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Lipopeptidophosphoglycan from *Trypanosoma cruzi*

Amide and Ester-Linked Fatty Acids

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Lipopeptidophosphoglycan, extracted from whole cells of epimastigote forms of *Trypanosoma cruzi*, has now been shown to contain 12.6% of fatty acids in addition to the previously identified content of neutral sugars (60%), glucosamine (0.8%), peptide (9.5%) and acid-hydrolyzable phosphate (2%). The main fatty acids are palmitic (6.9%) and lignoceric (4.6%) acids. Stearic (0.55%), oleic (0.15%) and myristic (0.18%) acids were also found. One third of the fatty acids are bound in the lipopeptidophosphoglycan as esters (14 mmol%) and two thirds as amides (28 mmol%). Lignoceric acid was found to be bound only as amide. Two ninhydrin-positive compounds, obtained by chloroform extraction of a total acid hydrolysate of the lipopeptidophosphoglycan, were tentatively identified as sphingosine bases.

We have recently described the purification and properties of a lipopeptidophosphoglycan from epimastigote forms of *T. cruzi*. This was the first example of such a complex carbohydrate isolated from a trypanosomatid. The inhibition of concanavalin-A-induced agglutination of epimastigotes [1] by low concentrations of lipopeptidophosphoglycan suggested a membrane location for the compound [2].

The following composition was reported for the lipopeptidophosphoglycan: 60% neutral sugars (mannose/galactose/glucose, mol ratio 35/22/1); 0.8% glucosamine; 9.5% protein and 2% phosphorus. Fatty acids were detected in a hexane extract after acid hydrolysis.

This paper describes the fatty acid composition of the lipopeptidophosphoglycan as well as the nature of their linkages in the macromolecule.

MATERIALS AND METHODS

Extraction of Lipopeptidophosphoglycan from T. cruzi

Lipopeptidophosphoglycan was purified by a modification of the described method [2]. Epimastigote forms were cultivated and extracted with phenol [3]. A carbohydrate complex was precipitated from the aqueous phase with 4 volumes of ethanol. After centrifugation the residue was resuspended in water, extracted with ether and reprecipitated with ethanol. 1.5 g of the dry precipitate were extracted with 90 ml of chloroform/methanol (2/1) to remove free lipids and the insoluble residue was twice extracted (1 g in 200 ml) with chloroform/methanol/water (10/10/3). The residue was removed by centrifugation and filtration. The lipopeptidophosphoglycan was precipitated from the clear solution by the addition of an equal volume of methanol. After standing overnight at − 20 °C the substance was collected by centrifugation. From 1.5 g of carbohydrate complex 61 mg of electrophoretically pure lipopeptidophosphoglycan was isolated.

Analytical Methods

Polyacrylamide gel electrophoresis was performed in 15% gels in the presence of sodium dodecylsulfate as described [4]. The gels were stained for carbohydrate with periodic acid-Schiff [3].

Determination of Fatty Acids

All solvents were redistilled before use. Fatty acid standards were obtained from Applied Sciences and Sigma. Methyl esters were prepared in two ways: (a) treatment with 14% (w/v) boron trifluoride in methanol for 2 min at 100 °C [5]; (b) heating for 3 h with methanol containing 1% sulfuric acid. The methyl esters were qualitatively and quantitatively analysed by gas-liquid chromatography with a Varian (model 1800) gas chromatograph equipped with a flame ionization detector. Heptadecanoic acid was used as internal standard. Peak area measurements were made with a Varian (model 480) electronic integrator. A 0.3 × 180-cm column packed with 3% SE-52 (Varian)

on 100–120 mesh Varaport 30 was used at 180 °C and 220 °C. Nitrogen was used as carrier gas.

Thin-layer chromatography was performed on 250-μm silica gel H plates (Merck) with light petroleum/diethyl ether/acetic acid (95/5/0.5) as solvent. Bands were detected with iodine vapor. After iodine evaporation the fatty acid methyl esters were recovered by elution with 5 ml of ethyl ether (3×) followed by 5 ml of chloroform/methanol (2/1).

Mass spectra were measured with a Varian Gas Micromass MM 12 F mass spectrometer at 70 eV, using direct inlet.

