PARADOXICAL ACTIVATION OF *LEPTOMONAS* NAD-LINKED α-GLYCEROPHOSPHATE DEHYDROGENASE BY ETHIDIUM AND ANTRYCIDE

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Received December 28,1976

SUMMARY. Antrycide and ethidium bromide -- 2 cationic trypanocides -- inhibited NAD-linked α-glycerophosphate dehydrogenase from *Leptomonas* sp. The kinetics of enzyme inhibition was determined by Lineweaver-Burk, Dixon, or direct linear plots. Inhibition by Antrycide was noncompetitive for dihydroxyacetone phosphate in the presence of saturating Mg^{2+} or spermidine. With dihydroxyacetone phosphate at saturation, Antrycide inhibition was also noncompetitive with respect to Mg^{2+} (K_i = 115 μM) and spermidine (K_i = 85 μM). Inhibition by ethidium in the presence of saturating dihydroxyacetone phosphate, was noncompetitive for Mg^{2+} (K_i = 400 μM) but mixed for spermidine (K_i = 495 μM); inhibition was noncompetitive for dihydroxyacetone phosphate in the presence of saturating Mg^{2+} or spermidine. Rabbit-muscle α-glycerophosphate dehydrogenase was inhibited at all concentrations of Antrycide and ethidium tested, but the *Leptomonas* enzyme was stimulated up to 3.5-fold by low concentrations of inhibitors in the absence of polyamine. New chemotherapeutic possibilities may thus be opened and an evolutionary distinction between trypanosomatid and mammalian enzyme.

The cationic trypanocide ethidium bromide, practical against *Trypanosoma congolense* of African cattle, was supposed to act by inhibiting DNA synthesis (1) by intercalating between base pairs of the template (2), especially to the circular DNA in the unique mitochondrial-kinetoplast organelle of trypanosomatid flagellates (3, 4). Information on quinapyramine [Antrycide; 4-amino-6-(2-amino-6-methyl-4-pyrimidinyl)amino-1-methylquinaldinium methosalt], another cationic trypanocidal dye, is sparse compared to the vast literature on ethidium. Newton (5), using the symbiont-bearing lower trypanosomatid *Crithidia oncopelti*, emphasized that Antrycide primarily aggregated and inactivated *C. oncopelti* ribosomes by displacing Mg^{2+} and polyamines from these particles but later noted (6) that binding of ethidium to DNA could not account for ethidium's chemotherapeutic specificity. The same presumably applies to Antrycide.

Purified NAD-linked α-glycerophosphate dehydrogenase from the insect trypanosomatid *Leptomonas* sp. appeared unique among similar enzymes from other

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sources in its activation by low levels of spermidine and spermine, replacing the Mg^{2+} cofactor requirement (7); this was likewise observed for the dehydrogenase from another trypanosomatid, Crithidia fasciculata (8). Since spermidine, spermine, and Mg^{2+} compete for binding sites on yeast tRNA (9) and rabbit-liver tRNA nucleotidyltransferase (10), we investigated the effects of ethidium and Antrycide on purified Leptomonas α-glycerophosphate dehydrogenase. We now report that ethidium and Antrycide at low concentrations stimulate and at higher concentrations inhibit the Leptomonas enzyme. Some chemotherapeutic possibilities are therefore suggested, as well as the possible differences in regulation between the Leptomonas and rabbit-muscle enzyme.

MATERIALS AND METHODS

Enzyme isolation: The Leptomonas (ATCC 30250) was mass-cultured in complex HY medium (8). Cytosolic α-glycerophosphate dehydrogenase (EC 1,1,1.8) was isolated by a 2-column purification procedure involving DEAE-cellulose chromatography, then affinity chromatography on unsubstituted agarose (7).

Substrates: Dihydroxyacetone phosphate was obtained and prepared according to instructions from Sigma Chemical Co. Spermidine (trihydrochloride, Sigma) was dissolved in 25 mM KPO_4 buffer (pH 6.3).

Drugs: Ethidium bromide (Sigma) and Antrycide methylsulfate (gift, Imperial Chemical Industries, England) were used as fresh aqueous solutions.

Enzyme and protein assays: Leptomonas α-glycerophosphate dehydrogenase activity was monitored at 25 C in 25 mM KPO_4 buffer (pH 6.3) in a final volume of 3.0 ml as described (7). Rabbit-muscle enzyme (Sigma) was dissolved and assayed in 50 mM Tris-HCl buffer (pH 7.4). Reactions were started by adding dihydroxyacetone phosphate. NADH oxidation was followed for 1 min by measuring decrease in absorbance at 340 nm. Protein concentration was estimated by the method of Lowry et al. (11) with bovine serum albumin (fraction V, Sigma) as standard.

