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KINETICS OF GOSSYPOL INHIBITION OF BOVINE LACTATE DEHYDROGENASE X

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Summary: Gossypol, a polyphenolic binaphthalene dialdehyde isolated from cotton meal is a potent inhibitor of lactate dehydrogenase-X purified from bovine testis. For the conversion of pyruvate to lactate the IC_{50} for gossypol is 200 μM for the reverse reaction the IC_{50} is 12 μM. Gossypol is a competitive inhibitor of NADH, Ki = 30 μM (Km = 17 μM), and NAD^+, Ki = 6 μM (Km = 130 μM), and noncompetitive for pyruvate, Ki = 220 μM (Km = 224 μM), and lactate, Ki = 52 μM (Km = 5.6 mM).

L-lactate dehydrogenase x (LDH-X) [EC 1.1.1.27] is an isozyme of LDH found exclusively in the gametogenic cells of mammalian testis (1). This isozyme is unique in that it has high affinity for long chain α-keto acids as well as lactate and pyruvate (2). Approximately 40 % of the cellular LDH-X is localized in the mitochondrial fraction of sperm, and the remainder is cytosolic (3). LDH-X activity is closely associated with sperm motility, and it has been proposed that the physiological role of this isozyme is to shuttle reducing equivalents from the cytosol to the mitochondrial respiratory chain similar to the malate shuttle (4).

Gossypol [1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-(2,2' binaphthalene)-8,8'-dicarboxaldehyde (Scheme I)] has been reported to be a potent non-steroidal male contraceptive agent (5). On the ultrastructrual level, gossypol has been shown to produce extensive damage to the sperm mitochondrial sheath (6), while on the biochemical level, gossypol inhibits several enzymes involved in energy metabolism including Mg^{2+}/Ca^{2+} ATPase and several dehydrogenases (7,8).

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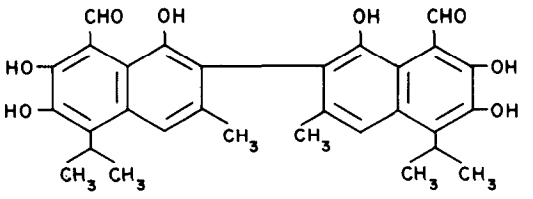

Scheme I

We have recently reported reversible competitive inhibition by gossypol of adenylate cyclase (9), and have posed the hypothesis that this compound is a general inhibitor of enzymes possessing the nucleotide binding cleft. The present study was undertaken to examine the inhibition pattern of gossypol inhibition at the nucleotide binding domain of a readily accessible purified protein whose structure has been well defined (10).

MATERIALS AND METHODS

Chemicals: Gossypol acetic acid, L(+)lactic acid, sodium pyruvate, NAD^+, NADH and imidazole were purchased from Sigma. All other reagents used were of the highest purity commercially available and were used without further purification.

Enzyme: LDH-X was purified from two kilograms of freshly frozen mature bovine testis (Arena & Sons, Hopkinton, MA) by the methods outlined by Goldberg (11), and was judged to be homogeneous using established criteria for this enzyme (12).

Assays: LDH-X was assayed at 25°C in a 1.0 ml reaction mixture containing 0.15 mM NADH, 2.0 mM pyruvate in 0.1 mM imidazole-HCl, pH 8.0, by measuring the decrease in absorbance at 340 nm. The reaction was initiated by addition of 1 µg of enzyme. The reverse reaction was measured in the same manner except the reaction mixture contained 5.6 mM NAD^+ and 77.5 mM sodium lactate.

Gossypol solutions were freshly prepared by dissolution in 10 mM sodium bicarbonate buffer, pH 8.3. Gossypol concentrations were measured spectrophotometrically at 372 nm using an absorptivity of 14.3 mM (13). Addition of gossypol to the LDH-X assay mixture did not significantly alter the pH.

Protein concentration was estimated by a modification of the Lowry method (14) usirg BSA as a standard.

All calculations and linear regression analyses were performed using a Tandy model TRS-80 III computer.

