Localization of the initial steps in alkoxyphospholipid biosynthesis in glycosomes (microbodies) of *Trypanosoma brucei*

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Cell fractionation of *Trypanosoma brucei* cultured procyclic stages showed that the key enzyme of glycerol-ether lipid synthesis, dihydroxyacetone-phosphate acyltransferase (EC 2.3.1.42) was exclusively associated with the microbody fraction. These organelles contained in addition 1-acyl glycerol-3-phosphate:$NADP^+$ oxidoreductase (EC 1.1.1.101) and acyl-CoA reductase and were capable of utilizing DHAP, but not G-3-P, as substrate for lysophosphatidic acid formation. It is concluded that in *T. brucei* the glycosomes are the exclusive site of the synthesis of precursors for glycerol-ether lipid synthesis and that they contain the entire pathway to form alkoxylipids from glycerol and acyl-CoA.

Alkoxyphospholipid *Glycerol-ether lipid* *Dihydroxyacetone-phosphate acyltransferase* *Acyl-CoA reductase* Trypanosoma *Microbody* *Glycosome*

1. INTRODUCTION

Glycerol phospholipids with ether linkages (alkoxyphospholipids) are found in nearly all animal and bacterial cells with the exception of most aerobic bacteria [1]. In several protozoans, like *Acanthamoeba, Tetrahymena* and the haemoflagellates belonging to the family of Trypanosomatidae, plasmalogens (alkenyl acyl glycerophospholipids) were detected [2] and an extensive study on the trypanosomatid species *Leishmania tarentolae* revealed the presence of considerable amounts of glycerol-ether lipids in the latter organism [3].

I present here evidence that in the haemoflagellate *Trypanosoma brucei*, the causative agent of nagana in domestic animals and intimately related to *T. rhodesiense* and *T. gambiense*, responsible for human sleeping sickness in East and West Africa, not only the essential steps involved in the biosynthesis of alkoxyphospholipids are associated with microbody-like organelles, as in mammalian cells [4–6], but also that these organelles contain the entire pathway to form alkoxylipids from glycerol and acyl-CoA. We have shown that the Trypanosomatid microbodies contain a number of glycolytic enzymes [7] but lack all classical marker enzymes of peroxisomes. These organelles which have been called glycosomes [7] were supposed to be unique to the Trypanosomatidae; however the association of the essential steps of ether-lipid biosynthesis with *T. brucei* glycosomes suggests that the microbodies of the Trypanosomatidae and the peroxisomes and microperoxisomes of mammals are related organelles.

Abbreviations: DHAP, dihydroxyacetone phosphate; G-3-P, *sn*-glycerol 3-phosphate; CoA, coenzyme A

2. MATERIALS AND METHODS

T. brucei stock 427 procyclic trypomastigotes were grown in SDM-79 medium at 28°C exactly as in [8]. Preparation of cell homogenates, cell fractionation by differential centrifugation, isopycnic sucrose-gradient centrifugation, and presentation of the results were exactly as in [8].

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Highly purified glycosomes were prepared by differential centrifugation of homogenates followed by sequential isopycnic centrifugation in a Percoll and a sucrose gradient, respectively, as in [9].

DHAP acyltransferase at pH 7.0 and 1-acyl G-3-P:$NADP^+$ oxidoreductase were assayed as in [10]. Acyl-CoA reductase activity was measured by following procedure A in [6].

Palmitoyl-CoA and clofibric acid were obtained from Sigma and bovine serum albumin (fraction V, fatty acid poor) was a product of Miles Laboratories. $H_2{}^{32}PO_4$ and [1-^{14}C]palmitoyl-CoA were obtained from New England Nuclear. Dihydroxyacetone [^{32}P]phosphate and glycerol 3-[^{32}P]phosphate were prepared as in [10]. All other chemicals were of the highest purity commercially available.