Hydroxylaminolysis

The lipopeptidophosphoglycan (10 mg) was treated with 2 ml of the alkaline hydroxylamine reagent prepared as described by Snyder and Stephens [6]. After heating for 5 min at 65 °C the suspension was centrifuged and half of the supernatant was analysed for hydroxamates with ferric perchlorate [6]. Tripalmitin was used as standard. The other half of the supernatant was hydrolysed by heating in 4 M HCl at 100 °C for 4 h.

To release the amide-bound fatty acids the de-*O*-acylated sediment was hydrolysed with 2 ml of 4 M HCl at 100 °C for 4 h.

The free fatty acids released from the supernatant and the sediment were twice extracted with hexane followed by ether. The solvents were evaporated under nitrogen and the fatty acids were analysed by gas-liquid chromatography after esterification by method (a). A quantitation of the fatty acids obtained from the sediment was carried out by the hydroxamate method.

Determination of Total Fatty Acids

The lipopeptidophosphoglycan (6 mg) was hydrolysed with 2 ml of 4 M HCl at 100 °C for 5 h and the hydrolysate was extracted and esterified by method (b). Total esters were quantitatively determined as hydroxamates and analysed by gas-liquid chromatography.

Methanolysis

The lipopeptidophosphoglycan (20 mg) was treated with 0.25 M KOH in methanol (7 ml) at 37 °C for 16 h. The insoluble residue was separated by centrifugation, washed with methanol and hydrolysed with 4 M HCl for 4 h at 100 °C. The fatty acids were extracted with hexane and esterified for gas-liquid chromatography.

The supernatant was acidified, extracted with hexane and the methyl esters analysed by gas-liquid chromatography.

Hydrogenation

Samples of methyl ester fatty acids were hydrogenated by bubbling hydrogen through the ethanol/hexane (2/1) solution in the presence of palladium on charcoal (5%). After 2 h the reaction mixture was centrifuged and the supernatant evaporated under nitrogen.

RESULTS

Isolation of the Lipopeptidophosphoglycan

The method previously reported for the purification of the lipopeptidophosphoglycan has been simplified by the omission of the gel filtration step [2]. The solubility of the compound in chloroform/methanol/water (10/10/3) allowed the complete separation of the lipopeptidophosphoglycan from the other three components of the glycoprotein complex (Fig. 1). The lipopeptidophosphoglycan could be recovered from the solution by precipitation with methanol. Although the compound could be directly extracted from a $105000 \times g$ cell particulate with the organic solvent, this method yielded a material that was contaminated by lipids (detected by thin-layer chromatography) rendering if difficult to purify the lipopeptidophosphoglycan further. In contrast, the compound isolated from the complex obtained by 44% phenol extraction of broken cells followed by ether extraction of the water phase, showed only one spot at the origin in thin-layer chromatograms run in several solvent systems commonly used for lipid identification.

Fatty Acid Composition of the Lipopeptidophosphoglycan

In order to determine the way fatty acids are bound in the lipopeptidophosphoglycan the ester-linked fatty acids were released by two methods of alkaline cleavage: hydroxylaminolysis and methanolysis, respectively.

The amount of fatty acids released as hydroxamates, as measured by the ferric perchlorate method [6], was found to be 14 mmol/100 g lipopeptidophosphoglycan. The de-*O*-acylated samples were then hydrolysed with acid in order to determine the amide-linked acids. After transformation of the latter in the hydroxamate derivatives a value of 28 mmol/100 g lipopeptidophosphoglycan was found. Total fatty acids, released from lipopeptidophosphoglycan by acid hydrolysis, gave a value of 43.3 mmol/100 g.

