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Molecular species analysis of phospholipids from *Trypanosoma brucei* bloodstream and procyclic forms

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We present a quantitative description of the molecular species composition of the major phospholipid classes in bloodstream and procyclic forms of *Trypanosoma brucei*. Phospholipid classes were resolved by 2-dimensional thin-layer chromatography. Diradylglycerols were released from individual phospholipid classes by phospholipases C, converted into benzoate derivatives and separated into diacyl, alkylacyl and alk-1-enylacyl subclasses. Individual molecular species were quantitated and identified by HPLC and the assignments were confirmed by mass spectrometry. Comparison of the diacyl species of PC, PE and PI in bloodstream trypanosomes showed major differences in the relative amounts of individual molecular species between the different classes but no striking changes in the degree of saturation or overall chain length. In contrast, in procyclic trypanosomes the relative amounts of diacyl molecular species with polyunsaturated fatty acyl chains decreased in the order of PC > PE ≫ PI. Also, the alkylacyl and alk-1-enylacyl subclasses of PC and PE in bloodstream trypanosomes comprised a single molecular species, 18:0 18:2. Such exclusivity was not observed in procyclic trypanosomes among the same phospholipid subclasses, although 18:0 18:2 was the predominant species. Almost all the PI of bloodstream forms contained one 18:0 acyl species, which is consistent with the composition of the PI used for glycosylphosphatidylinositol synthesis.

Key words: *Trypanosoma brucei*; Lipid composition; Phospholipid; Glycolipid anchor

Introduction

Historically, interest in the composition and metabolism of lipids in trypanosomatids has been driven by the desire to develop suitable media to support the growth of these parasites in vitro. Some of these studies have indicated that *Trypanosoma brucei* has a very limited ability to synthesize fatty acids de novo and is dependent upon an exogenous supply [1]. Insofar as fatty acids are critical intermediates for the synthesis of vital membrane components such as phospholipids, these studies have alluded to a possible biochemical basis for the obligate parasitism of these organisms [1,2]. A more specific impetus for the development of baseline data on the lipid composition of *T. brucei* stems from our ongoing interest in the biosynthesis of glycosylphosphatidylinositol (GPI) anchors of membrane proteins. GPIs serve as the sole means by which many eukaryotic proteins, including the variant surface glycoproteins (VSGs) of *T. brucei*, are

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Abbreviations: VSG, variant surface glycoprotein; GPI, glycosylphosphatidylinositol; GlcNAc, *N*-acetylglucosamine; PARP, procyclic acidic repetitive protein; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLC, phospholipase C; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry.

anchored to cell membranes [3–5].

The core structure of GPI anchors, which consists of ethanolamine-phosphate-6 mannoseα1–2 mannoseα1–6 mannoseα1 4 glucosamineα1 6 inositol1-phosphate-lipid, is conserved over large evolutionary distances [6–9], suggestive of a shared biosynthetic pathway. Additional sugar residues and/or phosphoethanolamine linked to the core mannose hydroxyls, or an additional fatty acid esterified to inositol, have been observed as elaborations of the basic structure. In addition, the VSG GPI anchor is unique in that the lipid moiety is exclusively 1,2-dimyristoylglycerol [10]. We and others have been interested in the biochemical basis of this remarkable specificity, which could provide a target for chemotherapy [11].

Biosynthetic intermediates in the construction of the GPI anchor have been identified in extracts of metabolically labelled trypanosomes and in experiments using trypanosome membrane preparations [12–20]. The structures of these intermediates suggest that GPI biosynthesis is initiated by the addition of *N*-acetyl-glucosamine (GlcNAc) to phosphatidylinositol (PI); subsequent steps include deacetylation of GlcNAc-PI and the sequential addition of mannose residues and phosphoethanolamine. None of the intermediates contain dimyristoyl-PI. Rather, the fatty acid moieties of the early intermediates (primarily $C_{18:0}$ at the 1-position of the glycerol and as yet unknown fatty acids at the 2-position) are replaced by myristic acid late in the biosynthetic pathway by an apparently developmentally regulated fatty acid remodelling reaction [19,20].

