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INHIBITION STUDIES ON LDH ISOENZYME PURIFIED FROM UROMASTIX TESTES

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An LDH isoenzyme was purified to homogeneity from uromastix testes and its inhibition spectrum towards known LDH isoenzyme inhibitors studied. Platinum compounds inhibited the enzyme in the forward reaction (pyruvate → lactate) only, *n*-hexanediol and colchicine showed no inhibition and gossypol acetic acid (GAA) strongly inhibited both the forward and reverse reactions and the reactions were time-dependent. Oxalate caused non-competitive inhibition (K_i app = IC_{50} = 0.15 mM) of the forward reaction, NADH was more effective in blocking inhibition by GAA than pyruvate. This enzyme was also unable to use ketocaproic acid as a substrate.

KEY WORDS: LDH, uromastix testes, ketocaproic acid, oxalate, uncompetitive inhibitor, gossypol acetic acid

INTRODUCTION

Lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27; LDH) is a glycolytic enzyme which catalyzes the interconversion of pyruvate and lactate. In most mammalian tissues it appears as five isoenzymes, each made up of four polypeptide chains.[1] In the mature testes and sperm of many species, a sixth isoenzyme is also present, known as LDH-X (or LDH-C).[2,3] Six isoenzymes of LDH were also detected in the gastrocnemius muscle of *uromastix hardwickii*.[4]

The properties of LDH-1 (heart-type) purified from the liver of same species were quite different as compared to the LDH-1 from other species. In our previous studies, we have purified an LDH isoenzyme from the testes of uromastix which showed certain properties like those of LDH-1 and some like those of LDH-X.[3] In this paper, we describe further characteristics of this isoenzyme using certain inhibitors of LDH which differentiate between the iso-enzyme forms of different species (see later).

MATERIALS AND METHODS

Materials

NAD^+, NADH, lithium lactate, sodium pyruvate, cis-platinum (II) diamine chloride (CP), transplatinum (II) diamine dichloride (TP), AMP, ketocaproic acid, *n*-hexanediol,

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colchicine, oxalic acid and gossypol acetic acid (GAA) were from Sigma Chemical Co. (USA). All other chemicals were of analytical grade.

Purification of LDH

LDH from the testes of uromastix was purified to homogeneity according to the method described earlier.[3]

LDH Activity

Enzyme activity was measured in the forward reaction (NADH to NAD^+) at 25°C in one ml of reaction mixture containing 50 mM phosphate buffer (pH 7.5), 0.18 mM NADH, 0.6 mM sodium pyruvate and the required amount of enzyme. The production of NAD^+ was followed by a decrease in absorbance at 340 nm using a Beckman DU 70 spectrophotometer. One unit of enzyme was defined as the amount of enzyme that produced one micromole of NAD^+ per minute under the assay conditions (molar absorption coefficient[5] of NADH being 6.22×10^3 liter mol^{-1} at 340 nm). For the reverse reaction (NAD^+ to NADH), the enzyme activity was determined by measuring an increase in absorbance at 340 nm. The final concentration of the reactants in one ml reaction mixture was 50 mM Tris-HCl buffer (pH 7.5), 50 mM lithium lactate and 0.1 mM NAD^+. It contained 50 μl of suitably diluted enzyme.

For inhibition studies, a known concentration of the test inhibitor (after adjusting to pH 7.5) was added to the reaction mixture. The activity of the enzyme was then measured by the forward or reverse reactions. The results are expressed as remaining activity (in %) compared to control activity (100%). The nature of the oxalate inhibition was determined using Lineweaver-Burk plots (LBPs) and the inhibition constant (K_i) was obtained from a Dixon Plot.[6]

Time-dependant inhibition by GAA

For time-dependent inhibition by GAA, an incubation mixture of the forward reaction system was prepared (25°C) by mixing 250 mM potassium phosphate buffer, pH 7.5 (2 ml), 0.3 U/ml enzyme (1 ml), 18 mM GAA in ethanol (10 μl), and water (3 ml). For control experiments, 10 μl of ethanol was added in the incubation mixture instead of GAA. Aliquots (600 μl) of the incubation mixture were transferred into a cuvette at different time periods (0, 2, 5, 10, 20, 30, 45 and 60 min) and the reaction was started by adding a mixture of 0.9 mM NADH (200 μl) and 3 mM pyruvate (200 μl). To test the protective effect of substrates, pyruvate or NADH was also added to the incubation mixture. In all cases the volume of incubation mixture was 6 ml. When the incubation mixture contained NADH, the assay of LDH was started by adding water (200 μl) and pyruvate (200 μl). Similarly, when the incubation mixture contained pyruvate, the reaction was started by mixing water (200 μl) and NADH (200 μl). The final volume of the reaction mixture in all cases was 1 ml. The activity of LDH in control experiments at zero time incubation was taken as 100%.

