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In vitro effects of gossypol on testicular lactic dehydrogenase-X and other dehydrogenases

N. GIRIDHARAN†, MAHTAB S. BAMJI and M. N. MADHYASTHA*

National Institute of Nutrition, Indian Council of Medical Research, Jamai Osmania, Hyderabad 500 007, India

*Department of Postgraduate Studies and Research in Biosciences, University of Mangalore, Mangalore 574 152, India

Abstract. The *in vitro* inhibition of several rat testis dehydrogenases by gossypol was examined. Inclusion of the coenzyme (substrate for $NADP^+$-isocitrate dehydrogenase) in the preincubation mixture containing the enzyme and gossypol, protected the enzymes against inhibition by gossypol. Lactic dehydrogenase-X was amongst the least protected enzymes. This, coupled with its low K_i for gossypol makes it one of the most vulnerable target enzymes *in vivo* for gossypol action.

The inhibition kinetics for lactic dehydrogenase-X were competitive when NADH was present during preincubation, but non-competitive when the coenzyme was excluded during preincubation. In the latter condition, the enzyme seems to undergo progressive inactivation with time causing a nonreversible type of inhibition.

Keywords. Gossypol; lactic dehydrogenase-X; malate dehydrogenase; testicular dehydrogenases.

Introduction

Gossypol is a potent antifertility agent for males of a variety of species (Sang, 1983). Its mechanism of action at molecular level, however, remains speculative. Recent studies from our laboratory and others (Giridharan *et al.,* 1982; Lee *et al.,* 1982; Tso and Lee, 1982) show that sperm-specific lactic dehydrogenase-X (EC 1·1·1·27) is inhibited by gossypol and this effect may be causally related to the antifertility action of gossypol. This enzyme is involved in sperm metabolism and provides energy for sperm motility (Burgos *et al.,* 1982; Hutson *et al.,* 1977).

Gossypol, has also been found to inhibit other forms of lactic dehydrogenase and other enzymes, (Lee *et al.,* 1982; Lee and Mailing, 1981; Tso *et al.,* 1982; Oligati *et al.,* 1984), suggesting that its effect on sperm lactic dehydrogenase-X may be non-specific.

There is considerable controversy in the literature regarding the characteristics of inhibition of lactic dehydrogenase-X by gossypol. While some workers found the inhibition to be reversible (Giridharan *et al.,* 1982; Oligati and Toscano, 1983; Burgos el *al.,* 1986), others found it to be non-reversible (Lee *et al.,* 1982).

The studies reported in this paper were aimed at examining the effects of gossypol on lactic dehydrogenase-X and some other key testicular dehydrogenases, under different conditions of enzyme assay, to clarify the controversies existing in the literature regarding the specificity and type of inhibition.

†To whom correspondence should be addressed.

Materials and methods

Enzyme preparation

For all studies described below, adult Wistar/NIN strain of rats were used. The testes from these animals were homogenized in 9 volumes of 0·25 M sucrose using polytron and the homogenate was subjected to differential centrifugation at 0–4 °C. The 100,000 *g* supernatant without any further treatment was used for assaying the following testis dehydrogenases: lactic dehydrogenase-X, sorbitol dehydrogenase (EC 1·1·1·14), α-glycerophosphate dehydrogenase (EC 1·1·1·8), glucose-6-phosphate dehydrogenase (EC 1·1·1·49), NADP+-isocitrate dehydrogenase (EC 1·1·1·42) and malate dehydrogenase (EC 1·1·1·37). Apart from these, lactic dehydrogenase-1 and 5 were also examined in the cytosolic preparation from rat heart and muscle, respectively.

For measurement of kinetic constants, lactic dehydrogenase-X was partially purified from 100,000 *g* supernatant by heat treatment and acidification with 1 N acetic acid, as described by Schatz and Seagal (1969). This 'heat-treated' preparation was readjusted to pH 7·3 for enzyme assay. It showed 3 fold increase in specific acti vity compared to the untreated supernatant.

Gossypol preparation

Purified cotton-seed gossypol was gifted by Dr. A. V. B. Sankaram, Regional Re search Laboratory, Hyderabad (Giridharan *et al.,* 1982).