In the procyclic insect mid-gut stage of its life-cycle, *T. brucei* expresses another abundant cell-surface protein (the procyclic acidic repetitive protein, PARP, or procyclin) that is glycolipid anchored [21]. Palmitoylation of inositol renders the PARP GPI anchor resistant to phosphatidylinositol-specific phospholipase C [21–24]. Furthermore, unlike the VSG GPI anchor in bloodstream forms, the mature anchor in procyclics does not contain dimyristoyl-PI. Intriguingly, the lipid moiety of the PARP anchor is a 1-stearoyl, 2-*lyso*-PI species that is identical to one of the intermediates of the GPI fatty acid remodelling reaction seen in bloodstream forms [19,20,22,23].

We have been interested in determining the molecular species of PI that are available to, or selected by, the GlcNAc transferase in constructing the first GPI intermediate, GlcNAc-PI. In the process of characterizing the PI molecular species in bloodstream and procyclic trypanosomes, we obtained detailed information on the phospholipid composition of these organisms as well as the molecular species spectrum for each of the major phospholipids.

Materials and Methods

Solvents and glassware. Solvents used were of HPLC grade. All glassware were baked at 535°C for 2 h and cooled to room temperature before use.

Trypanosomes. Bloodstream trypanosome clones of the Molteno Institute Trypanozoon antigenic type 1.5 (clone 118) of *T. brucei* strain 427 were purified from infected rat blood as previously described [25]. Briefly, infected rat blood with a parasite concentration of $5{\cdot}8 \times 10^8$ cells ml^{-1} was centrifuged at approximately $1000 \times g$ for 10 min. and 'buffy coat' trypanosomes were removed, suspended in buffer (44 mM NaCl/ 57 mM Na_2HPO_4/ 3 mM KH_2PO_4/ 55 mM glucose, pH 8.0), and purified by passage through a column of DEAE-cellulose. Culture-adapted procyclic forms of *T. brucei* strain 427 were grown at 27°C in SDM-79 medium [26] supplemented with 10% heat-inactivated fetal calf serum (Gibco) and 7.5 μg ml^{-1} hemin. Cells were maintained at a density of $5 \times 10^5 - 1 \times 10^7$ cells ml^{-1}.

Lipid extractions. Lipids were extracted as described previously [18]. Trypanosomes were centrifuged at approximately $1000 \times g$ for 10 min. and the supernatant was removed. 10 ml of chloroform/methanol (2:1, v/v) (referred to

as CM) was added to the cell pellets of approximately 10^{11} bloodstream forms or 10^{10} procyclics. The pellet was dispersed by stirring with a Pasteur pipette, followed by sonication in a bath sonicator. The tube was allowed to stand for 30 min following which it was centrifuged and the supernatant was removed and stored. The pellet was twice re-extracted with CM and the extracts were pooled. The pooled extracts were subjected to repeated Folch washings before analysis [18]. The combined lipid extracts were dried under nitrogen, resuspended in a small volume of CM and stored under nitrogen at −20°C.