3. RESULTS

3.1. *DHAP acyltransferase*

Table 1 shows that when a trypanosome homogenate or highly purified glycosomes were incubated in the presence of acyl-CoA, [^{32}P]DHAP and other co-factors, a ^{32}P-labelled lipid was formed which had the characteristics of an acyl [^{32}P]DHAP. The formation of acyl DHAP in a trypanosome homogenate required the presence of Mg^{2+}, NaF and bovine serum albumin, similar to what has been described for DHAP acyltransferase in rodent liver [10]. In total homogenates the enzyme had a specific activity of 0.09 nmol/min per mg protein and the reaction was only partly dependent on palmitoyl-CoA, probably due to the presence of endogenous CoA esters. By contrast, purified glycosomes which had a 17-fold higher specific activity (1.5 nmol/min per mg protein) showed an absolute dependence on the presence of palmitoyl-CoA. The incorporation of [^{32}P]DHAP into lipid was completely blocked by the addition of 0.1% Triton X-100. The activity displayed a rather sharp pH-dependency profile between pH 6 and 8 with an optimum around pH 7 (not shown). Citrate had an inhibitory effect (50% inhibition at 25 mM), as had clofibrate, an inhibitor of peroxisomal DHAP acyltransferase (table 1).

Table 1

Requirements for the acylation of DHAP in total *T. brucei* homogenate and in a glycosomal fraction

Conditions	Product formed (%)	
	Homogenate	Glycosomes
Whole system	100	100
Whole – palmitoyl-CoA	67	0
Whole – $MgCl_2$	26	–
Whole – NaF	60	–
Whole – bovine serum albumin	27	–
Whole with boiled enzyme[a]	1	–
Whole + 0.1% Triton X-100	–	0
Whole + clofibrate (1 mM)		29
Whole + clofibrate (5 mM)		0

[a] This enzyme fraction was heated for 10 min at 100°C before use

The whole system contained 60 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM NaF, 2 mg bovine serum albumin, 0.1 mg palmitoyl-CoA, 0.1 mM [^{32}P]DHAP (3×10^4 cpm/nmol) and 0.4 mg *T. brucei* homogenate or 0.03 mg glycosomal preparation in a final volume of 0.5 ml

3.2. *Acyl-CoA reductase*

T. brucei glycosomes contained a low activity of acyl-CoA reductase (0.08 pmol/min per mg protein) which was specific for NADPH, no activity being found with NADH as cofactor. The enzyme was inactivated by heating and inhibited by 50% by the presence of 0.1% Triton-100. The presence of bovine serum albumin resulted in stimulation of the enzyme activity.

3.3. *Subcellular fractionation*

When the large-particle supernatant fraction of a *T. brucei* homogenate after centrifugation for 10 min at $5000 \times g$ was subjected to further fractionation by density gradient centrifugation, the DHAP acyltransferase activity equilibrated together with the glycosomal enzymes glycerol kinase, G-3-P dehydrogenase and triosephosphate isomerase [7] at a density of 1.23 g/cm^3 (fig.1); there was no indication of the presence of DHAP acyltransferase activity in subcellular constituents other than the glycosome. Fig.1 shows that the majority of the acyl-CoA reductase activity also equilibrated at glycosomal density but in addition some activity was associated with other cell constituents, possibly the mitochondrion (cf. succinate dehydrogenase).

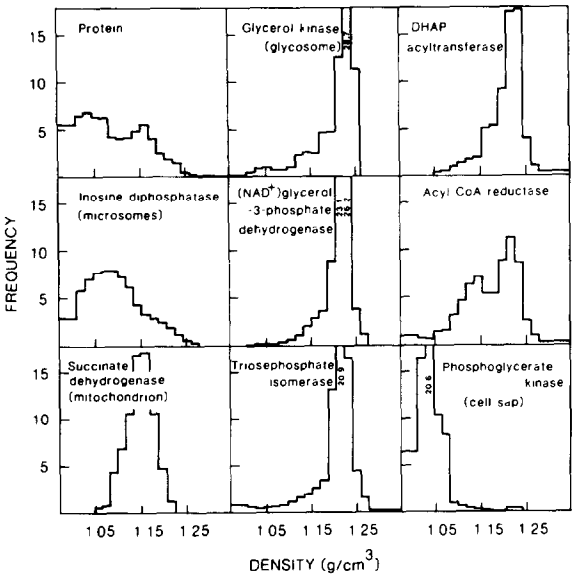

Fig.1. Distribution profile of enzymes present in a post-large granule extract of *T. brucei* procyclic trypomastigotes after isopycnic centrifugation on a linear sucrose gradient. The subcellular constituents for which some enzymes serve as markers are shown within brackets.

