THE POTENTIAL USE OF INHIBITORS OF GLYCEROL-3-PHOSPHATE OXIDASE FOR CHEMOTHERAPY OF AFRICAN TRYPANOSOMIASIS

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1. Introduction

The bloodstream forms of African trypanosomes, responsible for sleeping sickness in man, are completely dependent on glycolysis for their energy supply, because the biogenesis of a mitochondrial respiratory chain is suppressed in the vertebrate host [1–4]. The present ideas on trypanosome glycolysis are presented in a simplified form in fig.1. Grant and Fulton [5] showed that the flow of carbon is consistent with the view that the Embden-Meyerhof scheme is the only pathway of glucose utilization. Pyruvate is not further metabolized because of the absence of a functional Krebs cycle [6,7] and the lack of lactate dehydrogenase [8] and is excreted into the host blood. The NAD^+ reduced in the glyceraldehyde phosphate dehydrogenase step is reoxidized by a L-glycerol-3-phosphate oxidase system [9], unique to trypanosomes (boxed in fig.1). According to fig.1 this oxidase system is essential for survival of bloodstream trypanosomes. Since it is absent in the mammalian host, it should provide an ideal target for a selective chemotherapeutic agent.

This paper deals with the effect of salicylhydroxamic acid (SHAM), a powerful inhibitor of the glycerol-phosphate oxidase system in trypanosomes, on glycolysis and on the energy production of isolated, intact trypanosomes. Although the oxidase is completely blocked by 1 mM SHAM, we have found that motility and ATP production are not drastically affected. Therefore, we conclude that a functional oxidase is not essential for all energy production and that fig.1 presents an incomplete and misleading picture of glycolysis in trypanosomes.

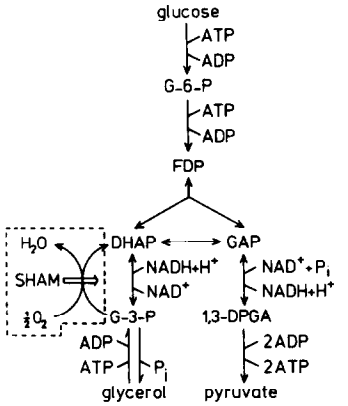

Fig.1. Glycolysis in bloodstream trypanosomes (after [4]). Abbreviations: G-6-P, glucose-6-phosphate; FDP, fructose-1,6-diphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate, G-3-P, L-glycerol-3-phosphate; 1,3-DPGA, 1,3-diphosphateglycerate.

Abbreviations: SHAM, salicylhydroxamic acid; *m*-CLAM, *m*-chlorobenzhydroxamic acid; ~P, energy-rich phosphate bond.

2. Materials and methods

Trypanosoma brucei EATRO 427 was maintained as a stabilate at −196°C or by syringe passage since its isolation. This strain does not show any pleomorphic characteristics [10] upon intraperitoneal inoculation of 10^6 trypanosomes in rats, where it produces

a fulminating infection resulting in death of the host in about 100 h. Nevertheless, occasional relapsing parasitaemias have been seen and this strain is able to infect the insect vector (Van Emden, H., personal communication). The trypanosomes were isolated from the blood of infected 300 g Wistar rats by filtration through a DEAE-cellulose column according to the method of Lanham [11], followed by washing with 50 mM sodium phosphate–45 mM sodium chloride–55 mM glucose (pH 8.0).

Glucose [12], glycerol [13], pyruvate [14] and ATP [15] were determined according to published procedures.

Oxygen consumption was measured polarographically with a Clark-type oxygen electrode [16].

SHAM, purchased from Aldrich Chemical Company, Beerse, Belgium, was added as a solution in dimethylformamide. Control experiments done in the absence of SHAM were always supplemented with the same amount of dimethylformamide.