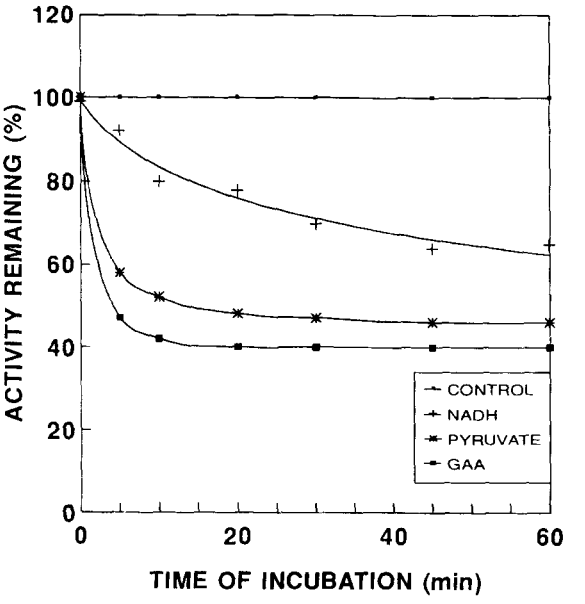

FIGURE 1 Rates of inactivation of LDH by reaction with GAA in the presence and absence of substrates. The enzyme (0.3 U/ml) was incubated with GAA (30 μM) at pH 7.5 and 25°C. Activity was measured as described in the Materials and Methods.

RESULTS

Table 1 shows the effect of the various test inhibitors, at the concentration quoted, on the activity of LDH purified from the testes of uromastix. The effect of platinum compounds was time-dependent and cis-platinum was a more potent inhibitor compared to trans-platinum in the forward reaction. Both platinum compounds showed very minor inhibition of LDH in the reverse-reaction. Ethanol and colchicine did not show any inhibitory effect on LDH for the forward reaction. AMP-inhibition was also time-dependent but showed a greater effect on the reverse reaction. The most effective inhibitors were oxalate and GAA which showed strong inhibition of LDH. In the absence of LDH, these compounds in the reaction mixture did not alter the absorbance of NADH or NAD^+. Thus, these modulators had no effect on the oxidation/reduction of NADH/NAD^+, and the effects were on LDH activity. At low concentration, the inhibition by GAA was time-dependent (Figure 1). In the forward-reaction NADH as a substrate partially protect inhibition of the enzyme. Its protection was stronger than that of pyruvate protection (Figure 1) and the apparent IC_{50} was about 24 μM (Figure 2). Oxalic acid caused non-competitive inhibition of pyruvate reduction since K_m remained constant (c. 48 μM). The apparent K_i for oxalate inhibition was 0.15 mM (Figure 3 inset).

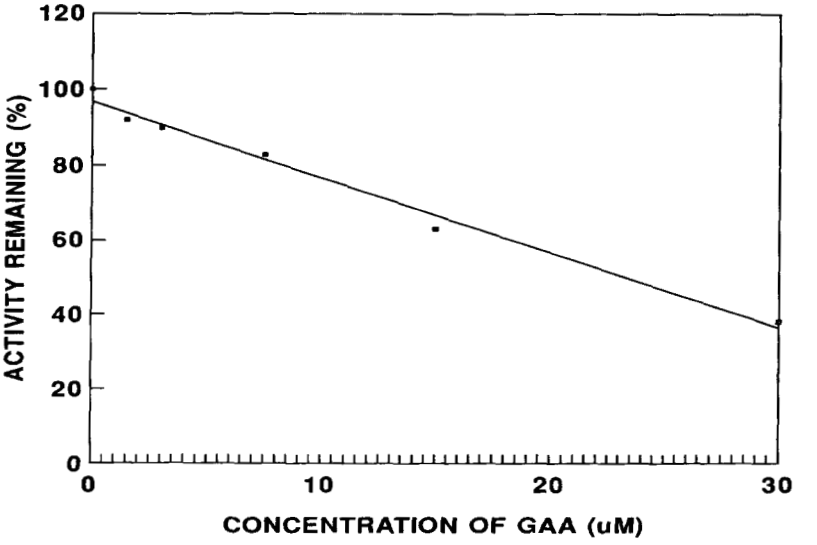

FIGURE 2 Inactivation of LDH by GAA. The LDH (0.3 U/ml) was incubated with desired amount of GAA at pH 7.5 and 25°C for about 20 minutes. The activity of the LDH was then measured for the forward reaction as described in the Materials and Methods.

