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INHIBITION BY GOSSYPOL OF OXIDOREDUCTASES FROM *TRYPANOSOMA CRUZI*

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Abstract—The effects of gossypol, a polyphenolic compound isolated from the cotton plant upon six oxidoreductases from cultured epimastigotes of *Typanosoma cruzi* were studied. Gossypol was a powerful inhibitor of the α-hydroxyacid and malate dehydrogenases, NAD-linked enzymes, and of glutamate dehydrogenase, malic enzyme and glucose-6-phosphate dehydrogenase, NADP-dependent enzymes. The drug did not have an effect on succinate dehydrogenase, a flavoprotein. The K_i values with respect to substrate were 0.73, 0.3 and 3.5 μM for α-hydroxyacid, malate and glutamate dehydrogenases, respectively, and 1.1, 0.19 and 7.8 μM with respect to the coenzyme. Inhibition was noncompetitive with respect to substrate and uncompetitive in relation to the coenzyme.

Evidence has accumulated during the last 10 years indicating that gossypol, a polyphenolic compound present in the cotton plant, acts as an antifertility agent in man and in males of several species of mammals [1]. Oral administration of the compound produces disturbances of spermatogenesis resulting in oligospermia and azoospermia [2, 3]. The drug is fairly well tolerated by humans at effective doses [2]. The possible use of gossypol as a male contraceptive prompted many studies trying to elucidate its mechanism of action [1, 4].

A preliminary observation by Lee and Malling [5] suggested that the drug inhibits selectively the lactate dehydrogenase isozyme X (LDH X or C_4), an enzyme specific to gametogenic cells and spermatozoa. The isozyme X of lactate dehydrogenase (EC 1.1.1.27, L-lactate:NAD oxidoreductase), first reported in 1963 by Blanco and Zinkham [6], is related to unique metabolic processes that provide energy for motility and survival of the male gamete [7–11]. Although we could not confirm selectivity of LDH X inhibition of gossypol, claimed by Lee and Malling [5], we found that the drug was a powerful inhibitor of the enzyme from humans; the K_i values were 11.0 μM with respect to substrate and 7.0 μM for the coenzyme NADH (unpublished observations).

Investigations, carried out in our laboratory, demonstrated that *Trypanosoma cruzi*, the flagellate parasite that causes Chagas' disease, possesses an enzyme similar to the LDH X from mammalian spermatozoa [12]. Total extracts of cultured epimastigotes of *T. cruzi* possess an NAD-linked oxidoreductase whose catalytic properties resemble those of mouse [8] and rat [13] LDH X. The enzyme, designated α-hydroxyacid dehydrogenase (HADH), exhibits two molecular forms, which have been purified and characterized from the point of view of substrate specificity and kinetic constants [14]. On account of the similarities between HADH and LDH X, it was assumed that these enzymes are functionally homologous. Thus, the α-hydroxyacid dehydrogenase may be integrated in metabolic pathways supplying energy for the motility of flagellum.

As gossypol is an effective inhibitor of LDH X, we decided to investigate the effect of the compound on HADH from *T. cruzi*. The first studies demonstrated that the drug is indeed a potent inhibitor of the enzyme [15]. This paper presents a more detailed account of the mechanism of inhibition by gossypol of several oxidoreductases of *T. cruzi*.

MATERIALS AND METHODS

Microorganism

Trypanosoma cruzi (Tulahuén strain) was cultivated in a modified Warren's liquid medium [16]. Parasites were harvested at day 7 (during the exponential phase of growth) by centrifuging at 3000 *g* for 15 min. The pellet was washed three times with about 20 parts of 0.15 M sodium phosphate buffer, 0.15 M NaCl, pH 7.2. The final pellet of epimastigotes was resuspended in 4 parts of the same buffer (1:5) and homogenized with a Sorvall microhomogenizer at 30,000 rpm for 3 min. All operations were carried out at 4°.