RESULTS

Gossypol inhibited LDH-X in a concentration dependent manner. Half-maximal inhibition of the reaction utililing pyruvate and NADH as substrates was observed at a gossypol concentration of 200 µM (Figure 1). Preincubation of the enzyme with NADH showed partial protection from inhibition, with an observed shift in IC_{50} to 300 µM. The reverse reaction, utilizing lactate and NAD^+ as substrates was also inhibited by gossypol in a concentration dependent manner as shown in Figure 2. The IC_{50} of 12 µM was 17 fold lower for this

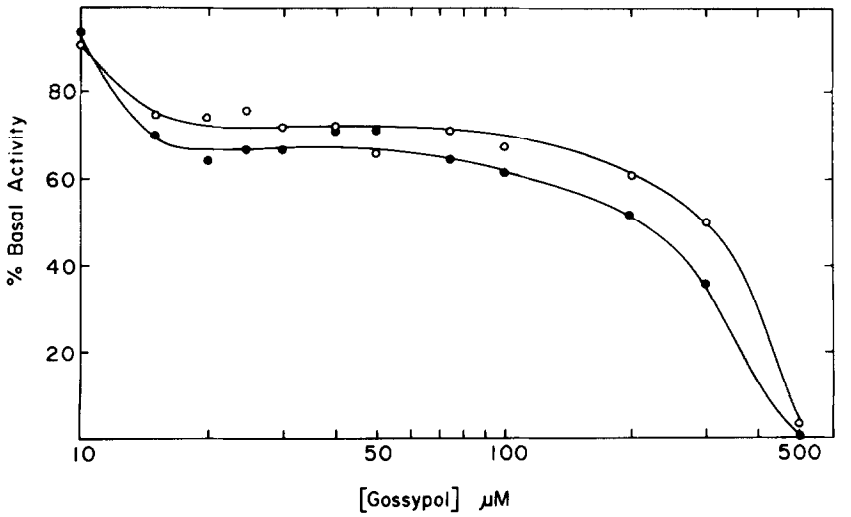

Figure 1. Inhibition of the conversion of pyruvate to lactate as a function of gossypol concentration.
Gossypol was preincubated with LDH-X at the concentrations shown above for 5 min at 37°C prior to assay (●-●) or in the presence of 0.15 mM NADH prior to the addition of gossypol (○-○).

reaction than for the oxidation of NADH, and preincubation of the enzyme with NAD^+ did not protect the enzyme from gossypol inhibition. Gossypol inhibition is readily reversible by passing the gossypol-LDH-X complex over a Bio Gel P4

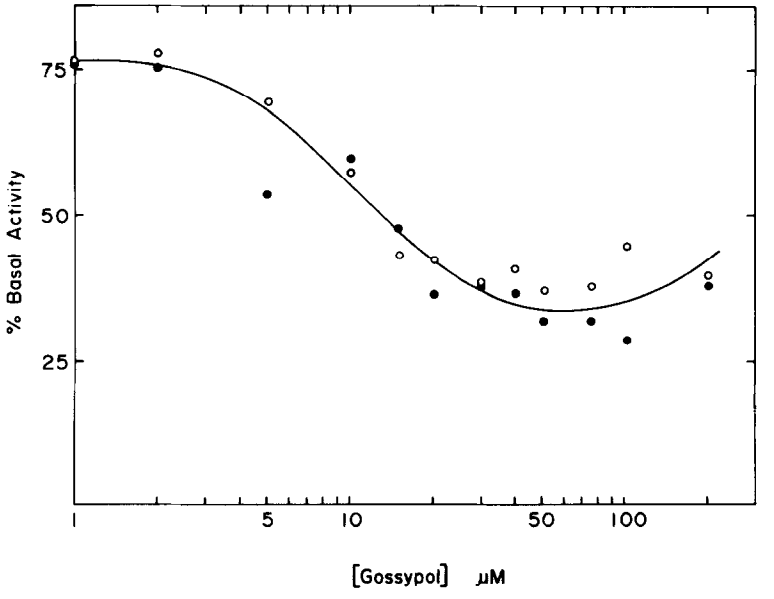

Figure 2. Gossypol inhibition of the conversion of lactate to pyruvate. The assay was carried out as described in METHODS and Figure 1. (●-●) preincubated in the presence of gossypol; (○-○) preincubated in the presence of NAD^+.

