THE FUNCTION OF SULFHYDRYL GROUPS IN LACTATE DEHYDROGENASE ISOLATED FROM SOY BEAN SEEDLINGS USING BLUE DEXTRAN-SEPHAROSE

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Received January 25th, 1977

The specific adsorption of lactate dehydrogenase from soy bean seedlings on Blue Dextran – Sepharose was used for its isolation. The function of sulfhydryl groups of the isolated enzyme was studied using N-ethylmaleimide and iodoacetamide which acted as weak inhibitors. In low concentrations, *p*-chloromercuribenzoate was a non-competititve inhibitor characterized by $K_i = 3{\cdot}4 \cdot 10^{-4}$ M both for NAD and lactate as substrates. The inhibition became irreversible when the concentration of the inhibitor exceeded 0·01 M. Rate constants were determined for the inactivation of lactate dehydrogenase by *p*-chloromercuribenzoate and iodoacetate. Preincubation of the enzyme with NAD decreased the inhibition of lactate dehydrogenase by *p*-chloromercuribenzoate and iodoacetate by 50% and 75%, respectively. No protective effect of NADH was found.

Sulfhydryl groups are essential for the function of the majority of dehydrogenases[1]. It is well known that lactate dehydrogenases of animal origin contain dodekapeptide of similar amino acid composition containing an essential sulfhydryl group[2]. This group is a part of the active centre of the enzyme and it is likely that it participates in the binding of the nicotinamide part of the coenzyme[3]. The data on plant lactate dehydrogenase are rare and sometimes contradictory. Lactate dehydrogenase from pea seedlings[4] is strongly inhibited by *p*-chloromercuribenzoate, iodoacetamide and N-ethylmaleimide.

Previously we established that lactate dehydrogenase from soy bean contained 24 SH-groups in one molecule[5] and was, as preliminarily shown, inhibited by SH-poisons, in the contrast to the enzyme King[6] worked with. In this paper we studied the role of sulfhydryl groups in catalytic function of lactate dehydrogenase from soy bean seedlings. The enzyme was isolated from plant material by the specific adsorption on Blue Dextran-Sepharose.

EXPERIMENTAL

Materials

We used 2-mercaptoethanol, *p*-chloromercuribenzoate (PCMB) and dithiotreitol (Koch-Light, England), tris/hydroxymethyl/aminomethane (Tris), natrium pyruvate and L-cystein (Lachema, Brno), NAD and Sevac-test-LDH (Immuna, Šárišské Michalany), NADH (Calbiochem, USA),

iodoacetamide (NBC, Cleveland), N-ethylmaleimide and iodoacetate (Aldrich, USA), Sepharose 4B and Blue Dextran (Pharmacia, Uppsala). All other chemicals were, if not otherwise indicated, of analytical grade.

Methods

Isolation of enzyme. The enzyme was isolated from soy bean seeds which had germinated 32 h in distilled water at 20°C. 400 g of germinating seeds were homogenised 5 min at 4°C in 400 ml of 20 mM Tris-acetate buffer pH 8·6 containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The homogenate was passed through four layers of gauze and the filtrate was centrifuged 2 h at 3000 *g* at 4°C. The supernatant obtained (crude extract) was fractioned by precipitation with ammonium sulphate. The fraction of proteins with lactate dehydrogenase activity was precipitated between 30 and 40% of saturation. This protein fraction was further purified by a procedure applied originally for the isolation of lactate dehydrogenase from rat liver[7]. For the isolation of soy bean lactate dehydrogenase, we partly modified the original procedure. The sulphate fraction (max. 0·8 g of protein) was applied to a column of Blue Dextran — Sepharose prepared according to Ryan and Vestling[7]. The column (1·8 x 10 cm) was equilibrated with 20 mM Tris-acetate buffer pH 8·6 containing 1 mM EDTA and 1 mM 2-mercaptoethanol. At first, the column was eluted by the same buffer at a rate of 0·4 ml/min until the extinction of the eluate decreased below 0·02 at 280 nm. The column was then washed through by the buffer containing 0·7M-KCl until the extinction of the eluate decreased below the value 0·02. The lactate dehydrogenase activity and protein concentration were determined in the individual fractions (4 ml). The diluted solutions of the enzyme were concentrated and desalted by ultrafiltration through a XM 50 membrane.

