The Phospholipase A₁ of *Trypanosoma brucei* Does Not Release Myristate from the Variant Surface Glycoprotein

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[3H]Myristoyl-labeled variant surface glycoprotein (VSG) has been isolated from *Trypanosoma brucei* by reverse phase high performance liquid chromatography and used as substrate for the conversion by trypanosomal enzymes of membrane-form VSG to soluble VSG. Conversion is detected by the release of myristoyl-containing lipids. The major lipolytic enzyme of *T. brucei*, phospholipase A₁, is effective for the hydrolysis of myristoyl esters of p-nitrophenol, in a colorimetric assay. However, the phospholipase is unable to cleave the myristoyl ester linkage of VSG. The phospholipase can be separated from the myristoyl-releasing activity of trypanosome homogenate by centrifugation, affinity chromatography, and anion-exchange chromatography. Elution profiles on anion-exchange high performance liquid chromatography also indicate that the phospholipase is inactive against VSG. A small amount of myristoyl-releasing activity associated with the purified phospholipase is probably due to contamination with a phosphodiesterase which releases myristoyl-containing diglyceride from VSG.

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African trypanosomes are the protozoan parasites responsible for causing African sleeping sickness and related animal diseases. The species *Trypanosoma brucei* includes trypanosomes which infect livestock in Africa as well as the human pathogens, *T. gambiense* and *T. rhodesiense*. In common with other salivarian trypanosomes, *T. brucei* has a tightly packed surface coat of a single glycoprotein or VSG which is periodically replaced by a different glycoprotein in a process known as antigenic variation. This appears to be an effective method of evading the immune response of the mammalian host. Upon lysis of trypanosomes, the VSG is rapidly released from the plasma membrane in a soluble form. This is due to the removal of a hydrophobic "anchor" from the membrane-form VSG by a VSG-releasing enzyme present in the trypanosomes (1). The hydrophobic anchor contains myristic acid, ester-linked to the protein via a complex containing ethanolamine, sugars, and phosphate (2). The major VSG-releasing enzyme is a phosphodiesterase, which acts on membrane form VSG to produce sn-1,2-dimyristoyl glycerol and soluble VSG (3, 4). However, a phospholipase A₁ is also present in *T. brucei* (5, 6) and appears to be mainly cytoplasmic although present also in a vesicular fraction (7). This enzyme is very active against phosphatidylcholine but, like other phospholipases A₁, has a broad specificity including lysophospholipids (8) and other esters such as fatty acyl esters of p-nitrophenol. To investigate the possible role of this enzyme in decylation of mVSG, we have examined the products of hydrolysis of mVSG by trypanosomal phospholipase A₁ preparations. In the process, we developed an efficient method of preparing mVSG by HPLC, and a rapid colorimetric assay for trypanosomal phospholipase A₁.

**MATERIALS AND METHODS**

[9,10-3H]Myristic acid was obtained from New England Nuclear. L-p-phenylphosphorylcholine di(1-14C)oleoyl was obtained from Amersham. Acetonitrile, HPLC grade, was obtained from Caledon Laboratories Ltd., Georgetown, Ontario. Acrylamide was from Bethesda Research Laboratories. All other chemicals were from Sigma.

**SDFS-PAGE**—Gel electrophoresis was carried out in 12% gels 0.7 mm thick by the method of Laemmli (9). Gels were fixed and stained with Coomassie Blue following the method of Steck et al. (10). Fluorography of stained gels was done using EN3HANCE (New England Nuclear).

**Preparation of [*H]Myristoyl-labeled Variant Surface Glycoprotein**—Trypanosomes (*T. brucei*, Shinyanga III, maintained by syringe passage in rats for several years) were isolated from infected rat blood by the method of Lasham and Godfrey (11) and labeled with [*H]myristate by the method of Ferguson and Cross (2). After labeling for 1 h, the trypanosomes (5 × 10⁸) were collected by centrifugation at 3000 × g for 4 min, washed once with RPMI 1640 medium, and extracted with 1.5 ml of 20% acetonitrile in 0.1% trifluoroacetic acid. Insoluble material was removed by centrifugation at 8000 × g for 4 min followed by filtration through a 0.45-μm filter (Millipore, Milipore). The extract was subjected to reversed-phase HPLC on a Bio-Gel TSK, Phenyl 5-PW column, 5 μl 7.5 mm (Bio-Rad) (Fig. 1). A gradient of 20-50% acetonitrile in 0.1% trifluoroacetic acid was applied over 20 min at a flow rate of 1.0 ml min⁻¹. Fractions of 0.5 ml were collected and samples of 16 μl from each fraction were assayed by liquid scintillation counting in 5 ml of a phase-combining scintillator (12). Samples were also removed for analysis by SDS-PAGE. Fractions corresponding to the first major protein peak (A₃₀₅), which coincided with the only *H* peak before elution of lipid, were combined. The preparation was almost pure VSG by SDS-PAGE, containing 1-2.5 × 10⁶ cpm. Myristylation was confirmed by release of [*H]*myristate by treatment of mVSG with alkali and by release of 1.2-[*H*]diglyceride from mVSG by phospholipase C (see Table II). Fluorography of the acrylamide gel located the *H* in the VSG band and showed no labeled contaminants (Fig. 2).

