ESSENTIAL ARGININE RESIDUES IN LACTATE DEHYDROGENASE FROM GERMINATING SOYBEAN

Jana BARTHOVA, Irena HULOVA and Miroslava BIRCAKOVA

Department of Biochemistry,
Charles University, 128 40 Prague 2, The Czech Republic

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The lactate dehydrogenase was isolated from soybean (*Glycine max.* L.) by a procedure that employed biospecific chromatography on a column of Blue-Sepharose CL-6B. The participation of the guanidine group of arginine residues in the mechanism of enzyme action was determined through kinetic and chemical modification studies. The dependence of enzyme activity on pH was followed in the alkaline region (pH 8.6 – 12.8). The p*K* values found were 12.4 for the enzyme–substrate complex and 11.1 for the free enzyme. The enzyme was inactivated by phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione and *p*-hydroxyphenylglyoxal reagents used in modification experiments. Kinetic analysis of the modification indicated that one arginine residue is modified when inactivation occurs. No effect was observed on the rate of inactivation upon addition of coenzyme. The extent of enzyme modification by *p*-hydroxyphenylglyoxal was determined. It appears there are at least two arginine residues in the active site of the enzyme.

Lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27, LDH) plays an important role in organism overcoming temporary oxygen insufficiency that takes place e.g. in hard working muscle[1] or during germination of seeds[2]. Information about plant enzymes is meager in comparison with those of animals[2]. The data available so far characterize mainly kinetic and also some structural properties of potato[3] and soy[4 – 6] LDH. It is known that soy LDH has a molecular weight 140 000 (ref.[4]) and consists of four electrophoretically identical subunits with M.w. 36 000 (ref.[7]). In structural studies it has been found that cysteine[8], tyrosine[9], histidine[10] and lysine[11] take part in substrate binding and in the catalytic function of soy LDH. The present paper is another contribution to the study of the soy LDH structure. It is concerned with the role of arginine residues.

EXPERIMENTAL

Lactate dehydrogenase was extracted by 0.01 M Tris-acetate buffer pH 8 containing 1 mM EDTA and 1 mM mercaptoethanol from soy seeds (*Glycine max.* L., cv. Polanka) germinating for 32 h in water. The enzyme was purified from the crude extract by fractionation with ammonium sulfate (the fraction of proteins precipitating between 20% and 40% saturation contained most of the LDH activity).

Sulfate fraction was applied on a column of Blue-Sepharose CL-6B equilibrated with 0.1 M sodium phosphate buffer pH 6.8 containing 1 mM mercaptoethanol. The active fractions eluted by 0.8 mM NADH in the buffer were pooled, desalted and concentrated by ultrafiltration.

The initial velocity of LDH-catalyzed pyruvate reduction was determined spectrophotometrically at 340 nm by measuring the NADH oxidation rate. The reaction was started by the addition of enzyme to a mixture of 0.125 – 1 mM pyruvate and 0.025 – 0.2 mM NADH in 0.05 M sodium phosphate buffer pH 7.2 at a temperature of 37 °C. The lactate oxidation rate was determined colorimetrically by monitoring the amount of pyruvate formed after reaction with 2,4-dinitrophenyl hydrazine at 505 nm. The reaction was started by the addition of enzyme to a mixture of 10 – 250 mM lactate and 0.4 – 2 mM NAD^+ in 0.1 M Tris-acetate buffer pH 8.8 at temperature 37 °C. In alkaline pH 0.05 M glycine–NaOH buffer (pH 8.6 – 12.8) was used for measurements.

Modification of the arginine residues in lactate dehydrogenase was performed using phenylglyoxal[12] (PGO), 2,3-butanedione[12] (BD), 1,2-cyclohexanedione[13] (CHD) and *p*-hydroxyphenylglyoxal[14] (HPGO). Modification by PGO was performed in 0.2 M sodium phosphate buffer pH 8 over a range of reagent concentrations (1 – 50 mmol l^{-1}), by BD (1 – 50 mmol l^{-1}) in 0.2 M sodium borate buffer pH 8.2, by CHD (10 – 50 mmol l^{-1}) in 0.2 mM sodium borate buffer pH 8.2 and by HPGO (0.5 – 20 mmol l^{-1}) in 0.2 mM sodium phosphate buffer pH 8 at 25 °C in darkness. Samples were taken from the reaction mixtures at different intervals and the LDH activity was determined. Pseudo-first-order rate constants of the inactivation reaction were determined from semi-logarithmical plots of residual activity vs time. Similar procedures were performed with the enzyme preincubated with 1 mM NADH or 25 mM NAD^+ for 10 min.