Analysis of phospholipid molecular species. Individual phospholipid classes were resolved by two-dimensional thin-layer chromatography (TLC) and identified by co-migration with pure lipid standards applied on the same TLC plates [27]. To determine the phospholipid composition of trypanosomes, lipids were visualized by iodine staining and the relative amounts of individual classes were measured by phosphorus analysis [28]. For analysis of molecular species, lipids were visualized by spraying with 1,6-diphenyl-2,3,5-hexatriene, scraped from the plates, and extracted from silica with chloroform/methanol/acetic acid/water (50:39:1:10; v/v/v/v). The combined extracts were washed once with 4 M NH_4OH and dried under nitrogen. Diradylglycerols of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) were obtained by treating the lipids with phospholipase C from *Bacillus cereus* (Boehringer Mannheim, Germany). Diradylglycerols of phosphatidylinositol (PI) were obtained by treating PI with PI-specific PLC from *Bacillus thuringiensis* (ICN Biochemicals, Cleveland, OH). The phospholipase treatments were carried out in a mixture of 2 ml buffer (100 mM Tris, pH 7.4/ 5 mM $CaCl_2$) and 2 ml ether at 37°C during vigorous mixing. Diradylglycerols were subsequently extracted with ether and immediately benzoylated as described by Blank et al. [29]. Diradylglycerobenzoates were separated into their subclasses (diacyl, alkylacyl, and alk-1-enylacyl types) by TLC in benzene/hexane/ether (50:45:4; v/v/v) and analyzed by combined HPLC / mass spectrometry using the instrumentation described in [30,31] or in [32]. Briefly, HPLC separation of molecular species was done on an C_{18} reverse-phase column using a solvent system of acetonitrile/isopropanol (80:20; v/v). Individual peaks were quantitated by measuring absorbance at 230 nm. After the UV detector, methanol/0.2 M aqueous ammonium acetate was added via a T-connector, and the total flow was introduced through a thermospray interface into a VG Masslab 30–250 quadrupole mass spectrometer [30,31], or a Finnigan MAT model TSQ70 mass spectrometer [32]. The M^+Na^+ and M^+NH4^+ ions of the diradylglycerobezoates were monitored by selected ion recording. The relative amounts of the subclasses of each phospholipid class were determined by measuring absorbance at 230 nm in ethanol [29], or were calculated by integrating the $A_{230\ nm}$ peaks after HPLC. Both methods essentially gave the same results. The positional distribution of the fatty acyl and fatty alcohol chains of individual diradylglycerol molecular species was not determined.

Results and Discussion

Although lipid compositional analyses of *T. brucei* membranes have been reported previously [2,33–35] the spectrum of individual phospholipid components, *i.e.* molecular species, has not been described. By using a combination of HPLC and mass spectrometry we have been able to separate, identify and quantitate the various molecular species of the major phospholipid classes in *T. brucei* bloodstream and procyclic forms.

Phospholipid composition. Analysis of the phospholipid composition of *T. brucei* showed that PC, PE and sphingomyelin were the most prominent classes in both bloodstream and procyclic forms, comprising about 80% of total membrane phospholipids (Table I). Our findings on the phospholipid composition of bloodstream form trypanosomes are in good

agreement with earlier results by Carroll and McCrorie [35]. Comparison of the phospholipid composition of bloodstream and procyclic trypanosomes showed no striking differences in the relative proportions of the major phospholipid classes, except the amount of sphingomyelin in procyclic forms was decreased as compared to bloodstream forms (Table I). Similar findings were reported previously on the comparison between bloodstream and procyclic forms of *Trypanosoma lewisi* and *Trypanosoma rhodesiense* [2].

The unknown lipid component seen after TLC separation of bloodstream and procyclic-form trypanosome lipids ("X" in Table I) was tentatively identified as inositol sphingophospholipid. This identification was based on its reactions on TLC with specific reagents (positive staining for lipids and phosphorus; negative reaction with ninhydrin); in addition, its migration on TLC relative to other phospholipids was identical to that of isolated inositol sphingophospholipid in a similar TLC system [36]. The presence of inositol sphingophospholipids in trypanosomes may not be surprising since these lipids have been identified in *Leishmania mexicana mexicana* [37].