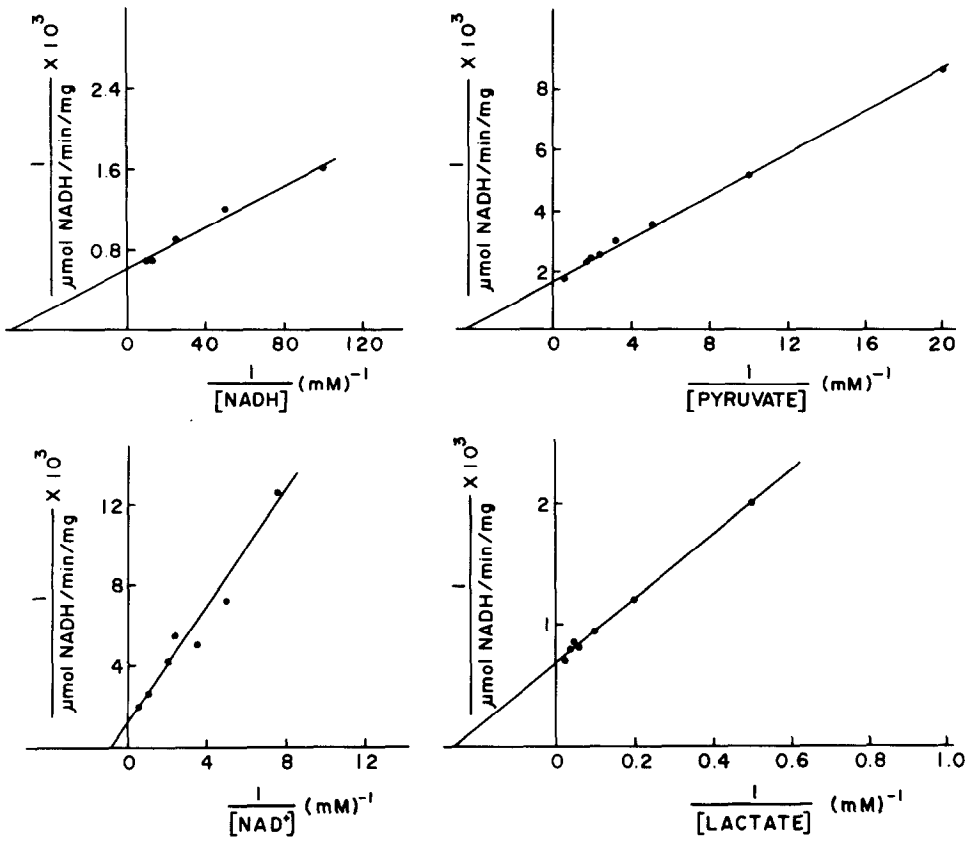

Figure 3. Kinetics of LDH-X with each of its four substrates. Initial rates of LDH-X activity were measured as described in METHODS, and the data plotted as the double reciprocal plots. The resulting lines were evaluated by linear regression analysis, r = 0.99 in each case.

column, and inihibition is apparently not due to formation of a Schiff base since the enzyme could not be rendered inactive by treatment with $NaBH_4$.

The kinetics of LDH-X was examined for each of its four substrates. The apparent Km values calculated from double reciprocal plots (Figure 3) are: pyruvate, 224 µM; NADH, 17 µM; lactate, 5.6 mM; and NAD^+ 130 µM. The kinetics of gossypol inhibition was examined with respect to each of the four substrates for LDH-X. When either NADH or NAD^+ was the varied substrate, competitive inhibition patterns were observed (Figure 4) with apparent Ki values of 30 and 6 µM respectively. Gossypol showed noncompetitive inhibition patterns when either pyruvate or lactate was the varied substrate, with apparent Ki values of 220 and 52 µM respectively.

