrast the ternary complex of soybean lactate dehydrogenase with NADH and pyruvate is inactivated up to six times faster than the apoenzyme. This again supports the view that the binding of NADH to the apoenzyme of lactate dehydrogenase is paralleled by change of conformation of the protein which makes its active center more accessible to chemical moification. A similar observation has been made with the sulfhydryl group which undoubtedly is also a part of the active site of soybean lactate dehydrogenase.[7]

We may conclude that the treatment of soybean lactate dehydrogenase with diethyl pyrocarbonate leads to ethoxyformalytion of 20 histidine residues in its molecule of which 8 are necessary for the activity of the enzyme. In analogy to the animal lactate dehydrogenase studied in more detail we may postulate that histidine is not only a part of the catalytic domain of the plant enzyme, where it accepts the proton from the substrates and then releases this proton into the medium, but that histidine obviously plays an important role also in the coenzyme domain where it participates in the binding of the oxidized coenzyme.

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