Phospholipid subclass composition. Determination of the subclass composition of the major phospholipid classes demonstrated that PC and PE in both bloodstream and procyclic trypanosomes contained significant amounts of species belonging to the three subclasses, diacyl, alkylacyl, and alk-1-enylacyl (Table I). In bloodstream forms, PI was almost exclusively composed of diacyl molecular species, while in procyclics 10% of PI was of the alkylacyl type. PS from bloodstream forms contained both diacyl and alkylacyl species while alk-1-enylacyl species were essentially absent. In contrast, procyclics contained all three subclasses of PS. Interestingly, the relative amounts of alkylacyl type species were considerably increased in all procyclic-form phospholipid classes as compared to the respective bloodstream-form classes. The reason for this and other differences between the phospholipids of bloodstream and procyclic trypanosomes are not known. However, they are likely to reflect biochemical changes associated with the transformation from one form to another. Also, since the synthesis of ether glycerophospholipids occurs through distinctly different pathways than that of the corresponding ester lipids [38-40], the above mentioned changes may not only be due to adaptations that occur through exchange of

TABLE I

Phospholipid composition of procyclic and bloodstream-form trypanosomes

Phospholipid class	Procyclic form trypanosomes				Bloodstream form trypanosomes			
	Mol% of total[a]	Subclasses (mol% of each class)[b]			Mol% of total[a]	Subclasses (mol% of each class)[b]		
		Diacyl	Alkylacyl	Alk-1-enylacyl		Diacyl	Alkylacyl	Alk-1-enylacyl
PC	57.0 ± 1.1	75.6	19.4	4.9	47.8 ± 0.9	87.4	7.0	5.5
PE	16.0 ± 1.1	16.3	25.6	58.1	20.7 ± 0.6	26.6	14.6	58.7
PI	8.3 ± 0.3	89.0	10.5	< 1.0	5.4 ± 0.2	99.0	< 1.0	< 1.0
PS	1.7 ± 0.1	12.3	77.1	10.6	3.0 ± 0.1	40.0	60.0	< 1.0
PA	0.4 ± 0.1	n.d	n.d	n.d	0.5 ± 0.3	99.0	< 1.0	< 1.0
CPL/PG	4.2 ± 0.1	n.d	n.d	n.d	1.1 ± 0.4	n.d	n.d	n.d
SM	5.2 ± 0.3				14.5 ± 0.6			
lyso PC	5.1 ± 0.4				0.5 ± 0.3			
lyso PE	1.6 ± 0.2				1.0 ± 0.2			
X	0.7 ± 0.1				2.5 ± 0.5			
Others	0.1 ± 0.1				1.6 ± 0.2			

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; SM, sphingomyelin; CPL/PG cardiolipin and phosphatidylglycerol; X, unknown. n.d., not determined.

[a]Mean ± S.D of 4–6 determinations.

[b]Mean of 2 determinations.

TABLE II

Molecular species composition of bloodstream form trypanosome diacyl glycerophospholipids

Species	Class		
	PC (%)	PE (%)	PI (%)
18:2 22:5 + *18:2 22:6*[a]	**5.3**	tr	n.d
[18:2 20:4 + 18:1 20:5][b]	**6.2**	0.9	0.7
18:1 22:6	0.6	1.5	0.6
16:0 22:6 + [*18:2 18:2 + 18:1 18:1*][b,a]	**12.3**	**11.3**	1.5
14:0 14:0	tr	n.d	tr
18:1 22:5	3.2	tr	n.d
[18:1 20:4 + 16:0 22:5][b]	3.0	1.4	tr
16:0 20:4 + 18:0 20:5[a]	0.9	1.4	tr
18:0 22:6 + *18:1 18:2 + 18:1 22:4*[a]	**11.3**	**7.7**	**15.0**
16:0 18:2	**8.6**	**17.8**	2.4
16:0 22:4	4.3	2.0	n.d
18:0 22:5	2.0	1.7	**7.5**
18:0 20:4	4.7	3.6	**16.5**
18:0 18:2 + *16:0 18:1*[a]	**23.0**	2.9	**28.1**
18:0 22:4	**6.6**	**36.8**	**18.7**
18:0 18:1	2.9	0.5	**6.4**
16:0 18:0	0.1	3.3	tr
Others	**5.4**	**7.1**	1.2

Molecular species are listed in order of their elution from the HPLC column. Individual peaks were quantitated at the picomole level by measuring absorbance at 230 nm. The numbers represent mean values from 2–3 determinations.
[a]These species co-elute from the HPLC column; MS analysis indicates the presence of both (or all three) species. Italics indicate the less abundant species among the co-eluting group.
[]^{b}, these species co-elute from the HPLC column; MS analysis does not distinguish between the individual species.
tr, trace amounts. n.d, not detected. Major (>5%) components of each class are in bold type.

lipids between the trypanosomes and their environment but also involve regulation of different pathways of lipid synthesis.