Enzyme assays

Lactic dehydrogenase-X was assayed by the method of Schatz and Seagal (1969) using a final concentration of 4·1 mM α-ketoglutarate and 0·17 mM NADH in the assay mixture. Rest of the testicular dehydrogenases were assayed as described by Shen and Lee (1976). The final concentration of the substrate and the coenzyme for these enzyme assays were as follows: 66·6 mM fructose and 0·19 mM NADH for sorbitol dehydrogenase, 0·78 mM dehydroxyacetone phosphate and 0·22 mM NADH for α-glycerophosphate dehydrogenase, 3·33 mM glucose-6-phosphate and 0·19 mM NADP for glucose-6-phosphate dehydrogenase, 0·5 mM isocitrate and 0·13 mM NADP for isocitrate dehydrogenase and 0·5 mM oxaloacetic acid and 0·2 mM NADH for malate dehydrogenase. Lactic dehydrogenase-1 and 5 were assayed as described by Bergmeyer *et alb.* (1974) using a final concentration of 0·6 mM pyruvate and 0·18 mM NADH in the assay mixture.

All activity measurements were done in Gilford spectrophotometer at 340 mm and 37°C. Enzyme velocity was expressed as changes in absorbance per min (Δ absorbance/min).

For inhibition studies, alcoholic solution of gossypol was used. The solvent, alcohol at the concentration used in the study had no effect on the activity of the enzymes studied. The preincubation of enzymes with gossypol in the presence or absence of either the substrate or the coenzyme was done under conditions described in the results section. All the assays were done in duplicate.

In all experiments protein was estimated by the method of Lowry *et al.* (1957).

Determination of kinetic constants

Kinetic constants like K_m, V_{max} and K_i for lactic dehydrogenase-X and malate dehydrogenase were calculated as described by Segel (1975) and Dixon and Webb (1979). For these measurements, the range of NADH concentration used was 0·05–0·2 mM and 0·0l–0·2mM, respectively, for lactic dehydrogenase-X and malate dehydro genase.

Results

Effect of inclusion of substrate or coenzyme in the preincubation mixture containing enzyme and gossypol

In the absence of both the substrate as well as the coenzyme in the preincubation mixture, all the enzymes were markedly inhibited by gossypol. Under this condition the inhibition was most marked for glucose-6-phosphate dehydrogenase (IC_{50} - 8 μm) and least for sorbitol dehydrogenase (IC_{50} > 80 μm). Inclusion of coenzyme in the preincubation mixture protected all the enzymes (except isocitrate dehydrogenase) against gossypol inhibition. In the case of isocitrate dehydrogenase, isocitrate conferred greater protection (IC_{50}>80μm) than NADP (IC_{50}-36μm). Apart from the coenzyme, glucose-6-phosphate dehydrogenase was protected by its substrate as well. Amongst the dehydrogenases studied, the coenzyme-mediated protection was least for malate dehydrogenase and lactic dehydrogeneous-X, being 29 and 43%, respectively (figure 1). Lactic dehydrogenase 1 and 5 were also protected against inhibition with gossypol in the presence of NADH in the preincubation mixture.

Effect of NADH in the preincubation mixture on the kinetic properties of lactic dehydrogenase-X and malate dehydrogenase

The data in table 1 suggest that when NADH was present in the preincubation mixture, the inhibition characteristic of lactic dehydrogenase-X was of the reversible competitive type, where V_{maxapp} was not altered but the K_{mapp} was increased. On the other hand, when NADH was not present in the preincubation mixture and the reaction was started with NADH, the inhibition acquired a non-competitive character where K_{mapp} was unaffected but V_{maxapp} was reduced. Similar trends were observed for malate dehydrogenase as well.

Values for K_{iapp} for the enzymes under conditions where NADH was omitted from the preincubation mixture, were erratic and hence not reported. In the presence of NADH, the K_{iapp} of lactic dehydrogenase-X and malate dehydrogenase were 1·94 and 5·3 μm, respectively.

Effect of enzyme concentration and duration of preincubation on the inhibition of lactic dehydrogenase-X by gossypol

In this experiment, the activity of lactic dehydrogenase-X at different concentrations of the enzyme and different times of preincubation, in the presence and absence of coenzyme was measured, using a fixed concentration of 10 μM gossypol.