Kinetic measurements: Inhibition constants (K_i) were determined from either Lineweaver-Burk (12), Dixon (13) or linear plots (14).

RESULTS

Fig. 1 depicts a striking differential effect of ethidium and Antrycide upon the activity of the Leptomonas as compared with the rabbit-muscle α-glycerophosphate dehydrogenase. The rabbit-muscle enzyme was inhibited linearly as a function of increasing Antrycide concentrations, 150 μM Antrycide being totally inhibitory (Fig. 1A). In contrast, without added cofactor (i.e., no Mg^{2+} or spermidine), low concentrations of Antrycide stimulated the activity of the Leptomonas enzyme up to 3.5-fold at a concentration of 75 μM (Fig. 1A).

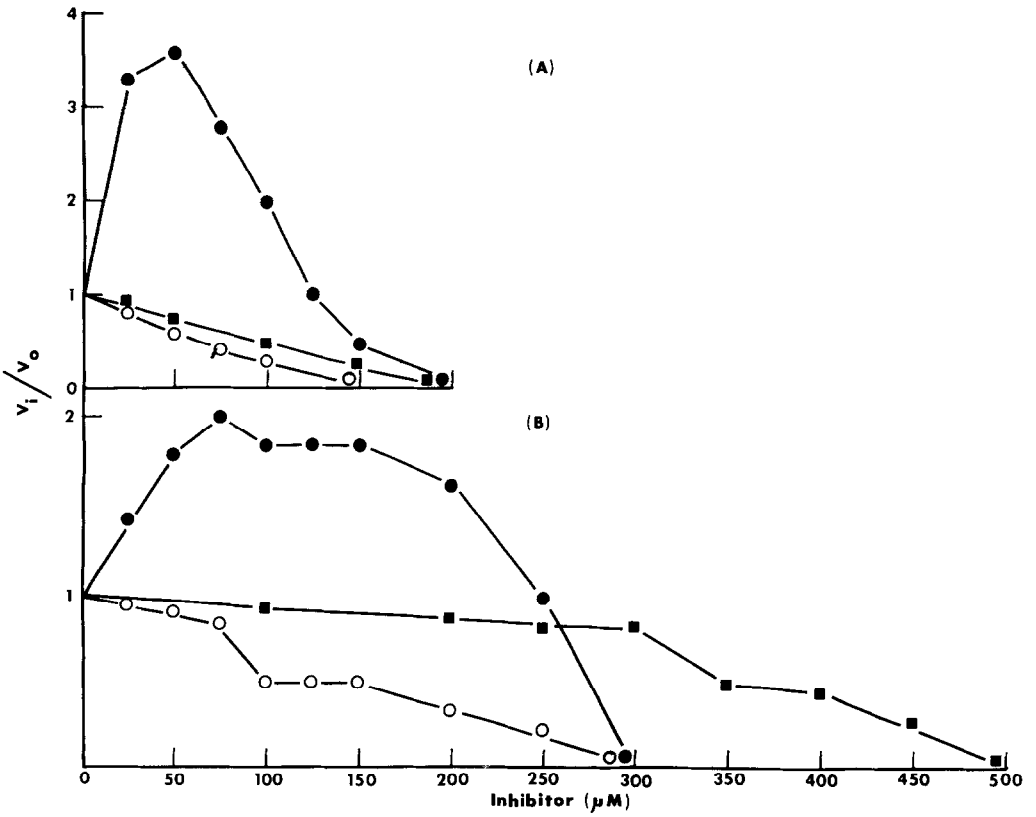


Fig. 1. Effect of Antrycide and ethidium on Leptomonas and rabbit-muscle α-glycerophosphate dehydrogenase. (A) Antrycide: ●—●, Leptomonas enzyme in the absence of cofactor; ■—■, Leptomonas enzyme in the presence of saturating spermidine; O——O, rabbit-muscle enzyme. (B) ethidium: legend remains the same as for Antrycide, V_0 = initial rate without drug, V_i = inhibitory rate. Reaction mixture contained: Leptomonas, 0.01 ml of enzyme (44 μg protein/ml); rabbit-muscle, 0.01 ml of enzyme (150 μg protein/ml).