Enzyme preparations

The isozyme II of HADH was purified as described by Coronel *et al.* [14]. Malate dehydrogenase (EC 1.1.1.37, L-malate:NAD oxidoreductase) was partially purified by column chromatography on DEAE cellulose by using the same system proposed for the HADH. The enzyme eluted with the first peak of protein, just before the isozyme II of HADH [14]. Glutamate dehydrogenase (EC 1.4.1.4, L-glutamate:NADP oxidoreductase, deaminating),

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glucose-6-phosphate dehydrogenase (EC 1.1.1.49, D-glucose-6-phosphate:NADP oxidoreductase), and malic enzyme (EC 1.1.1.40, L-malate:NADP oxidoreductase, decarboxylating) were assayed in crude preparations. Succinate dehydrogenase (EC 1.3.99.1, succinate:(acceptor) oxidoreductase) was purified by the method of Weeger *et al.* [17], except that the epimastigotes were suspended in 100 mM sodium phosphate buffer, pH 7.4, homogenized, and then centrifuged for 30 min at 10,000 *g*. The pellet was washed three times with distilled water and finally suspended in 20 mM phosphate buffer, 1 mM EDTA, pH 7.6. Then, the procedure was continued as indicated by Weeger *et al.* [17].

Enzyme assays

Alpha-hydroxyacid dehydrogenase, isozyme II, activity was determined by using as substrate α-ketoisocaproate (4-methyl-2-oxopentanoate) as indicated previously [14]. Malate dehydrogenase was assayed by the method of Yoshida [18], glutamate dehydrogenase by the technique described by Juan *et al.* [19], malic enzyme by the method used by Cannata *et al.* [20], glucose-6-phosphate dehydrogenase, by the procedure of Funayama *et al.* [21], and succinate dehydrogenase by the technique of Singer [22]. All enzyme assays were performed at 37°.

Determination of K_i *values*

The enzymes were incubated with the buffer used in the assay, the inhibitor (gossypol-acetic acid), and the coenzyme for 10 min at room temperature (20–23°) before starting the reaction by adding the substrate. Values of K_i were calculated from those of K_m and V obtained with and without gossypol added to the assay mixture or from replots of reciprocals of K_m values against inhibitor concentrations. Concentrations of substrates, coenzymes and gossypol that were used are given in Results.

Chemicals

Gossypol [1,1′, 6,6′, 7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-(2,2′-binaphtalene)-8,8′-dicarboxaldehyde]-acetic acid was supplied by Dr. A. V. Graci, Agricultural Research, Southern Region, U.S. Department of Agriculture, New Orleans, LA, U.S.A. All other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.).

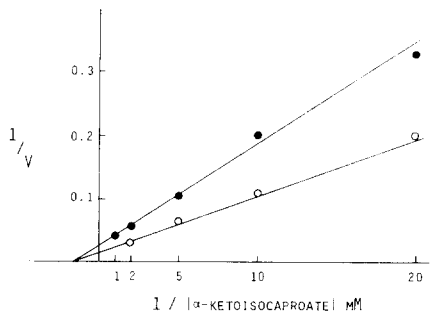

Fig. 1. Effect of α-ketoisocaproate on the inhibitory activity of gossypol on α-hydroxyacid dehydrogenase from *T. cruzi*. Reciprocals of V (expressed as ΔE_{340} per min) are plotted against reciprocals of α-ketoisocaproate concentration. The concentrations of substrate used were 0.05, 0.1, 0.2, 0.5 and 1.0 mM; NADH concentration was kept constant at 0.10 mM. Key: (○) assays without gossypol, and (●) assays with 0.5 μM gossypol added to the reagent mixture. The K_m for α-ketoisocaproate was 0.41 mM.

RESULTS

Isozyme II of α-hydroxyacid dehydrogenase

Figure 1 shows double-reciprocal plots of initial velocities at different substrate concentrations and the effect of gossypol. The inhibition was noncompetitive; the K_i value with respect to α-ketoisocaproate was 0.73 μM.

Figure 2 presents the same type of plots of activity at various coenzyme concentrations and the effect of different concentrations of gossypol. The K_m for NADH was 0.025 mM. Gossypol at 2.0, 3.0 and 4.0 μM concentrations produced reductions of K_m values to 0.014, 0.0075 and 0.0058 mM respectively. Graphic representation indicates that the inhibition