**Assay for Phospholipase A₁**—Two assay methods were used; one used radiolabeled phosphatidylcholine di(1-14C)oleoyl as substrate and has been described previously (13). The other assay used p-nitrophenyl palmitate as substrate and, although less specific, gave results far more rapidly. It was used for screening large numbers of fractions. The major soluble trypanosome enzyme hydrolyzing this substrate was the phospholipase A₁, which hydrolyzed p-nitrophenyl...
palmitate at about 1% of the rate for phosphatidylcholine. Fatty acyl esters of p-nitrophenol with a variety of acyl chain lengths were tested as substrates in this assay. Spontaneous hydrolysis was found to be excessive with chain length less than 6. In addition, nonspecific hydrolysis was catalyzed by a variety of common substances including amines, azide, and free amino or phenolic groups of proteins (14) and this made the shorter chain substrates of little value. Specificity for phospholipase A₁ was greatest for the C-12-C-18 substrates, but solubility problems made the C-18 substrate difficult to use.

p-Nitrophenyl palmitate (10⁻⁶ mol) was dissolved in 0.5 ml of methanol with warming to 40 °C and stirring (too much heat causes the substrate to undergo non-enzymic hydrolysis). To this methanolic solution was rapidly added a mixture of 0.5 ml of 10% Triton X-100, 0.5 ml of 20 mM EDTA, 20 mM dithiothreitol, and 3.5 ml of 0.1 M sodium phosphate buffer, pH 7.5. This pH was chosen to minimize non-enzymic hydrolysis of the substrate while giving sufficient sensitivity in a rapid assay. If this solution was warm (40 °C) before adding to the methanolic p-nitrophenyl palmitate, a clear solution was obtained. This substrate was mixed with an equal volume of enzyme solution and incubated either at room temperature or at 37 °C for 0.5-24 h before determining the absorbance at 405 nm. Usually this incubation was performed in a 0.1-ml volume in a 96-well microtiter plate, absorbance being determined with a Titrtek Multiscan enzyme-linked immunosorbent assay reader.

**Assay For Release of Myristoyl-containing Lipids from [³H]Myristoyl-labeled VSG—**The procedure is similar to that for the phospholipase A₁ assay (15). The incubation mixture contained [³H]VSG (approximately 1 μg, 10,000 cpm), EDTA (1 mM), dithiothreitol (1 mM), bovine serum albumin (1 mg ml⁻¹), Triton X-100 (1 mg ml⁻¹), sodium phosphate buffer, pH 7.5 (50-100 mM) and enzyme in a total volume of 100 μl. Incubation was at 37 °C for 20 min. The reaction was stopped by adding 50 μl of 20% trichloroacetic acid and 900 μl of hexane:ethanol (5:4), vortexed, and centrifuged at 8,000 g, 30 min. A 200-μl sample of the upper phase was assayed for tritium by liquid scintillation counting. The reaction was first order with respect to enzyme concentration up to 20% hydrolysis of substrate.

**Partial Purification of Phospholipase A₁—** This was accomplished by the method described previously (6) using phosphatidylcholine-Sepharose and DEAE-cellulose column chromatography.