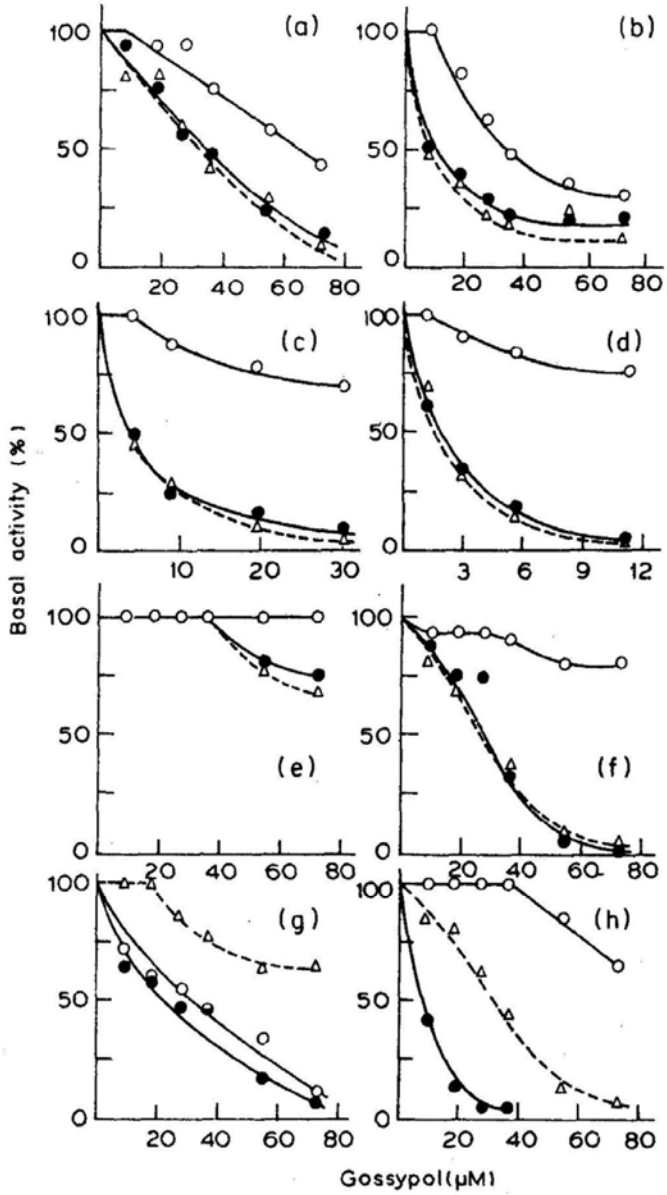

Figure 1. Effect of inclusion of substrate or coenzyme in the preincubation mixture containing enzyme and gossypol.
(a), Lactic dehydrogenase-X; (b), malate dehydrogenase; (c), lactic dehydrogenase-1; (d), lactic dehydrogenase-5; (e), sorbitol dehydrogenase; (f), α-glycerophosphate dehydrogenase; (g), isocitrate dehydrogenase; (h), glucose-6-phosphate dehydrogenase.

Buffered solutions of the enzymes and varying concentrations of gossypol were preincubated with, (•) neither substrate nor coenzyme, (Δ) only substrate, (O) only coenzyme, for 15 min and the reactions initiated appropriately. The concentration of the substrate and coenzyme were as described in 'materials and methods' section. The protein content per 100 μl of the cytosol in the assay mixture were a, e, f, g and h, 400–440 μg; b, 44 μg; c and d, 3 μg. Inhibition of the enzyme activity was expressed as percentage of basal activity, *i.*e., activity remaining after inhibition.

Table 1. Effect of NADH in the preincubation mixture on the V_{max} and K_m for lactic dehydrogenase-X.

NADH in the preincubation mixture	Concentrations of gossypol (μM)	Kinetic constants	
		V_{max} (Δabsorbance)	K_m (mM) NADH
Present	0·0	0·049	0·0051
	0·625	0·050	0·0061
	1·25	0·050	0·0073
	2·5	0·049	0·0083
	5·0	0·049	0·012
	10·0	0·050	0·027
	20·0	0·032	0·029
Absent	0·0	0·057	0·004
	0·625	0·049	0·0039
	1·25	0·047	0·004
	2·5	0·041	0·004
	5·0	0·034	0·0035
	10·0	0·016	0·0036
	20·0	0·008	0·0038

The protein content in the assay mixture for the enzyme preparation was 32μg.

The data in figure 2A show that in the presence of NADH, the plot of enzyme concentration against enzyme activity passed through the origin, regardless of the duration of the preincubation of the enzyme with the inhibitor, suggesting a typical reversible type of inhibition. However, by omitting the coenzyme from the prein cubation mixture, the inhibition characteristics appeared to be non-reversible type, wherein the plots of varying concentrations of the enzyme against enzyme activity did not pass through the origin, except, when the duration of preincubation was very short (figure 2B).

The duration of preincubation had no effect on the enzyme activity without the inhibitor and hence only a representative plot is presented in the figure 2.

Discussion

The earlier suggestion of Lee *et al.* (1982) that the inhibition of sperm-specific lactic dehydrogenase-X by gossypol may be the basic molecular mechanism of gossypol mediated infertility in males was questioned recently, when it was observed that other enzymes are also inhibited by gossypol. The data presented here show that though a variety of dehydrogenases are indeed inhibited by gossypol in the absence of the coenzyme and substrate in the preincubation mixture, the inhibition was markedly reduced by including the substrate in. the preincubation mixture of isocitrate dehydrogenase and coenzyme for all the other dehydrogenases.