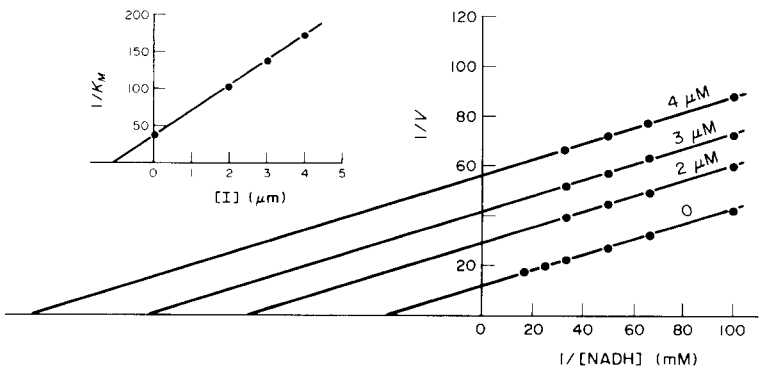

Fig. 2. Effect of NADH on the inhibitory activity of gossypol on α-hydroxyacid dehydrogenase from *T. cruzi*. Reciprocals of V (expressed as ΔE_{340} per min) are plotted against reciprocals of NADH concentration. The concentrations of NADH were 0.01, 0.015, 0.02, 0.03, 0.04 and 0.06 mM; α-ketoisocaproate concentration was 5.0 mM in all assays. The concentration of inhibitor (gossypol-acetic acid) is indicated on each curve. Upper left: replot of reciprocals of K_m values against inhibitor concentration.

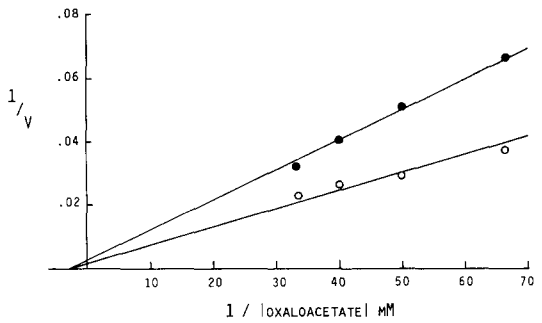

Fig. 3. Double-reciprocal plots of the effect of opaloacetate on the inhibitory activity of gossypol on malate dehydrogenase from *T. cruzi*. The concentrations of oxaloacetate were 0.015, 0.02, 0.025 and 0.03 mM; NADH concentration was 0.10 mM in all assays. Key: (○) assays without gossypol, and (●) assays with 0.3 μM gossypol added to the reagent mixture. The K_m for oxaloacetate was 0.21 mM.

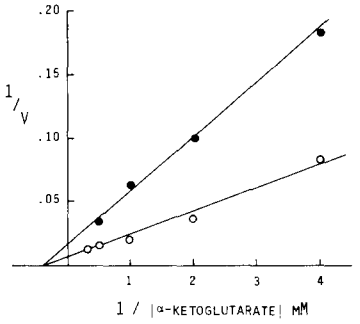

Fig. 5. Double-reciprocal plots of the effect of α-ketoglutarate on the inhibitory activity of gossypol on glutamate dehydrogenase from *T. cruzi*. The concentrations of α-ketoglutarate were 0.25, 0.5, 1.0, 2.0 and 3.0 M; an NADPH concentration of 0.10 mM was used in all assays. Key: (○) assays without gossypol, and (●) assays with 5.0 μM gossypol added to the reagent mixture. The K_m for α-ketoglutarate was 2.7 mM.

was uncompetitive. Replots of reciprocals of the K_m values against inhibitor concentration gave a K_i with respect to NADH of 1.1 μM.

Malate dehydrogenase

Figure 3 shows reciprocal plots of enzymic activity at different substrate concentrations, with and without gossypol. The inhibition was noncompetitive with respect to oxaloacetate, with a K_i of 0.3 μM.

Figure 4 presents the plots of activity at various coenzyme concentrations and the effect of different concentrations of gossypol. The K_m for NADH was 0.066 mM. With gossypol concentrations of 0.4, 0.6 and 0.8 μM, the K_m values were 0.021, 0.016 and 0.013 mM respectively. The inhibition was uncompetitive, and the K_i in relation to NADH was 0.19 μM.

Results with crude extracts for malate and α-hydroxyacid dehydrogenases were the same as those presented for purified enzymes.

Glutamate dehydrogenase

Figure 5 shows double-reciprocal plots of activity at different substrate concentrations with and without gossypol. Inhibition was noncompetitive, and the K_i with respect to α-ketoglutarate was 3.5 μM.