The determination of lactate dehydrogenase activity was performed using the Sevac-test-LDH in which the reaction of pyruvate with 2,4-dinitrophenylhydrazine is measured colorimetrically.

The concentration of proteins was determined by the method of Lowry and coworkers[8].

Determination of Michaelis constants. Michaelis constants for NAD and lactate were calculated according to the rate equation for a two-substrate reaction[9].

$$v = \frac{V \,.\, [\mathrm{A}] \,.\, [\mathrm{B}]}{K'_{\mathrm{A}} K_{\mathrm{B}} + K_{\mathrm{B}} \,.\, [\mathrm{A}] + K_{\mathrm{A}} \,.\, [\mathrm{B}] + [\mathrm{A}] \,.\, [\mathrm{B}]}$$

where [A] and [B] are the concentrations of NAD and lactate resp., K_{A} is the Michaelis constant for NAD at saturation of the enzyme by lactate, K_{B} that for lactate at saturation of the enzyme by NAD and K'_{A} is the dissociation constant of the enzyme–NAD complex.

RESULTS AND DISCUSSION

Isolation of enzyme. Affinity chromatography on a column of Sepharose 4B with bound Blue Dextran was used for the isolation of lactate dehydrogenase from soy bean seedlings. This carrier interacts specifically with all proteins having a so-called "dinucleotide fold" in their structure[10]. Blue Dextran-Sepharose was prepared according to Ryan and Vestling[7]. For the purpose of our work the chromatographic procedure was modified, as the crude enzyme extract from plant tissue contained a great deal of ballast material of non-protein nature. Only the fraction of proteins

precipitating between 30 and 40% of ammonium sulphate saturation was subjected to chromatography on the column of Blue Dextran-Sepharose. As the desorption of enzyme was not affected substantially by the presence of NADH in the elution buffer, we used a solution of high ionic strength for eluting the enzyme. Lactate dehydrogenase was enriched 16 times using this simple step. A survey of the efficiency of the individual isolation steps is given in Table I. The simple purification procedure lasting 12 h resulting in an enzyme preparation with 80 times higher specific activity than that of the crude extract.

The study of sulfhydryl groups in the molecule of soy bean lactate dehydrogenase. The important role of SH-groups in the molecule of lactate dehydrogenase[11-13] and other dehydrogenases[14] was proved several times. We studied the role of these groups in plant lactate dehydrogenase. We followed the influence of several inhibitors of SH-groups on the enzyme activity and found that *p*-chloromercuribenzoate, iodoacetate, iodoacetamide and N-ethylmaleimide inhibited lactate dehydrogenase irreversibly. The first two were strong inhibitors, whereas iodoacetamide and N-ethylmaleimide seemed to act weakly, even a 600 fold molar excess of inhibitor resulted in very slow inhibition. We observed the same properties in the case of lactate dehydrogenase from pea plants[4]. However, King[6] observed no inhibition of the soy bean lactate dehydrogenase after an 11 hours' treatment of the enzyme with *p*-chloromercuribenzoate, iodoacetate and arsenite.

The action of the strong inhibitors PCMB and iodoacetate was studied in detail. First we determined the dependence of the inactivation rate on the inhibitor concentration. At low concentrations – max. 0·01M PCMB (molar ratio of enzyme to inhibitor 1 : 4) the compound acted as a reversible inhibitor of lactate dehydrogenase (Fig. 2).

TABLE I

Isolation of Lactate Dehydrogenase from Soy Bean Seedlings, Values of Michaelis Constants

Fraction	Proteins mg	Activity		$K_{lac.}$ M	K_{NAD} M
		total U	specific U/mg		
Crude extract	4 740	$275 . 10^3$	43·7	—	—
30—40% saturation by ammonium sulphate	438	$96 . 10^3$	219	$2·1 . 10^{-2}$	$7·2 . 10^{-4}$
Affinity chrom. Blue Dextran-Sepharose	11	$39 . 10^3$	3 540	$2·1 . 10^{-2}$	$6·8 . 10^{-4}$

The character of the inhibition was non-competitive and the values of the inhibition constants for both NAD and lactate were almost identical, namely $3{\cdot}4 \cdot 10^{-4}$M.