**RESULTS**

**Preparation of [³H]Myristoyl-labeled Variant Surface Glycoprotein—** Ferguson and Cross (2) were the first to describe a method for the preparation of mfVSG in which they isolated the protein from a boiled detergent digest of T. brucei with a concanavalin A-Sepharose column. This method is, however, difficult to apply to small amounts of material, particularly the solvent precipitation and washing steps which remove contaminating lipids. Clarke et al. (15) showed the feasibility of using reverse phase HPLC to purify soluble form VSG from trypanosome lysates. We modified this technique to allow purification of mfVSG. To prevent conversion of mfVSG to soluble form VSG with loss of myristate, trypanosomes were extracted directly with the acidic starting solvent for HPLC. Little VSG was extracted by 0.1% trifluoroacetic acid, but if the extraction medium also contained 20-50% acetonitrile, then approximately 80% of the total trypanosomal proteins were solubilized. Use of 20% acetonitrile in 0.1% trifluoroacetic acid allowed direct application of the extract to a reverse phase column and elution with an increasing concentration of acetonitrile (Fig. 1). The mfVSG was the first major protein peak to elute, being identified both by SDS-PAGE, and by [³H] label when [³H]myristic acid-labeled trypanosomes were used. No other protein peak was labeled. The yield of mfVSG as a percentage of the initial total protein was approximately 8%, based on the Bradford method (16). The apparent molecular mass of 66 kDa (Fig. 2) is higher than that previously reported for other T. brucei strains (2, 17). Membrane-form VSG prepared from our strain (Shinyanga III, by the method of Ferguson and Cross (2)) also gave an apparent molecular mass of 66 kDa by SDS-PAGE.

**Separation of VSG-releasing Enzyme from Phospholipase A₁—** Phospholipase A₁ was partially purified from trypanosome homogenate as described previously (6). The levels of VSG-releasing enzyme at each step were measured by the ability of the fractions to release hexane-soluble myristic acid-containing residues from VSG labeled with [³H]myristate. The assays throughout this experiment were done with a single preparation of labeled VSG and so are comparable. The
results (Table I) show that the phospholipase and myristoyl-releasing activities were separated during the purification, with the phospholipase activity being almost completely soluble and the myristoyl-releasing activity being mostly membrane-bound. As the purification progressed, the ratio of phospholipase A₁ activity to myristoyl-releasing activity increased until it was 27,000 times that of the homogenate. The phospholipase A₁ apparently has relatively little ability to release myristic acid from mVSG. The small amount (0.5%) of phospholipase A₁ activity which may be retained by the column is probably some of the membrane-bound enzyme which is either in a natural soluble form, or solubilized by the homogenization procedure.

Ion-Exchange Chromatography of Trypanosome Supernatant—An attempt was made to separate phospholipase A₁ activity from soluble VSG-releasing enzyme by chromatography of trypanosome supernatant on a Pharmacia Mono-Q HPLC anion exchange column. Much of the VSG-releasing activity was not retained by the column. The remainder came off in a broad peak trailing into the phospholipase A₁ peak (Fig. 3). The lack of a subsidiary peak of VSG-releasing enzyme corresponding to the phospholipase A₁ peak confirms that the phospholipase A₁ has little activity against mVSG, at least under the assay conditions used.

Identification of the Lipid Products of Degradation of mVSG—[3H]Myristoyl-labeled VSG was incubated with three separate trypanosome enzyme fractions: partially purified phospholipase A₁ (307 μmol min⁻¹ mg⁻¹), the unretarded fraction during the DEAE-cellulose step of phospholipase purification, and the 150,000 × g pellet from trypanosome homogenate (washed twice and resuspended in 10 mM phosphate, pH 7.5, containing bovine serum albumin (1 mg ml⁻¹)). This membrane preparation catalyzed hydrolysis of the VSG to give a labeled product which co-migrated with 1,2-diglyceride during thin layer chromatography on silica gel in two different solvent systems: petroleum ether-diethyl ether-acetic acid, 70:30:2. Spots were visualized with iodine vapor, scraped into scintillation vials, mixed with 0.5 ml of water and counted in 5 ml of Anderson's scintillator (12).

When larger amounts of membranes were used, less diglyceride was obtained while significant amounts of labeled free fatty acid were obtained (Table II). This free fatty acid is probably the result of hydrolysis of diglyceride by low levels of lipase (or perhaps phospholipase A₁) present in the membrane preparation.

The soluble enzyme fraction unretarded by DEAE-cellulose also released diglyceride from VSG, but in this case no free fatty acid was observed. Since this fraction contains no detectable phospholipase A₁, this result is consistent with the

**TABLE I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1,2-Diglyceride</th>
<th>1,3-Diglyceride</th>
<th>Free fatty acid</th>
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</thead>
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<tr>
<td>Control</td>
<td>6.0</td>
<td>0.6</td>
<td>4.5</td>
</tr>
<tr>
<td>NaOH (0.5 M)</td>
<td>1.2</td>
<td>0.6</td>
<td>98.3</td>
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<td>Phospholipase C (60 units)</td>
<td>69.5</td>
<td>1.6</td>
<td>5.7</td>
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<tr>
<td>Membranes (60 μg protein)</td>
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<td>1.9</td>
<td>6.6</td>
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<tr>
<td>Membranes (300 μg protein)</td>
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<td>10.1</td>
<td>19.5</td>
</tr>
<tr>
<td>S (2.9 μg protein)</td>
<td>46.2</td>
<td>1.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Phospholipase A₁ (29 μg)</td>
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<td>0.5</td>
<td>23.9</td>
</tr>
</tbody>
</table>