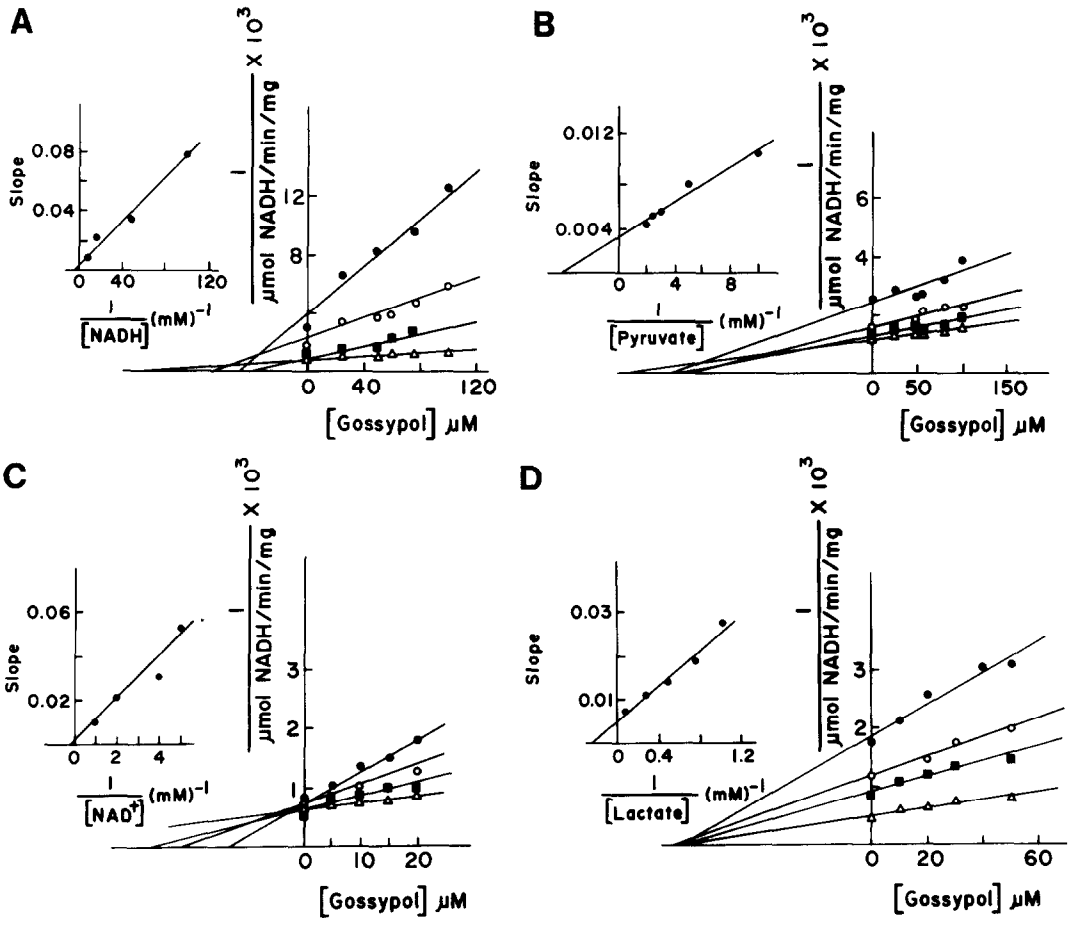

Figure 4. Kinetics of gossypol inhibition of LDH-X for each of its four substrates. Dixon plots of the substrate dependent kinetics of LDH-X in the presence of varying concentrations of gossypol are shown above. Insets are replots of the slope vs the reciprocal of substrate concentration. Apparent Ki values were estimated from the respective replot. A. The concentrations of pyruvate were: 0.1 mM (●-●); 0.20 mM (○-○); 0.3 mM (■-■); 2.0 mM (Δ-Δ). NADH was constant at a concentration of 0.15 mM. B. NADH concentrations used were: 0.01 mM (○-○); 0.02 mM (●-●); 0.08 mM (■-■); and 0.15 mM (Δ-Δ). Pyruvate concentration was 2.0 mM. C. Sodium lactate concentrations used were 1.0 mM (●-●); 1.33 mM (○-○); 8.0mM (■-■) and 20 mM (Δ-Δ). NAD^+ concentration was constant at 2.0 mM. D. NAD^+ concentrations used were 0.20 mM (●-●); 0.25 mM (○-○); 0.5 mM (■-■); and 1.0 mM (Δ-Δ). Lactate was held constant at a concentration of 20.0 mM.

DISCUSSION

Gossypol has been shown to be an effective inhibitor of LDH-X in relatively impure systems (8). In this communication, we report the first detailed study of gossypol inhibition of purified LDH-X. Gossypol is a competitive inhibitor of both the oxidation and reduction of NADH, but is most potent in inhibiting the reduction of NAD^+ to NADH. Gossypol is noncompetitive with respect to the keto- and hydroxy acid substrates.

We have recently reported the competitive inhibition of adenylate cyclase by gosyspol (9), and postulated that gossypol may exert its inhibitory effects by acting as an ATP analog and therefore binding at the ATP binding site. All enzymes which utilize either pyridine nucleotide or ATP have been shown to share a structural similarity known as the nucleotide binding domain (15). The competitive inhibition patterns observed for pyridine nucleotide in LDH-X and for ATP in adenylate cyclase suggests that gossypol may be a useful general nucleotide binding domain probe for the study of dehydrogenases or other enzymes which require nucleotides as substrates.

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