3. Results

Aromatic hydroxamic acids are well known inhibitors of cyanide-insensitive respiration in plants [17] and micro-organisms [18]. Evans and Brown [19,20] recently reported that this class of compounds, especially *m*-chlorobenzhydroxamic acid (*m*-CLAM), blocks the oxygen consumption of bloodstream trypanosomes by inhibition of glycerolphosphate oxidase. We have found that another of these compounds – SHAM – inhibits both glucose- and glycerol-induced respiration of intact cells >99% at a concentration of 1 mM; only 15 μM was required for half-maximal inhibition of glucose-induced O_2 uptake [21]. To determine whether 1 mM SHAM also stops ATP synthesis, we studied the motility of the trypanosomes and cellular ATP levels (table 1). In the absence of substrate the trypanosomes become immobile within 5 min and their ATP content falls to zero, showing that substrate or energy-rich phosphate stores are negligible. With glycerol as substrate, motility and ATP synthesis are blocked by SHAM, as expected from fig.1. With glucose as substrate, however, motility is not abolished and ATP levels remain at about half those found in the absence of SHAM.

This observation cannot be reconciled with the scheme in fig.1, because in this scheme net ATP synthesis is zero when the glycerol-phosphate oxidase is blocked. We therefore checked the carbon balance of glucose and glycerol metabolism in our *T. brucei* strain to verify whether it conforms to the stoichiometry expected from fig.1. The result in table 2 shows that this is the case. In the absence of SHAM, glucose

Table 1
Effect of salicylhydroxamic acid (SHAM) on the motility and ATP content of bloodstream form *T. brucei*, incubated with glucose or glycerol

Trypanosome samples	Substrate present	SHAM added (1 mM)	Motility		ATP concentration (nmoles/mg protein)	
			5 min incubation	15 min incubation	5 min incubation	15 min incubation
1	None	–	– – –	– – –	0.1	0.0
2	Glucose	–	+++	++	2.8	2.4
2	Glucose	+	+	++	1.2	1.0
3	Glycerol	–	+++	++	1.0	1.0
3	Glycerol	+	– – –	– – –	0.1	0.2

Trypanosomes were divided into three portions and taken up in a mixture containing 50 mM sodium phosphate and 45 mM NaCl (pH 8.0) (PS) with either no addition (sample 1); 55 mM glucose (sample 2), or 55 mM glycerol (sample 3). The various samples were incubated in PS at 37°C with or without 180 mM substrate, as indicated, with shaking, in a total volume of 0.8 ml in flat-bottom liquid scintillation vials (diameter 26 mm) to provide a high surface/volume ratio. Samples were taken at the times indicated and examined by phase contrast microscopy (within 30 sec) for motility oi deproteinized with 5% perchloric acid for ATP determination. The motility was scored by an observer, unaware of the incubation conditions used. The low motility after 5 min in the incubation with glucose plus SHAM was not observed in several other experiments and is presumably an artefact.

Table 2
Comparison of the products formed by incubation of *T. brucei* with glucose or glycerol under aerobic conditions in the presence and absence of SHAM

Substrate	Metabolite	Control		1 mM SHAM	
		Δ (μmoles)	Carbon yield (%)	Δ (μmoles)	Carbon yield (%)
Glucose	Glucose	−1.68	100	−1.54	100
	Pyruvate	+3.08	92	+1.55	50
	Glycerol	+0.05	1.5	+1.55	50
Glycerol	Glycerol	−1.71	100	−0.06	
	Pyruvate	+1.81	106	+0.05	

Trypanosomes (10 mg wet weight/assay) were incubated, with shaking, in a flat-bottom liquid scintillation vial in a volume of 1 ml for 30 min at 37°C. The reaction mixture contained 50 mM sodium phosphate–45 mM NaCl (pH 8.0), supplemented with either 5.8 mM glucose or 5.0 mM glycerol, as indicated. Similar results were obtained in two other separate experiments. After deproteinization the supernatants were assayed for metabolites.