The amount of fatty acids released by methanolysis (Table 1) was calculated from the peak areas obtained by gas-liquid chromatography analysis. The total (14.2 mmol %) is in good agreement with the

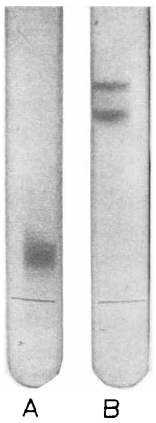

Fig. 1. *Sodium dodecylsulfate/polyacrylamide gel electrophoresis of the glycoprotein complex fractions from* T. cruzi *(cf. [2,3])*. The ethanol-precipitated complex was extracted with chloroform/methanol/water (10/10/3) after a previous extraction with chloroform/methanol (2/1) as described in Materials and Methods. Staining was performed with periodic acid-Schiff. (A) Lipopeptidophosphoglycan, soluble in chloroform/methanol/water; (B) insoluble in chloroform/methanol/water

Table 1. *Fatty acid composition of the lipopeptidophosphoglycan*
The amounts of the individual fatty acids in the first three columns are given as percentages of the total. Each figure is the mean of three independent determinations. The amounts released by methanolysis are given in percentage of the lipopeptidophosphoglycan (mmol %). tr means fatty acids detected in less than 1% from total

Fatty acid	Total	Ester (NH_2OH)	Amide	Ester (CH_3ONa)
	%			mmol %
$C_{14:0}$	1.4	7.5	0	1.2
Unknown	tr	1.2	0	tr
$C_{16:0}$	55.5	81.0	44.0	11.5
$C_{17:0}$	tr	tr	tr	tr
$C_{18:0}$	4.4	6.8	7.0	1.0
$C_{18:1}$	1.2	3.5	tr	0.5
$C_{24:0}$	37.0	0	49.0	0

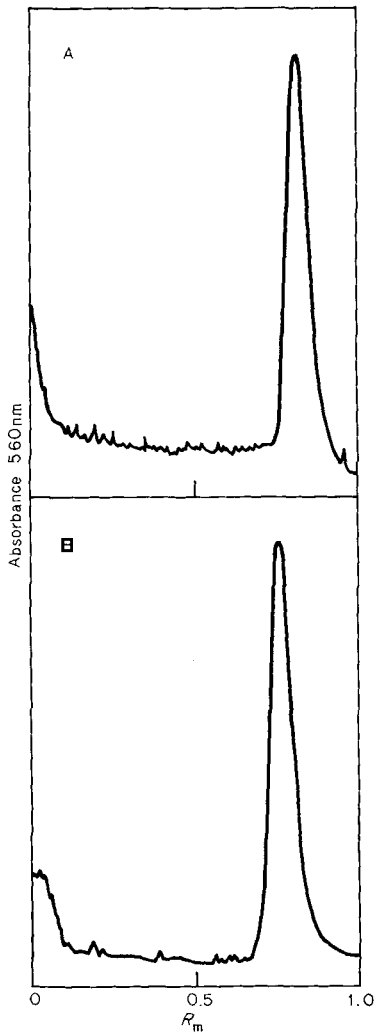

Fig. 2. *Spectrophotometric profiles of pure lipopeptidophosphoglycan (A) and de-O-acylated lipopeptidophosphoglycan (B) after sodium dodecylsulfate/polyacrylamide gel electrophoresis.* Scanning was performed at 560 nm after staining with the periodic acid-Schiff reagent

above value for the ester-linked acids liberated as hydroxyamates.

These results show that one third of the fatty acids were ester linked, the remaining being bound as amides.

Identification of the Ester and Amide-Linked Fatty Acids

Total fatty acids were identified as the methyl ester derivatives by gas-liquid chromatography following acid hydrolysis of the lipopeptidophosphoglycan (Table 1). Approximately 92% of the fatty acids present were accounted for by palmitic acid (55.5%) and lignoceric acid (37%). Smaller amounts of stearic (4.4%), oleic (1.2%) and myristic (1.4%) acids were also present. The acids were identified by cochromatography with authentic reference compounds. Due to its higher retention time (25 min approximately at 220 °C) lignoceric acid appeared as a broad peak. Therefore its identity was further confirmed by mass spectrum. The results, showing a molecular ion $(M)^+$ of *m/e* 382 and major fragments at *m/e* 74 and 87 (which rule out branching in α and β positions of the ester), were identical to those obtained with an authentic sample of methyl tetracosanoate. Analysis of the fatty acid methyl esters after catalytic hydrogenation of the sample showed the only disappearance of the peak corresponding to oleic acid with the concomitant increase of the stearic acid peak.