TABLE II

The Effect of Inhibitor Concentration on the Inactivation Rate of Soy Bean Lactate Dehydrogenase

The dependence is expressed by the rate constant of the first order.

Inhibitor concentration mM	k min^{-1}	Inhibitor concentration mM	k min^{-1}
p-chloromercuribenzoate		iodoacetate	
0·3	0·026	1·7	0·063
0·6	0·032	3·3	0·322
1·0	0·068	6·6	0·481
1·2	0·086	13·0	0·598
2·4	0·109		

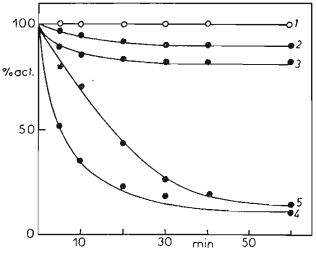

FIG. 1

The Inactivation of Lactate Dehydrogenase (LDH) from Soy Bean Seedlings by Compounds Reacting with Sulfhydryl Groups

Abscissa: time of incubation, min; ordinate: changes of activity at 20°C, %; *1* LDH, *2* LDH + 1·7 mM iodoacetamide, *3* LDH + + 1·7 mM N-ethylmaleimide, *4* LDH + + 1·7 mM iodoacetate, *5* LDH + 0·3 mM PCMB.

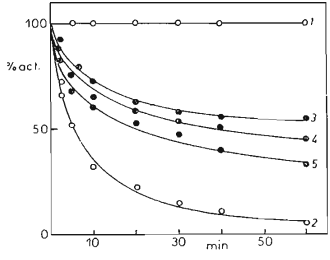

FIG. 2

The Action of PCMB on the Binary Enzyme–NAD Complex of Soy Bean Lactate Dehydrogenase

Abscissa: time of incubation, min; ordinate: changes of activity at 20°C, %; *1* LDH, *2* LDH + 2 mM PCMB, *3* LDH + 9 mM NAD + 2 mM PCMB, *4* LDH + 6 mM NAD + 2 mM PCMB, *5* LDH + 3 mM NAD + 2 mM PCMB.

Increasing the inhibitor concentration led to the irreversible inactivation of the enzyme which could not be suppressed either by a 10 fold molar excess of SH-compounds such as 2-mercaptoethanol, L-cystein or dithiotreitol, or by dialysis against 20 mM Tris-acetate buffer, pH 8·6, containing 1 mM 2-mercaptoethanol. The same holds for iodoacetate which is a strong inhibitor of soy bean lactate dehydrogenase. The effect of PCMB and iodoacetate is dependent on the dose and on the duration of the treatment. The effect of the inhibitor concentration on the inactivation rate of soy bean lactate dehydrogenase by iodoacetate and PCMB is shown in Table II.

In comparison with iodoacetate, the inhibitory effect of iodoacetamide and N-ethylmaleimide was weaker. Both compounds are specific alkylating agents of SH-groups which efficiently inactivate the enzyme isolated from pea plants[4]. The alkylating effect of iodoacetate is preceded by alterations of the tertiary structure of the proteins which render the SH-groups of soy bean lactate dehydrogenase accessible to alkylation. This explanation is supported by experiments in which the enzyme is inactivated by two inhibitors. When lactate dehydrogenase was preincubated 120 min with

TABLE III

The Combined Effect of Two SH-Inhibitors of Lactate Dehydrogenase (LDH) from Soy Bean Seedlings

Time min	% Activity		
	native LDH	LDH modified by 9 mM iodoacetate	LDH modified by 9 mM N-ethylmaleimide
	p-chloromercuribenzoate, 1·7 mM		
0	100	100	100
10	79	69	56
20	68	50	42
30	58	41	30
40	52	33	22
60	42	28	15
90	32	23	16
120	26	21	13
	iodoacetate, 8·3 mM		
0	100	100	100
10	30	11	9
20	12	4	3
30	6	0	0