Activity was lessened above this concentration, with complete inhibition occurring at 200 μM Antrycide. Similar results were obtained with ethidium, which produced a maximal stimulation (2.0-fold) of the Leptomonas enzyme at 75 μM (Fig. 1B). Inhibition by ethidium required higher concentrations of drug than with Antrycide; not until 200 μM was inhibition significant, with complete inhibition at 300 μM ethidium. As with Antrycide, rabbit-muscle α-glycerophosphate dehydrogenase was not stimulated by any concentration of ethidium (Fig. 1B). Complete inhibition of the rabbit-muscle enzyme was achieved at

TABLE 1. K_i values for Antrycide and ethidium tested against components of the Leptomonas α-glycerophosphate dehycrogenase assay.

Drug	Variable	Constant substrate/cofactor	Inhibition Type	K_i (uM)
Antrycide	DHAP*	Mg^{2+} (25 mM)	Noncompetitive	75
	DHAP*	Spermidine (2 mM)	"	60
	Mg^{2+}	DHAP*	"	115
	Spermidine	DHAP*	"	85
Ethidium	DHAP*	Mg^{2+} (25 mM)	"	210
	DHAP*	Spermidine (2 mM)	"	200
	Mg^{2+}	DHAP*	"	400
	Spermidine	DHAP*	Mixed	495

*Dihydroxyacetone phosphate

250-300 μM ethidium. In the presence of saturating spermidine, a much higher level of ethidium was required for total inhibition of the Leptomonas enzyme (Fig. 1B); no upward shift in inhibitory concentration was observed for Antrycide (Fig. 1A).

In the presence of spermidine or Mg^{2+}, Antrycide or ethidium inhibited the Leptomonas enzyme (Table 1). As seen, lower K_i values for both trypanocides were obtained with dihydroxyacetone phosphate treated as the variable in the presence of saturating Mg^{2+} or spermidine. On the average, K_i values for Antrycide were 3-fold lower than those calculated for ethidium.

Fig. 2A presents the mixed-type inhibition characterizing the action of ethidium vs. spermidine in the presence of saturating dihydroxyacetone phosphate as plotted by the direct linear method (14). A replot of the Lineweaver-Burk plot for this interaction yielded a K_i value of 495 μM (Fig. 2B).

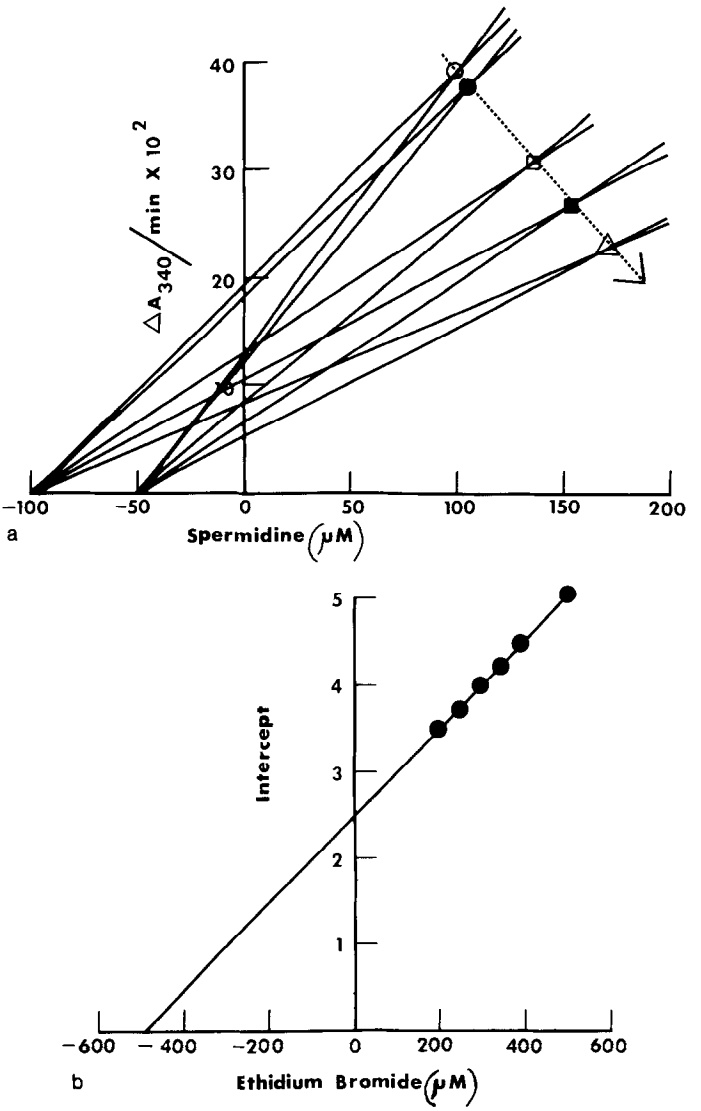
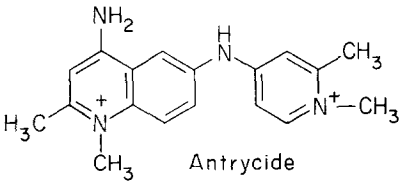