Molecular species composition. Analysis by combined HPLC/mass spectrometry of the benzoate derivatives of the different phospholipid subclasses demonstrated the presence of a large variety of individual species. Although comparison of the diacyl species of PC, PE and PI in bloodstream trypanosomes revealed major changes in the relative amounts of individual species between the different classes, we found no striking differences in degree of saturation or overall chain length between the classes (Table II). In contrast, in procyclic trypanosomes the relative amounts of diacyl molecular species with polyunsaturated fatty acyl chains clearly decreased in the order of PC > PE ≫ PI, while the sum of the species, 16:0 18:1 + 18:0 18:2 + 18:0 18:1, increased from 13.8% in PC to 30.4% in PE and 79.3% in PI (Table III). This result is surprising since PI generally contains high amounts of molecular species with polyunsaturated fatty acyl chains, with the predominant constituent usually containing 18:0 20:4 [39,41].

Most interestingly, molecular species analyses of alkylacyl and alk-1-enylacyl subclasses of PC and PE in bloodstream trypanosomes demonstrated the presence of a single species only, i.e., 18:0 18:2 (Table IVA). To the best of our knowledge, this is the first example of an individual molecular species making up an entire phospholipid subclass. On the other hand, in procyclic trypanosomes we detected no such selectivity in the molecular species composition of alkylacyl and alk-1-enylacyl type phospholipid subclasses, although 18:0 18:2 was clearly the predominant species comprising 50–80% of total molecular species in each individual subclass (Table IVB). Interestingly, alkylacyl and alk-1-enylacyl subclasses of both bloodstream and procyclic trypanosomes were completely deficient in long chain (>C-18) polyunsaturated (>three double bonds) acyl and alkyl chains (Table IV).

TABLE III

Molecular species composition of procyclic-form trypanosome diacyl glycerophospholipids

Species	Class		
	PC (%)	PE (%)	PI (%)
[18:2 20:4 + 18:1 20:5][b]	**6.7**	1.6	0.4
18:1 22:6	1.8	0.6	n.d
16:0 22:6	4.7	0.8	n.d
16:1 18:2 + [18:2 18:2 + 18:1 18:3][b,a]	**15.7**	**11.0**	1.8
14:0 14:0	tr	tr	tr
16:0 18:3	tr	tr	n.d
14:0 18:2	n.d	n.d	tr
18:1 22:5	2.1	0.4	n.d
[18:1 20:4 + 16:0 22:5][b]	1.9	0.7	n.d
18:1 18:2 + *16:1 18:1* + *18:0 22:6*[a]	**29.6**	**19.4**	**5.9**
[14:0 18:1 + 16:0 16:1][b]	tr	n.d	tr
18:1 22:4	tr	n.d	n.d
16:0 18:2	**7.1**	**6.1**	4.8
16:0 22:4	1.1	n.d	n.d
14:0 16:0	n.d	n.d	tr
18:0 22:5	n.d	1.0	0.7
18:0 20:4 + *17:0 18:2*[a]	tr	**14.4**	2.0
18:1 18:1	**9.2**	4.8	2.6
16:0 18:1 + 18:0 18:2[a]	**9.5**	**17.5**	**42.2**
18:0 20:3	tr	tr	tr
16:0 16:0	tr	tr	tr
17:0 18:1	tr	tr	0.8
18:0 18:1	**5.8**	**12.9**	**37.1**
16:0 18:0	tr	n.d	tr
Others	4.3	**8.6**	2.1

Legend as in Table II.