N-ethylmaleimide or iodoacetamide, the result was a 10% decrease of activity. The enzyme thus modified was treated with PCMB or iodoacetate which had a strong inhibitory effect on the intact lactate dehydrogenase. The time course of the inhibition of both intact and modified enzymes by PCMB or iodoacetate is presented in Table III. The enzyme pretreated with N-ethylmaleimide or iodoacetate is inhibited more efficiently than the intact enzyme. This finding confirms the original presumption, *i.e.* that the strong inhibitors intervene in the tertiary structure and thus make other SH-groups accessible to alkylation. From the slight differences in the inactivation rates of modified and intact enzymes by strong inhibitors one can see that the alkylation of the enzyme by weak inhibitors led also to the destabilisation of the tertiary structure of the enzyme. (Fig. 3, 4).

Protection of the sulfhydryl groups of lactate dehydrogenase. We studied how preincubation of the enzyme with NAD affected the inhibition of enzymic activity by PCMB or iodoacetate. The protection of the enzyme by NAD is dependent on the concentration of the coenzyme, and its presence protects the enzyme against both PCMB and iodoacetate. However, an 800 : 1 molar ratio of coenzyme to enzyme decreases PCMB inhibition by only 50% and that of iodoacetate by 75%. This

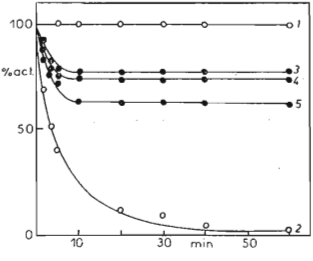

FIG. 3

The Action of Iodoacetate (*IA*) on the Binary Enzyme–NAD Complex of Soy Bean Lactate Dehydrogenase

Abscissa: time of incubation, min; ordinate: changes of activity at 20°C, %; *1* LDH, *2* LDH + 10 mM *IA*, *3* LDH + 9 mM NAD + 10 mM *IA*, *4* LDH + 6 mM NAD + + 10 mM *IA*, *5* LDH + 3 mM NAD + + 10 mM *IA*.

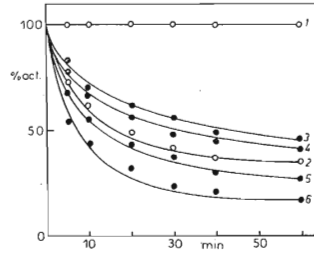

FIG. 4

The Action of PCMB on the Binary Enzyme–NADH Complex of Soy Bean Lactate Dehydrogenase

Abscissa: time of incubation, min; ordinate: change of activity at 20°C, %; *1* LDH, *2* LDH + 2 mM PCMB, *3* LDH + 20 μM NADH + 2 mM PCMB, *4* LDH + 50 μM NADH + 2 mM PCMB, *5* LDH + 100 μM NADH + 2 mM PCMB. *6* LDH + 400 μM NADH + 2 mM PCMB.

finding indicates that both inhibitors also modify SH-groups that do not participate in the binding of the coenzyme.

The preincubation of the enzyme with the reduced form of coenzyme (NADH) has a somewhat complicated effect. In comparison with NAD, NADH has only a low protective effect. Moreover, this effect is observable only when the concentration of NADH is lower than 0·1 mM. Increasing the concentration of NADH above this value resulted in inhibition instead of protection of the enzyme. We verified that this inhibition was not of the product inhibition type. NADH in 0·4 mM concentration had no effect on the rate of pyruvate formation from lactate. It therefore seems probable that the group attacked by PCMB is not in the vicinity of the binding site for NADH. We could even assume that the binary enzyme–NADH complex is more accessible to PCMB than the free enzyme. Anderson and coworkers[12], who studied the inactivation of animal lactate dehydrogenase by derivatives of maleimide, found that NADH protected only the H_4 and not the M_4 isoenzymes. In this respect, soy bean lactate dehydrogenase is similar to the M_4 isoenzyme of animal origin.

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Translated by T. Barth.