*The protein fraction from trypanosomes which was not retained by DEAE-cellulose in 10 mM Tris, pH 8.0, during purification of phospholipase A₁ according to Ref. 6.*

**TABLE II**

**Products of hydrolysis of [3H]myristoyl-labeled VSG under different conditions**

1.4 μg of [3H]myristoyl-labeled VSG (8000 cpm) was incubated with the enzyme preparations listed below for 20 min at 37 °C in a volume of 100 μl. Reaction was in 0.1 M sodium phosphate, pH 7.0, with 0.1% Triton X-100, 1 mM EDTA, and 1 mM dithiothreitol except for the phospholipase C (Bacillus cereus) incubation which was in 0.1 M Tris-HCl, pH 7.2, with 0.5 M CaCl₂. Reaction was terminated by adding 50 μl of 2-butanol containing 2.5 μg of trichloroacetic acid, 3 μg of oleic acid, 14 μg of 1-palmitoyl 2-oleoyl diglyceride, and 18 μg of 1,3-diolein. After vortexing, the butanol phase was spotted on a Whatman K₅ silica gel thin layer plate and developed in petroleum ether-diethyl ether-acetic acid, 70:30:2. Spots were visualized with iodine vapor, scraped into scintillation vials, mixed with 0.5 ml of water and counted in 5 ml of Anderson's scintillator (12).
suggestion that phospholipase A₁ is responsible for converting diglyceride to free fatty acid when using the membrane fraction.

The phospholipase A₁ preparation was also able to degrade mVSG, but in this case the labeled product was almost entirely free fatty acid. This could be due to direct action of phospholipase A₁ on mVSG, or alternatively to production of diglyceride from VSG by a small amount of contaminating membrane enzyme, followed by conversion of diglyceride to free fatty acid by phospholipase A₁, which is present in vast excess although its activity against diglyceride is unknown.

**DISCUSSION**

These studies have clearly shown that phospholipase A₁ is distinct from the major VSG-releasing enzyme of *T. brucei*. The phospholipase A₁ is soluble, whereas the VSG-releasing enzyme activity (measured as myristoyl-releasing activity) is mostly sedimented by 6 × 10⁶ g·min. During each step in the purification of phospholipase A₁, the amount of VSG-releasing enzyme decreases significantly. The partially purified phospholipase A₁ has an activity against VSG of 0.013 arbitrary units/mg, which is approximately 0.009 nmol min⁻¹ mg⁻¹ under the conditions used. The figure for phosphatidylcholine is higher by a factor of 15 × 10⁶. It seems likely that the small activity against VSG is due to contamination of the phospholipase preparation with another enzyme rather than an intrinsic property of the phospholipase itself. Ferguson et al. (3) found mVSG to be resistant to hydrolysis by pancreatic lipase, another enzyme with phospholipase A₁ activity; although snake venom (*Crotalus atrox*) phospholipase A₁ and phospholipase C (*Bacillus cereus*) did release myristate or myristoyl-containing lipids, the structure of the terminal glycolipid, or steric hindrance by the VSG protein itself, apparently prevents some phospholipases from acting on the mVSG. Unless the trypanosomal phospholipase A₁ functions to hydrolyze diglyceride released from VSG by the membrane-bound enzyme, it would not appear to be involved in the VSG release mechanism.

As reported previously (3) most of the VSG-releasing enzyme was membrane-bound, but a small amount appeared to be soluble. This fraction, like the membrane-bound enzyme, released diglyceride from VSG. It probably represents a portion of the membrane-bound enzyme which was solubilized during sonication, but this speculation should be tested by experiment.

The rapid method we have described for isolation of mfVSG by HPLC has advantages over some previous methods. Because of the presence of the VSG-cleaving enzyme (a phosphodiesterase) which is activated on disruption of trypanosomes, mfVSG can be isolated only if this enzyme is immediately inactivated. This has been done previously by boiling in detergent (2) or by disrupting trypanosomes in the presence of Zn²⁺ ions (17). The latter method leaves the VSG resistant to hydrolysis by the phosphodiesterase and so is of no value for enzymic studies. The present method of inactivation with the starting solvent for reversed-phase HPLC is convenient and allows for rapid isolation of small amounts of enzyme-sensitive mVSG. Another potentially useful and rapid method has recently been proposed based on a different solvent extraction system (4).

**REFERENCES**