Figure 6 presents the plots of reciprocals of enzymic initial velocity against those for concentrations of NADPH and the effects of different concentrations of gossypol. The K_m for NADPH was 0.21 mM. With the inhibitor at 9.0, 11.0 and 15.0 μM concentrations, the K_m values were 0.008, 0.007 and 0.006 mM respectively. The inhibition was uncompetitive, and the K_i value for NADPH was 7.8 μM.

Other dehydrogenases

Glucose-6-phosphate dehydrogenase and malic enzyme in crude extracts were also inhibited by gossypol. Succinate dehydrogenase was not affected by the drug up to 100 μM concentration. Table 1

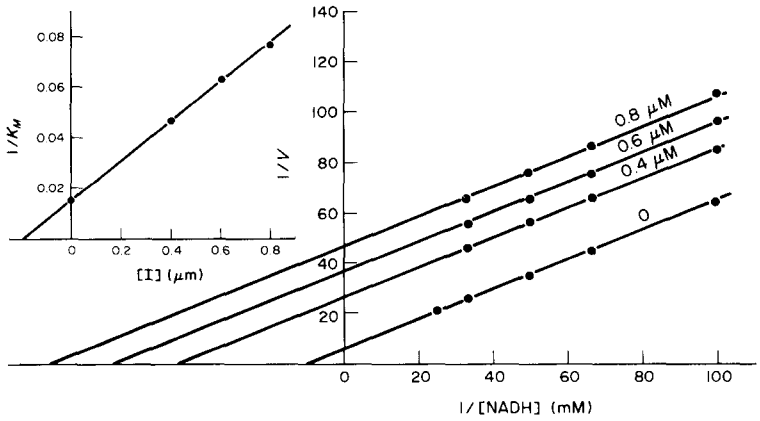

Fig. 4. Double-reciprocal plots of the effect of NADH on the inhibitory activity of gossypol on malate dehydrogenase from *T. cruzi*. NADH concentrations were 0.015, 0.02, 0.03, 0.04, 0.06 and 0.08 mM; oxaloacetate concentration was 0.5 mM in all assays. The concentration of inhibitor is indicated on each curve. Upper left: replot of reciprocals of K_m values against inhibitor concentration.

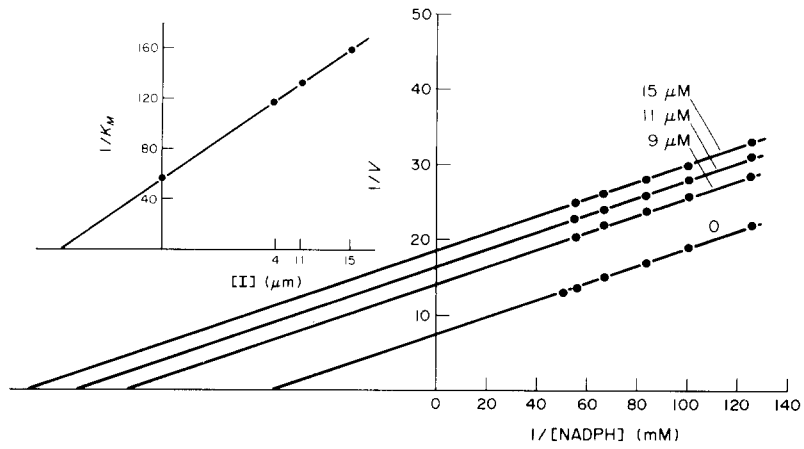

Fig. 6. Double-reciprocal plots of the effect of NADPH on the inhibitory activity of gossypol on glutamate dehydrogenase from *T. cruzi*. The concentrations of NADP were 0.008, 0.010, 0.012 and 0.015 mM; the α-ketoglutarate concentration was 4.0 mM in all assays. The concentration of inhibitor is indicated on each curve. Upper left: replot of reciprocals of K_m values against inhibitor concentration.

summarizes the results of the inhibitory action of gossypol on the six enzymes studied.