The ester-linked fatty acids were identified after hydrolysis of the hydroxamates. Palmitic acid accounted for 81% of the mixture (Table 1). The same acids

were released by methanolysis. The residue obtained by the latter procedure showed a greater water solubility. Sodium dodecylsulfate/polyacrylamide gel electrophoresis of the residue showed only one band with a slightly lower mobility than the lipopeptidophosphoglycan ($R_m = 0.79$ and 0.83, respectively) (Fig. 2). This behaviour can be explained by the fact that although the molecular size of the de-*O*-acylated lipopeptidophosphoglycan is lower than that of the original compound, the removal of fatty acids would have an opposite effect on its mobility. Jann *et al.* [7] have found a linear relationship between the mobility and the lipid A content of bacterial lipopolysaccharides.

The amide-bound fatty acids were identified and quantitated after hydrolysis of the de-*O*-acylated lipopeptidophosphoglycan. Lignoceric acid was found to be the main component (49%). These results show that lignoceric acid is all in amide linkages (Table 1).

A chloroform extract from a total acid hydrolysate was esterified and separated in three bands by thin-layer chromatography (band I at the origin, band II $R_f = 0.1$ and band III $R_f = 0.65$). The methyl esters eluted from band III were the same as in the original sample as identified by gas-liquid chromatography. No significant peaks could be detected in the material eluted from bands I and II by gas-liquid chromatography in 3% SE-52 at 180 °C or 220 °C.

The material from band I gave two ninhydrin-positive spots with R_f similar to those obtained from a sphingomyelin hydrolysate when chromatographed in thin-layer plates with chloroform/methanol/2 M NH_4OH (40/10/1), a solvent recommended for the separation of sphingosines [8]. Hydrolysis of the lipopeptidophosphoglycan with 6 M HCl, followed by an extraction procedure indicated for the analysis of sphingosines [9], yielded the same two ninhydrin-positive spots. Furthermore, formaldehyde was detected by the chromotropic acid reaction [10] when the extract was subjected to periodate oxidation. As sphingosine bases with similar structures but different chain lengths may have similar mobilities, and since acid hydrolysis may ensue isomer formation [8], further work is in progress to establish the structure of this component of the lipopeptidophosphoglycan.

DISCUSSION

The results shown in this paper further contribute to the identification of the components of lipopeptidophosphoglycan, a complex macromolecule isolated from epimastigote forms of *T. cruzi*. Palmitic acid was found to be the main fatty acid component. Von Brand also found palmitic acid as the main fatty acid in glyceride and phospholipid fractions from *T. cruzi* and that stearic acid was only poorly represented [11]. To our knowledge, lignoceric acid was never reported as a component of *T. cruzi* lipids.

From the results it was possible to calculate that 12.6% of the lipopeptidophosphoglycan is represented by fatty acids distributed as follows: 6.9% C_{16}, 4.6% C_{24}, 0.7% C_{18}, and 0.18% C_{14}. This value taken together with those reported for the other components of lipopeptidophosphoglycan [2] account for approximately 86% of the molecule.

One third of the fatty acids in the lipopeptidophosphoglycan are bound as esters and two thirds as amides. These two types of linkages were also found in lipopolysaccharides of *Salmonella* [12], in the lipoprotein linked to murein from *Escherichia coli* outer membrane [13] and in the lipophosphonoglycan of the plasma membrane of *Acanthamoeba castellanii* [14].

Considering that 0.8% (4.4 mmol %) of the lipopeptidophosphoglycan is represented by glucosamine [2], the molar ratio of amide-linked fatty acids to this amino sugar is 6.3. This means that even if part of the acids were to be linked as amide to glucosamine as in lipopolysaccharides [12] the major part must be linked to other amino-containing components of the lipopeptidophosphoglycan. Probably sphingosine is involved in the linkage taking into consideration that lignoceric acid, the main amide-linked fatty acid in the molecule, is a known component of ceramide moieties. Further work is in progress to elucidate this possibility.

While this manuscript was in preparation the presence of two phytosphingosine bases in a lipophosphonoglycan from the plasma membrane of *A. castellanii* was reported [14]. This feature and other similarities in the chemical composition of both complex carbohydrates suggest that they might play similar functions in both protozoa.

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