3.4. *Incorporation of DHAP by glycosomes*

When highly purified glycosomes [9] were incubated in the presence of palmitoyl-CoA and [^{32}P]DHAP, only one product, acyl DHAP, was formed. However, when NADPH was added to the incubation mixture acyl DHAP as well as acyl G-3-P was formed, indicating the presence of significant 1-acyl G-3-P:NADP$^+$ oxidoreductase activity in glycosomes. Almost no incorporation of G-3-[^{32}P]P into lipid was found suggesting the absence of G-3-P acyltransferase from glycosomes. A further conversion of the monoacyl derivatives into phosphatidic acids (diacyl *sn*-glycerol 3-phosphate) occurred only to a very low extent, in contrast to the mammalian liver system [10].

Table 2

Radioactive products formed from [^{32}P]DHAP or G-3-[^{32}P]P by glycosomes

Additions	Product formed (nmol)		
	Acyl DHAP	Acyl G-3-P	Diacyl G-3-P

Incubation was carried out in the standard DHAP acyltransferase mixture at 25°C for 30 min containing 60 μg glycosomal protein. The entire organic layer was evaporated and spotted on a silica gel TLC plate and developed in chloroform–methanol–acetic acid–5% aqueous sodium bisulfite (25:10:3:1). Spots identified by autoradiography of TLC plates with the help of non-radioactive standards were cut out and counted. ^{32}P-labelled dihydroxyacetone phosphate (DHAP) and glycerol 3-phosphate (G-3-P) were prepared as in [10].
n.d., not detected

4. DISCUSSION

The lipids of several representatives of the Trypanosomatidae have been characterized and found to contain ether lipids [2,3,11–17]. Authors in [2] demonstrated the presence of phosphatidylethanolamine plasmalogen in *Crithidia, Herpetomones, Trypanosoma* and *Leishmania* spp., and those in [3] carried out a detailed analysis of ether lipids and ether phospholipids of *L. tarentolae*, grown in lipid-free media. They found that 12% of all phospholipids and 32% of the phosphatidylethanolamine contained ether linkages. Direct incorporation of 1-*O*-alkyl *sn*-glycerols and octadecanol into ether lipids and plasmalogens has been demonstrated in the case of *L. donovani* promastigotes [14,16], whereas *C. fasciculata* has been shown to be capable of synthesizing alkyl DHAP from 1-*O*-alkyl *sn*-glycerols [12]. The above data not only indicate that the family of the Trypanosomatidae is capable of incorporating ether lipids from their environment but that they are also actively engaged in the synthesis of such lipids.

Phospholipid biosynthesis in mammals involves two pathways: the acyl DHAP and G-3-P pathways (cf. fig.2). Although both pathways probably contribute equally to the formation of phosphatidate it has clearly been shown that alkyl and alkenyl lipids are exclusively formed via the acyl DHAP route [18]. The results presented show that several key enzymes of the acyl DHAP pathway (i.e., acyl-CoA:DHAP acyltransferase, resembling the peroxisomal DHAP acyltransferase of mammals in its inhibition by clofibric acid [18] and 1-acyl/alkyl G-3-P:NADP$^+$ oxidoreductase, which is responsible for the reduction of both acyl