DISCUSSION

The results presented demonstrate that gossypol is a powerful inhibitor of the *T. cruzi* α-hydroxyacid and malate dehydrogenases, NAD-linked enzymes. Glutamate and glucose-6-phosphate dehydrogenases and malic enzyme, which utilize NADP as coenzyme, were also inhibited, although to a lesser degree. Gossypol did not affect the activity of succinate dehydrogenase, a flavoprotein. In the cases in which the mechanism of inhibition was analyzed, it was shown to be noncompetitive with respect to substrate and uncompetitive for the coenzyme.

It had been reported previously [15] that exposure to gossypol produces immobilization of epimastigotes and marked reduction of growth in cultures. Probably the blockage of metabolic pathways comprising NAD- and NADP-linked oxidoreductases can explain those effects.

It is possible that the inhibitory action of gossypol is not restricted to *T. cruzi* but could also be exerted upon enzymes of other flagellates as well. Working with *T. conorhini* and *Crithidia fasciculata*, Bacchi *et al.* [23] reported the existence of an enzyme with properties comparable to those of α-hydroxyacid dehydrogenase. We have confirmed this finding in *C. fasciculata* (unpublished observations). It appears that this enzyme, which could be the equivalent of LDH X in spermatozoa, may be a part of the metabolic machinery of flagellates. It will be of interest to investigate its existence in other species of *Trypanosomatidae*.

Studies so far conducted refer to the *in vitro* action of gossypol on enzymes of *T. cruzi* epimastigotes and on entire parasites [15]. Direct incubation with the drug reduces motility and produces marked ultrastructural damage [24]. Further investigations are needed to ascertain whether the trypomastigote (stage found in the gut of the insect vector and body fluids of the mammalian host) and the amastigote (stage seen in the mammalian host) are also susceptible to the compound. Preliminary observations in our laboratory showed that exposure to gossypol produces reduction of motility and morphological alterations on circulating trypomastigotes from

Table 1. Effect of gossypol on oxidoreductases from *Trypanosoma cruzi*

Enzyme	Substrates (mM)	Gossypol (μM)	Inhibition (%)
α-Hydroxyacid dehydrogenase	α-Ketoisocaproate (5) + NADH (0.115)	0.5	45
Malate dehydrogenase	Oxaloacetate (0.5) + NADH (0.115)	0.3	38.2
Glutamate dehydrogenase	α-Ketoglutarate (8) + NADPH (0.119)	5.0	66.8
Malic enzyme	L-Malate (9) + $MnCl_2$ (2) + NADP (0.15)	10.0	37.3
Glucose-6-phosphate dehydrogenase	Glucose-6-phosphate (2.5) + NADP (0.25)	20.0	57.6
Succinate dehydrogenase	Succinate (20.0)	100.0	0

infected mice, suggesting that these forms are also affected (unpublished results). Additional studies on the action of gossypol on experimental infections of mammalian cell cultures and of laboratory animals are necessary to test its possible activity as a therapeutic agent in trypanosomiases. The results presented encourage the conduct of these investigations. Since there is presently no satisfactory drug for use in Chagas' disease, a very serious endemic affecting millions of people in South America, the search for new pharmacological agents is amply justified.

Investigations with a large number of human volunteers in China have demonstrated that oral administration of gossypol for long periods (several years), in doses able to produce marked oligospermia and azoospermia, is tolerated without significant undesirable effects [2]. Furthermore, the disturbance produced in spermatogenesis is not permanent. In most cases, 2 months after discontinuation of the drug, gametogenesis and sperm counts return to normal [2]. It appears, then, that chronic treatment with the compound can be conducted safely.

There is no indication of selective accumulation of the drug in testis [2]. In any case, the effect of gossypol on the seminiferious epithelium indicates that it can penetrate through cellular membranes and other very restrictive permeability barriers (blood–testis). Levels of gossypol in the serum of men receiving the compound at effective doses have not been reported; thus, it is impossible to establish how they compare with the K_i values of the drug for *T. cruzi* enzymes. However, if the effect of gossypol is due to inhibition of sperm-specific enzymes, it is possible that the antifertility levels of the drug would be even more toxic for *T. cruzi*. Our data indicate that K_i values for the NAD-linked dehydrogenases of epimastigotes are much lower than those found for the equivalent human enzymes.

Since there is experience on the use of gossypol as a male contraceptive, a wealth of information is available on toxicity, side-effects, doses, long-term treatments, etc., which eventually would simplify clinical trials of the drug.

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