To characterize the extent of the reaction of HPGO with the enzyme, 1 ml of solution of lactate dehydrogenase (approximately 0.1 mg of protein) in 0.2 mM sodium phosphate buffer pH 8 was treated with 2 mM or 5 mM HPGO in darkness. The reaction was stopped after 20 or 40 min by dilution and the excess reagent was removed by ultrafiltration. The amount of arginines modified was determined in the protein fraction spectrophotometrically at 340 nm using the molar absorption coefficient $1.83 \cdot 10^7$ l mol^{-1} cm^{-1} (ref.[14]).

The protein concentration was determined by the Bradford[15] method using bovine serum albumin as a standard.

RESULTS

Experiments were produced with enzyme purified to homogeneity. The homogeneity of an enzyme preparation was tested by the Fast Protein Liquid Chromatography method (system Waters 625LC, column Mono Q HR 515). The specific activity of LDH was 1.14 mol s^{-1} l^{-1} which is 600 times higher than the specific activity of the crude extract.

The kinetic parameters of the enzyme reaction were determined to characterize the enzyme. Values of K_m, V_{max} and the dissociation constants of the binary complexes apoenzyme–coenzyme are presented at Table I.

The dependence of enzyme activity on pH was followed in the region of pH 8.6 – 12.8. The values obtained for K_m are presented in Fig. 1. This plot indicates the dependence of catalytic activity on the ionization of an essential group at the active site of the enzyme. The pK value of the essential group in the enzyme–substrate complex is 12.4 and of that in the free enzyme is 11.1.

The participation of guanidine groups of arginine residues in the mechanism of catalysis was investigated using phenylglyoxal (PGO), 2,3-butanedione (BD), 1,2-cyclohexanedione (CHD) and *p*-hydroxyphenylglyoxal (HPGO). The modification of lactate dehydrogenase by each of these reagents resulted in a loss of catalytic activity. The loss of activity was time- and reagent concentration-dependent and the inactivating reaction

TABLE I
Kinetic parameters of soy LDH. For details see Experimental

Parameter	Value, mol l^{-1}
K_m of lactate	$4.7 . 10^{-2}$
K_m of NAD^+	$9.0. 10^{-4}$
Dissoc. const. of LDH–NAD^+ complex	$1.2. 10^{-3}$
K_m of pyruvate	$3.0. 10^{-4}$
K_m of NADH	$1.0. 10^{-5}$
Dissoc. const. of LDH–NADH complex	$2.0. 10^{-5}$

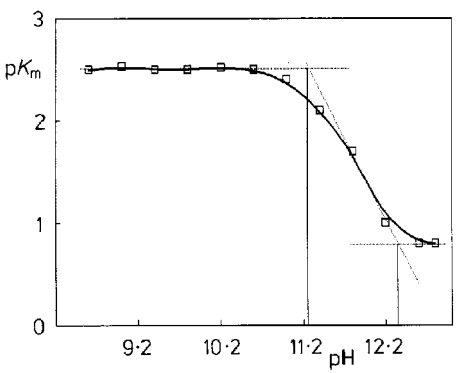

FIG. 1
Dependence of pK_m of lactate on pH for soy LDH. Values of K_m were obtained from Lineweaver–Burk plots determined with 25 mM NAD^+. For other details see Experimental

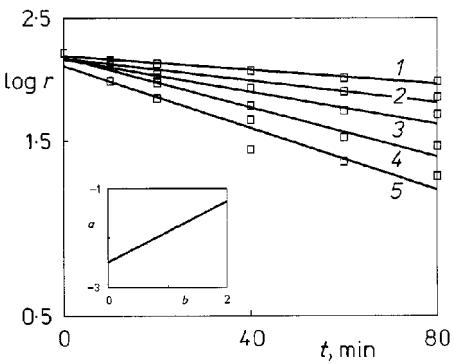

FIG. 2
Inactivation of soy LDH by 2,4-butanedione (BD). The enzyme was incubated with different concentrations of reagents: 1 mmol l^{-1} (*1*), 5 mmol l^{-1} (*2*), 10 mmol l^{-1} (*3*), 20 mmol l^{-1} (*4*), 50 mmol l^{-1} (*5*). Relative residual activity (*r*, %) was determined at the indicated times as described in the Experimental. Inset: Dependence of log k_1 (*a*) on log c_{BD} (*b*). The pseudo-first-order rate constants (k_1) were calculated from the slopes of the data in the figure

followed pseudo-first-order kinetics in every case. Examples of the inactivation of soy LDH by BD and HPGO are given in Figs 2 and 3, respectively. The second-order rate constants obtained from the slopes of linear plots of pseudo-first-order rate constants vs reagent concentrations are given in Table II. Analysis of the order of inactivation with respect to reagent concentration according to Levy et. al.[16] yielded a slope of 0.84 for CHD, 1.1 for BD, 1.05 for PGO, 0.75 for HPGO. Experiments were conducted when the enzyme was preincubated with a coenzyme (NADH, NAD^+). The coenzyme was not observed to have any effect on the rate of inactivation. The extent of enzyme modification by HPGO was determined (Fig. 4). It appears there are at least two easily modified arginine residues in the lactate dehydrogenase subunit.