TABLE IV

Molecular species composition of bloodstream and procyclic-form trypanosome alkylacyl and enylacyl glycerophospholipids

Species	PC		PE		PI	
	Alkylacyl (%)	Enylacyl (%)	Alkylacyl (%)	Enylacyl (%)	Alkylacyl (%)	Enylacyl (%)
A. Bloodstream form trypanosomes						
18:0 18:2	**100**	**100**	**100**	**100**	n.d	n.d
B. Procyclic form trypanosomes						
[18:2 18:2 + 18:1 18:3][b]	0.5	1.0	n.d	n.d	n.d	n.d
18:1 18:2	0.7	2.6	n.d	0.2	n.d	n.d
[16:0 20:3 + 18:0 18:3][b]	tr	n.d	n.d	n.d	n.d	n.d
16:0 18:2	4.9	**10.8**	1.8	2.4	3.6	**6.2**
17:0 18:2	tr	n.d	n.d	tr	n.d	n.d
18:1 18:1	0.3	0.7	n.d	tr	n.d	n.d
16:0 18:1 + 18:0 18:2[c]	**81.4**	**64.5**	**67.1**	**63.5**	**78.8**	**48.9**
18:0 18:1	**9.4**	**12.4**	**30.6**	**30.4**	**16.7**	**38.3**
18:0 18:0	n.d	n.d	n.d	1.4	n.d	n.d
Other	2.7	**7.8**	0.5	2.1	0.6	**6.6**

[c]The two species co-elute from the HPLC column; MS analyses indicate that the UV peak consists almost exclusively of 18:0 18:2.

For other explanations see Table II.

As a great deal of information has been obtained on the biosynthesis of GPI anchors in *T. brucei*, a discussion of this aspect of lipid metabolism in light of the present data is warranted. In bloodstream trypanosomes, the final products of GPI biosynthesis (GPI anchor precursors P2 and P3 (refs. 13 and 17); protein GPI anchor) [10] contain fatty acids that are not derived directly from the pool of PI. Rather, the glyceride fatty acids are remodelled by the sequential removal of the *sn*-2 fatty acid (to yield an intermediate termed glycolipid θ [19]) replacement with myristate (14:0) from myristoyl-CoA, and then removal and replacement of the *sn*-1 fatty acid [17,19]. This remodelling takes place prior to the addition of the GPI moiety onto VSG [42]. Analysis of glycolipid θ has shown that the original *sn*-1 fatty acid is stearate (18:0) [19]. The identity of the *sn*-2 fatty acid(s) remains unknown. In procyclics, a major product of the GPI-pathway is the PARP membrane anchor. This structure contains stearate at the *sn*-1 position and no substituent at the *sn*-2 position [22,23] and thus resembles the PI moiety of glycolipid θ seen in bloodstream forms. There is no evidence for the addition of a fatty acid to the glyceride of the protein-linked GPI or its precursor by a mechanism analogous to that seen in bloodstream forms [23], but the first step in remodelling probably takes place as the precursor of the PARP anchor, a lyso species (termed PP1 [22]), is derived from a diacyl form [20]. Thus, in bloodstream and procyclic trypanosomes, the starting material for GPI biosynthesis is a species of diacyl-PI that has stearate at its *sn*-1 position. Bloodstream trypanosomes contain only diacyl-PI. Of this more than 90% of the molecular species contain C18:0. In procyclics, stearate is a component in more than 80% of the molecular species of diacyl-PI. Therefore, in both stages, the PI utilised for glycosylation is probably drawn from a major subpopulation of diacyl-PI.

Although we did not determine the positional distribution of the acyl chains in the individual molecular species, it is probable that the more saturated chains, like stearate, predominantly occupy the *sn*-1 position at the glycerol backbone, whereas the more unsaturated chains are at the *sn*-2 position [40]. It will be interesting to determine if the acyl chain at the *sn*-2 position determines the potential precursor for GPI synthesis or if any PI molecular species with stearate at the *sn*-1 position can serve as a GPI precursor.

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