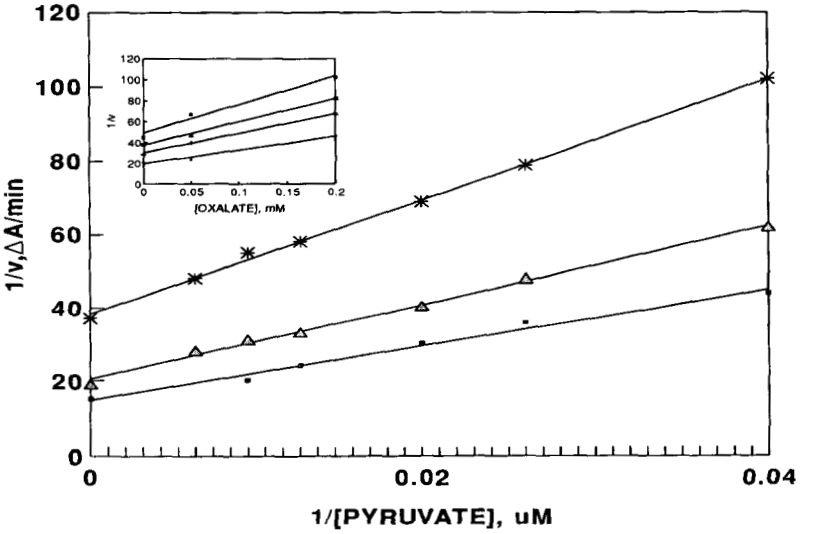

FIGURE 3 Lineweaver-Burk Double Reciprocal Plot of the LDH Activities in the presence and absence of oxalate. The desired amount of oxalate was added in the reaction mixture and the activity of LDH for the forward reaction was measured immediately as described in the Materials and Methods. □, Control (no oxalate); Δ, oxalate (0.05 mM); *, oxalate (0.2 mM). Inset: Dixon plot for oxalate inhibition of LDH. 1/v versus [oxalate] in the presence of different fixed concentrations of pyruvate (□ 20 μM; *, 38.5 μM; Δ, 50 μM and ⊠, 100 μM).

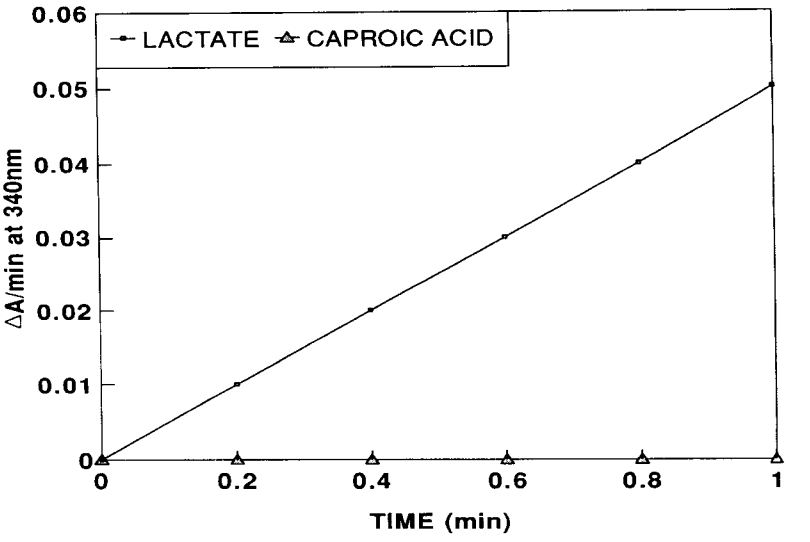

FIGURE 4 The determination of LDH activity in the Reverse Reaction. The activity was measured as described in the Materials and Methods ☒, lactate (50 mM); Δ, ketocaproic acid (130 mM).

Ketocaproic acid as substrate

Ketocaproic acid was tested as a substrate instead of lactate for the reverse reaction. Ketocaproic acid could not replace lactate as a substrate even at 130 mM concentration (Figure 4).