DISCUSSION

The kinetic parameters found for soy LDH presented in Table I are in good agreement with those found for LDHs from other species[1]. It was discovered that there are 12 arginine in the soy LDH subunit[6]. Their function in enzyme action was investigated.

The dependence of enzyme activity upon pH was followed in the alkaline region where dissociation of the guanidine group occurs. We have found that there is an ionizable group in the active site with a p*K* value of 12.4 in the enzyme–substrate complex and 11.1 in the free enzyme. These values are in good agreement with those reported in the literature[17] for guanidine groups in proteins.

Modification experiments were performed and the kinetics of inactivation was followed. All reagents modifying specifically the guanidine group caused a loss in LDH catalytic activity. The reactivity of individual reagents is apparent from the second-order rate constants. The most effective is PGO or its derivative HPGO, the lowest

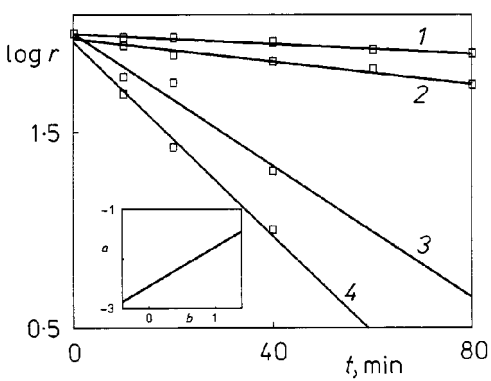

FIG. 3
Inactivation of soy LDH by *p*-hydroxyphenylglyoxal (HPGO). The enzyme was incubated with different reagent concentrations: 0.5 mmol l^{-1} (*1*), 1 mmol l^{-1} (*2*), 10 mmol l^{-1} (*3*), 20 mmol l^{-1} (*4*). Relative residual activity (*r*, %) was determined at the indicated times as described in the Experimental. Inset: Dependence of log k_1 (*a*) on log c_{pHPGO} (*b*). The pseudo-first-order rate constants (k_1) were calculated from the slopes of the data in the figure

effectivity has got CHD. The yield order of inactivation is nearly 1 in every case, indicating that one arginine residue is modified in the active site of the enzyme when inactivation occurs.

It is evident from experiments with HPGO that there are at least two arginine residues on the surface of each LDH subunit. We assume they are in the active site area and that modification of each of them would lead to the inactivation of the enzyme. Since this is true of animal LDH (ref.[1]) we assume it applies to the soy enzyme as well.

In order to investigate a probable function of the arginine residues in the active site of the enzyme, experiments were carried out when the enzyme was preincubated with an excess of coenzyme both oxidized and reduced (NADH, NAD^+). The coenzyme was not observed to have any effect on the rate of inactivation. This indicates that an arginine residue is involved in substrate binding (lactate, pyruvate). It is supposed that the interaction has the character of ion interaction between a positively charged guanidine group of the enzyme and a negatively charged carboxyl group of the substrate.

TABLE II
Modification of arginine residues of soy LDH. For details see Experimental

Modification agent	Second-order rate constant $l\ mol^{-1}\ min^{-1}$
PGO	1.38
CHD	0.30
BD	0.55
HPGO	3.60

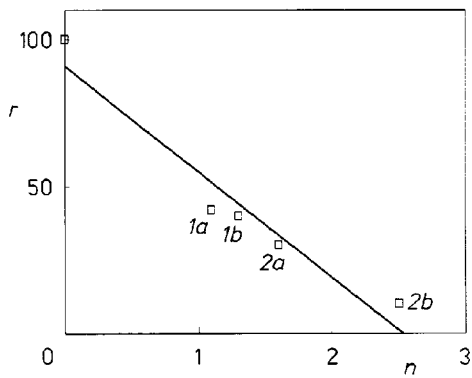

FIG. 4
Correlation between the number of arginine residues in soy LDH modified by HPGO (*n*) and the relative residual enzyme activity (*r*, %) with 2 mmol l^{-1} (*1*) or 5 mmol l^{-1} (*2*) HPGO. The relative residual activity and the number of arginines modified in one LDH subunit were determined after 20 (*a*) or 40 (*b*) min of incubation as described in the Experimental

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