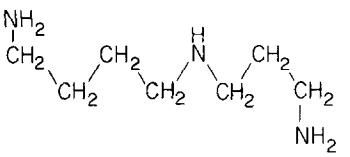
Fig. 2. (A) Direct linear plot of ethidium vs. spermidine in the presence of saturating dihydroxyacetone phosphate. Ethidium concentrations (μM): ○, 0; ●, 200; □, 250; ■, 300; △, 350. Each intersecting point is the average of 4 determinations. Arrow indicates direction of points to give mixed-type inhibition. (B) Replot of ordinate intercepts from Lineweaver-Burk plots as function of ethidium. Each point is the average of 3 determinations. Leptomonas reaction mixture contained 0.01 ml of enzyme (44 μg protein/ml).

DISCUSSION

Little is known about the specific mode of action of cationic trypanocides, their obvious activity as antagonists of nucleic acid metabolism does not suffice to explain their trypanocidal specificity. This paper presents the first



Antrycide



Spermidine

Fig. 3. Structures of Antrycide (top) and spermidine (bottom). Spermidine is drawn so as to parallel the structure of Antrycide.

known activation of an enzyme by DNA intercalator. Evidence for a functional as well as structural analogy between Antrycide and spermidine is Antrycide's activation of Leptomonas' ∝-glycerophosphate dehydrogenase. This may depend on similarities in spacing between key nitrogens of trypanocide and polyamine (Fig. 3). Sakai et al. (9) sketched this structural similarity between ethidium and spermidine. The presence of 2 protonated nitrogens having the same spacing as the spermidine terminal end groups may account for lower non-competitive K_i values obtained with Antrycide. Although ethidium induced a 2.0-fold stimulation of the Leptomonas enzyme, Antrycide, because of structural differences, as noted, may bind differently to the enzyme.

The presence or absence of spermidine invokes conditions for Antrycide to act either as inhibitor or activator of the Leptomonas enzyme. The two together could elicit a "shoving" of each other -- a reciprocal competition -- forcing Antrycide molecules to bind to the enzyme at a site other than the substrate (or cofactor) site, as indicated by noncompetitive inhibition. This type of interaction may apply, although to a lesser extent, to ethidium + spermidine since a mixed-type inhibition may not be inconsistent with noncompetitiveness. Antrycide also gave lower K_i values when spermidine was used as a cofactor or

as a factor opposing Mg^{2+} under similar conditions. Again, structural similarities between Antrycide and polyamine may be responsible.

Rat liver (8) and rabbit-muscle α-glycerophosphate dehydrogenase neither required Mg^{2+} nor were stimulated by spermidine. Therefore the cationic binding site(s) for spermidine or Mg^{2+} adduced for the Leptomonas dehydrogenase seemingly lacks a mammalian counterpart. Presumably, too, stimulation of the Leptomonas enzyme by low concentrations of Antrycide or ethidium is mediated through the cationic binding site.

Hope for chemotherapy comes from inhibition of the α-glycerophosphate oxidase portion of the trypanosome α-glycerophosphate shuttle with m-chlorobenzhydroxamic acid (15), salicylhydroxamic acid (16), and the apparently synergistic combination of salicylhydroxamic acid + glycerol (17). Our laboratory has found polyamine replacement of Mg^{2+} for α-glycerophosphate dehydrogenase from Crithidia fasciculata (8), Leptomonas sp. (7), and Trypanosoma brucei (Bacchi, unpubl.). Because polyamine stimulation is currently uniquely limited to the trypanosomatid enzyme the use of specific polyamine antagonists, eg. diacridines, should be examined (18); several of them inhibit tumor cells in vitro (19). The hope for a more specific chemotherapy is encouraged by an additional differential in polyamine metabolism: trypanosomatids, unlike mammals, lack spermine (Bacchi et al., submitted for publication). Analysis of both portions of the α-glycerophosphate shuttle may provide a lead to development of drugs badly needed for Chagas' disease (20), the African trypanosomiases (21, 22), and leishmaniases (23).

Acknowledgements

This work was supported by GRS USPHS grant FR-05596 (F.S. Cooper, Haskins Laboratories), a Pace Scholarly Research Award (C.J.B.), a Dr. G. Papanicolaou Graduate Scholarship Award (C.L.), and the S. and W.T. Golden Foundation.

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