DISCUSSION

Lactate dehydrogenase from various mammalian species consists of five isoenzymes.[1,2] We have reported that LDH from skeletal muscle and liver of uromastix are quite different from mammalian LDH. They could not be separated into isoenzymes by electrophoretic systems normally capable of separating mammalian LDH isoenzymes.[4,5] We have also shown that the LDH purified from testes of uromastix has some unique properties.[3] It has been demonstrated that LDH-X utilizes ketocaproic acid as a substrate from mouse and human spermatozoa.[7,8] The enzyme purified from uromastix testes was unable to utilize ketocaproic acid as a substrate. Thus, it may be different from the LDH-X from mouse and human spermatazoa.

Inhibition studies

One of the methods to characterize the LDH isoenzymes is to use specific inhibitors,[5,9,10] and the results obtained here for the LDH of uromastix testes are summarised in Table 1. *n*-Hexanediol has been shown to inhibit the LDH isoenzyme-5 (muscle type).[11]

TABLE 1
Effect of various agents on the activity of LDH from uromastix testes.

Reagents	Activity (%)		
	Forward Reactions ($NADH \rightarrow NAD^+$)		Reverse Reaction ($NAD^+ \rightarrow NADH$)
	0 min	60 min	20 min
Control	100	100	100
Ethanol (4%)	104	107	N.D.
n-hexanediol (0.4 M)	79	91	N.D.
Colchicine (50 μM)	79	104	N.D.
Cis-platinum (0.25 mg/ml)	88	13	97
Trans-platinum (0.25 mg/ml)	99	48	90
Gossypol acetic acid (30 μM)	29	26	28
AMP (2 mM)	86	66	28
Oxalic acid 0.2 mM)	20	N.D.	N.D.

For the zero time determinations, the desired inhibitor was added to the reaction mixture and the LDH activity was determined immediately as described in the Materials and Methods. At 20 or 60 min incubation, the enzyme (0.3 U/ml) was preincubated with desired inhibitor in buffer of reaction mixture at 25°C before activity measurements were made. The activity of the enzyme was then measured by adding pyruvate and NADH. All tests were performed in duplicate and the average values are shown. ND= not determined.

In a separate study we have shown that *n*-hexanediol strongly inhibits the LDH-4 and LDH-5 from varanus liver.[12] The inhibition of LDH by platinum compounds was according to previous findings where CP has been shown to inhibit the activity of enzymes containing sulfhydryl-groups at the active site.[9,13] However, in the case of rat kidney LDH, TP was a more effective inhibitor than CP.[13] Similarly, CP was ineffective even after 4 hours pre-incubation whereas TP almost completely inhibited the LDH-1 of sheep brain.[9] In beef heart LDH, TP also inhibited the enzyme much more efficiently than the cis-isomer.[14] Thus, LDH purified from uromastix testes may be different from LDH-1 of other mammals. However, for the reverse-reaction, both isomers of platinum were unable to block the activity of LDH (Table 1) thus it would seem that platinum compounds bind with LDH at the NADH or pyruvate binding sites but do not interfere with binding of NAD^+ or lactate to LDH purified from uromastix testes.

The inhibition of LDH from uromastix testes by oxalate was in accordance with previous reports on other organisms[9] as well as on LDH-1 of liver of the same species.[5] Oxalate was shown to be a competitive inhibitor of LDH for lactate oxidation and a non-competitive inhibitor of pyruvate reductions.[9]

In the case of LDH from *Plasmodium knowlesi* (a malarial parasite) oxalic acid caused competitive inhibition of pyruvate reduction.[15]

AMP and colchicine have both been reported to be strong inhibitors of LDH from other sources.[18] The LDH from uromastix testes probably is different from LDH of other animal

species or it needs higher concentrations of AMP and colchicine to inhibit the enzyme activity. Gossypol acetic acid (GAA) is a known male sterilizing agent and has been shown to be a potent inhibitor of all types of LDH isoenzymes.[19,20] GAA inhibited LDH isoenzymes from different tissues of goat, monkey and bull in a concentration and time-dependent manner in both forward and reverse reactions.[19]

Rapid inactivation is thought to be due to specific modification of critical residues at or near the active site of the LDH purified from uromastix testes. After about 20 minutes incubation, the inhibition remains the same for 60 minutes. It may be possible that the LDH contains two different groups necessary for activity. One group is modified with GAA while the second remains unaffected. The rate of inactivation was markedly slowed down in the presence of NADH, whereas, a smaller protective effect was observed in the presence of pyruvate. These results are indicative that the residue(s) modified is presumably located at or near the binding site for NADH rather than that for pyruvate in the enzyme. This type of protection has also been observed in LDH from other sources.[9,19]

Considering the above properties of purified LDH from uromastix testes, we suggest that this isoenzyme of LDH does not belong to either LDH-1 or LDH-X families of LDH dehydrogenase.[3]

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