Judging from the extent of coenzyme-mediated protection, lactic dehydrogenase-X and malate dehydrogenase appear to be more vulnerable to gossypol effect than the other enzymes which were more effectively protected. Between these two enzymes, the concentration as well as K_i of malate dehydrogenase in the testis is much higher

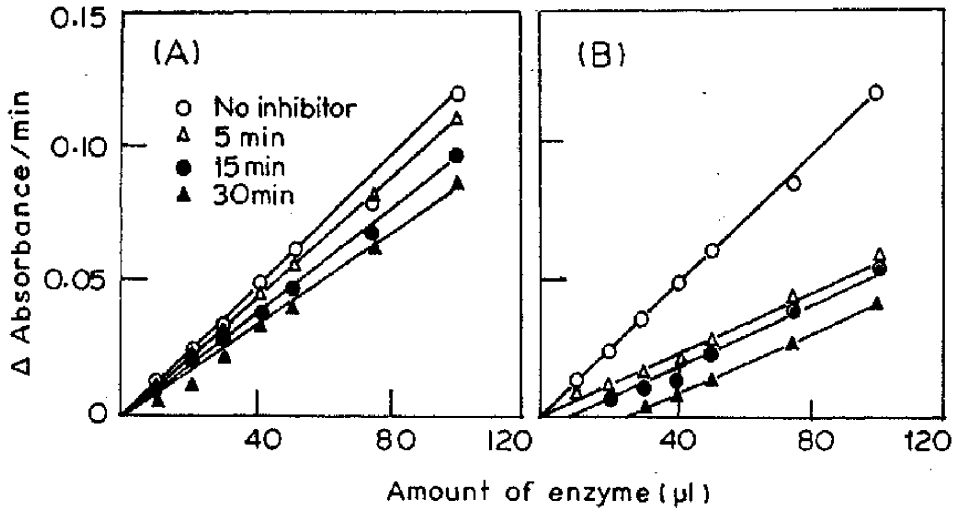

Figure 2. Effect of enzyme concentration and duration of preincubation on the inhibition of lactic dehydrogenase-X by gossypol.

(A), Bufered solution of the enzyme were preincubated with 10 μM gossypol and 0·17 mM NADH for the times indicated in the graph. At the end of each time point, reaction was started with 4·1 mM, α-ketoglutarate; (B), buffered solution of the enzyme was preincubated with 10 μM gossypol and 4·1 mM α-ketoglutarate for times indicated in the graph. At the end of each time point, reaction was started with 0·17 mM NADH.

The protein content of the heat-treated lactic dehydrogenase-X preparation in the assay mixture was 32 μg.

than that of lactic dehydrogenase-X. This may make lactic dehydrogenase-X a more vulnerable target for gossypol action.

The marked controversy in the literature regarding the type of inhibition of lactic dehydrogenase-X with gossypol (reversible or irreversible) appears to be due to the conditions of preincubation. A true reversible inhibitor is expected to show same inhibiton characteristics regardless of the sequence of addition. But the data presented in table 1 show that by omission of NADH from the preincubation mixture, the inhibition pattern changes from competitive to non-competitive type.

Segel (1959) has suggested using plots of varying enzyme concentration against enzyme activity in the presence or absence of the inhibitor to distinguish between reversible and irreversible inhibition. The data in figure 2 suggest that in the absence of NADH in the preincubation mixture, the inhibition of lactic dehydrogenase-X by gossypol is non-reversible.

Apart from the presence or absence of NADH, the duration of preincubation also influences the characteristics of the inhibition (figure 2). At the shorter time interval of 5min, the inhibition was reversible, regardless of the presence or absence of the coenzyme in the preincubation mixture. This would explain the observations of Oligati and Toscano (1983). The irreversible inhibition seen by Lee *et al.* (1982) was due to the exclusion of the coenzyme from the preincubation mixture and prolonged period of 20 min preincubation.

In general, from the data presented here and other workers, it appears that gossypol binds to NADH binding site in an irreversible manner, except when the

duration of preincubation is less than 5 min. The enzyme is possibly inactivated with time by gossypol or some breakdown products of gossypol, causing a non-reversible type of inhibition. NADH is able to prevent this partially by reducing the velocity constant of the reaction as suggested by Dixon and Webb (1979) for some irreversible inhibitors. Under such conditions the inhibitor combines with the substrate-binding group at the active centre, producing a competitive effect similar to a reversible type of inhibition.

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