is converted into pyruvate, only small amounts of glycerol being formed. In the presence of SHAM, however, equimolar amounts of pyruvate and glycerol are formed. In both cases >90% of the glucose carbon can be accounted for as pyruvate and glycerol. Table II shows in addition that glycerol alone is quantitatively converted into pyruvate and that this process is completely inhibited by SHAM. These data are in complete agreement with those of Grant and Fulton [5], who used the L-strain of *Trypanosoma rhodesiense* rather than *T. brucei* and anaerobiosis to block the glycerolphosphate oxidase. This shows that SHAM effectively mimics anaerobiosis in its effect on carbon flow and that our *T. brucei* strain, isolated from a fulminating infection, does not significantly metabolize pyruvate even though it still has the potential to make functional mitochondria, because it can still infect the insect vector (see Materials and methods).

4. Discussion

Already in 1959 Fulton and Spooner [6] questioned whether a drug inhibiting the glycerolphosphate oxidase could be of value in trypanosomiasis, as they had found [22] that *T. rhodesiense* can survive anaerobically for at least 30 min. Our experiments indeed show that a complete block of the glycerolphosphate oxidase system does not abolish trypanosome motility and only leads to a partial decrease of cellular ATP levels. This is not due to an intra-cellular store of utilizable energy, because added glucose is required to maintain ATP production. It can also not be attributed to further metabolism of pyruvate, because glucose is quantitatively converted into pyruvate and glycerol in the presence of SHAM (Table 2).

These results appear to differ from those of Evans and Brown [19] with *m*-CLAM, because 3.0 mM of this compound immobilized *T. brucei* bloodstream forms within 10 min. 0.3 mM *m*-CLAM was much less effective, moreover, although 0.1 mM completely blocked O_2 uptake. In view of this discrepancy and our results with SHAM we think that the effect on motility, observed with the very high doses of *m*-CLAM used, is a side effect not related to the inhibition of glycerolphosphate oxidase.

We conclude, therefore, that the scheme in fig.1 cannot be correct. There are several alternative schemes, however, that could account for our results. One (theoretical) alternative involves a transphosphorylation between glycerolphosphate and a hexosemonophosphate, avoiding the loss of one ~P/glucose incurred in fig.1, when glycerolphosphate is hydrolysed to glycerol and inorganic phosphate. Another alternative involves the splitting of a hexosemonophosphate into a triosephosphate and a triose. This would create a hydrogen acceptor (the triose) to re-oxidize NADH without the investment of a ~P. Both pathways would

allow glycolysis to operate anaerobically with a yield of one ~P/glucose, rather than the 2 ~P/glucose obtained aerobically.

Some evidence for the second alternative can be extracted from the older literature: Grant and Fulton [5] have shown that carbon 1 of glucose does not end up equally in glycerol and pyruvate under anaerobic conditions; this is the result expected if a hexose-monophosphate is split rather than a hexosediphosphate. Ryley [23] has made the interesting observation that under anaerobic conditions glucose utilization is strongly inhibited by glycerol. This is again the expected result if a glycerol dehydrogenase is responsible for the re-oxidation of glycolytic NADH. Both observations, however, can (and have, cf. [5]) been explained differently and we have been unable to demonstrate the presence of the requisite glycerol dehydrogenase in *T. brucei* extracts. Further experiments are, therefore, required to determine which alternative glycolytic scheme is the correct one.

These speculations nevertheless suggest that SHAM or its analogues can maximally decrease ATP synthesis in bloodstream trypanosomes by a factor two. If there is reserve capacity of the glycolytic pathway, the effect will even be less. It seems unlikely that effective chemotherapy could be based on this and, in fact, no effect of SHAM was observed on the course of *T. brucei* infection in rats (using a strain that kills the host in 4 days), although effective blood concentrations of 0.5 mM were reached [21]. SHAM might still be useful, however, in combination with other drugs that block the main route from glyceraldehydephosphate to pyruvate, or the enzyme(s) responsible for re-oxidation of NADH when the glycerolphosphate oxidase system is blocked. More detailed knowledge of glycolysis in trypanosomes is clearly required to asses the therapeutic potential of such combinations.

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