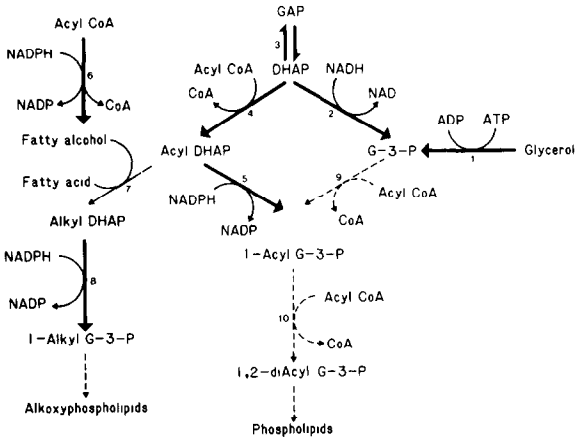

Fig.2. Biosynthetic pathways for phospholipids and alkoxyphospholipids. Thick arrows, reactions catalyzed by enzymes present in glycosomes; broken arrows, reactions either absent or not yet demonstrated in glycosomes. (1) Glycerol kinase, (2) G-3-P dehydrogenase, (3) triosephosphate isomerase, (4) DHAP acyltransferase, (5) 1-acyl G-3-P:$NADP^+$ oxidoreductase, (6) acyl-CoA reductase, (7) alkyl DHAP synthase, (8) 1-alkyl G-3-P:$NADP^+$ oxidoreductase, (9) G-3-P acyltransferase, (10) monoacyl G-3-P acyltransferase.

and alkyl DHAP [19]) are associated with the glycosomes of *T. brucei*. It is clearly demonstrated that when isolated glycosomes were incubated with DHAP, acyl-CoA, NADPH and other cofactors, lysophosphatidic acid and acyl DHAP were formed. The formation of phosphatidic acid occurred only to a minimal extent whereas G-3-P apparently could not be used as substrate by the glycosomes. These observations together with the fact that the DHAP acyltransferase activity was exclusively found in the microbodies and not in other subcellular constituents suggest that the biosynthetic pathway for glycerol-ether lipids in *T. brucei* is exclusively associated with the microbodies. The lack of formation of significant amounts of alkyl G-3-P from G-3-P and of phosphatidate from either DHAP or G-3-P also suggests that glycosomes are not involved in the synthesis of the precursors of normal diacyl phospholipids. In addition to the acyl DHAP pathway the glycosomes also contain trace amounts of acyl-CoA reductase, another enzyme of the ether-lipid pathway converting long-chain fatty acids into their corresponding alcohols. The latter serve as substrates for alkoxyphospholipid synthesis. The fact that the activity in *T. brucei* is at least 2 orders of magnitude lower than that in rat brain [6] could indicate that *T. brucei* procyclic trypomastigotes preferentially take up long-chain alcohols from the medium rather than synthesize them from fatty acids. Since in addition to the above-mentioned enzymes glycosomes also contain glycerol kinase, G-3-P dehydrogenase and triosephosphate isomerase [7] it seems that the entire machinery to synthesize alkoxylipids from glycerol and acyl-CoAs is associated with the microbodies of *T. brucei*.

The entire pathway for the biosynthesis of ether lipids is most likely associated with the glycosomes of all stages of the life cycle of *T. brucei* since, firstly, the presence of plasmalogens has been described for bloodstream forms of the closely related *T. rhodesiense* [15] and, secondly, DHAP acyltransferase is also present in the glycosomes of bloodstream-form stages of *T. brucei* stock 427 (unpublished). In addition we have found a similar location for DHAP acyltransferase in the promastigote stages of *L. major* and *L. donovani* (unpublished), suggesting that alkoxyphospholipid biosynthesis is associated not only with the glycosomes of *T. brucei* but also with those of all Trypanosomatidae.

In addition to ether-lipid biosynthesis mammalian peroxisomes are also involved in other functions like β-oxidation of fatty acids [20], metabolism of bile acids [21,22] and production and degradation of H_2O_2 [23]. Although the glycosomes of Trypanosomatidae are organelles highly specialised in carbohydrate metabolism and pyrimidine biosynthesis [7,24] and were thought to be a unique type of organelle, there are now strong indications that they share several pathways with the peroxisomes of other eukaryotes. In addition to the enzymes involved in the formation of alkoxyphospholipids we have found at least one enzyme of β-oxidation of fatty acids (3-β-hydroxyacyl-CoA dehydrogenase; unpublished) in *T. brucei* glycosomes and several others in the microbodies of *Leishmania* spp. [25]. Also there exists evidence for the presence of catalase activity in the microbodies of some representatives of the Trypanosomatidae as discussed in [9].

From the biochemical observations presented here, together with the morphological resemblance of glycosomes and peroxisomes, I conclude that

glycosomes and peroxisomes are related organelles. The fact that glycosomes are also involved in glycolysis [7], carbon dioxide fixation [26] and de novo pyrimidine biosynthesis [24] might have some important implications for our understanding of the functional role of peroxisomes in general.

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