A Phospholipase C from *Trypanosoma brucei* which Selectively Cleaves the Glycolipid on the Variant Surface Glycoprotein*

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The surface coat of *Trypanosoma brucei* is composed of 10⁷ molecules of the variant surface glycoprotein (VSG). Each VSG molecule is tethered to the cell membrane by a glycolipid moiety which contains 1,2-dimyristoyl-sn-phosphatidylglycerol (Ferguson, M. A. J., Low, M. G., and Cross, G. A. M. (1985) *J. Biol. Chem.* 260, 14547–14555). Following cell lysis, an endogenous phospholipase C cleaves dimyristoyl glycerol from the glycolipid, releasing soluble VSG.

We have purified this enzyme, which we designate VSG lipase, by detergent extraction, (NH₄)₂SO₄ fractionation, hydrophobic chromatography, and cation exchange chromatography. It is purified 2600-fold and is virtually homogeneous. Based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the apparent molecular mass is 37 kDa. In solutions containing the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), the Stokes radius (2.6 nm), s₂₀,w (3.7 S), and v (0.77 cm²/g) of VSG lipase suggest a molecular mass for the native enzyme of about 47 kDa, part of which may be due to bound VSG. Each VSG molecule is tethered to the cell membrane and the mechanisms by which it can be released. It was clear that the VSG binds tightly to the surface of the living trypanosome, yet after cell lysis it is released rapidly as a soluble protein which has no apparent affinity for membranes. Cardoso de Almeida and Turner (6) resolved this paradox when they discovered that boiling trypanosomes in SDS allows isolation of an amphiphilic or membrane form of VSG (mfVSG). In contrast, lysis under nondenaturing conditions causes release of a hydrophilic or soluble form of VSG (sVSG) which lacks a hydrophobic moiety present in the C-terminal region of mfVSG. Cardoso de Almeida and Turner presented evidence that conversion of mfVSG to sVSG is enzyme catalyzed.

Recent structural studies in several laboratories have revealed that mfVSG contains a glycolipid covalently linked to its C terminus which anchors the protein to the plasma membrane. This glycolipid is composed of myristic acid (7), glycerol (8, 9), phosphate (6, 10), inositol (11), several sugars (12–14), and ethanolamine (15). Part of the glycolipid is in the form of 1,2-dimyristoyl-sn-phosphatidylglycerol which is glycosidically bonded to non-acetylated glucosamine (11, 12). Consequently, nitrous acid treatment liberates dimyristoyl phosphatidylglycerol (11). The structure of the C-terminus of the moiety, which contains the sugars and ethanolamine, is not yet known. The glycolipid is linked to the VSG polypeptide through an amide bond between the ethanolamine and the α-carboxyl group of the C-terminal amino acid (15).

Conversion of mfVSG to sVSG during cell lysis involves cleavage of a phosphodiester bond and release of 1,2-dimyristoyl-sn-glycerol from the glycolipid moiety (9, 16). Therefore, the enzyme involved in this reaction has the specificity of a phospholipase C.

Little is known about this phospholipase. It is present in a particulate fraction of lysed cells (17), and it seems to be inhibited by mercurial compounds and Zn²⁺ (1, 6, 18–20). The enzyme activity is absent in cultured trypanosomes which resemble the coatless procyclic forms found in the gut of the tsetse fly vector (1, 18).

The biological role of the enzyme is not yet known, but Turner and co-workers have discussed several possibilities (1). The most exciting are: (a) the enzyme removes the surface coat when the parasite enters the gut of the tsetse, (b) it participates in antigenic variation by disposing of the old surface coat. In any event, it would seem likely that the enzyme is subject to regulation. It is relatively quiescent in living bloodstream trypanosomes as there appears to be little, if any, VSG release (21). However, the enzyme is present in a latent form as lysis of the cell results in conversion of all of

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*Trypanosoma brucei*, an African trypanosome, has a surface coat composed of about 10⁷ molecules of a single variant surface glycoprotein, or VSG (1). This parasite evades its host's immune response by antigenic variation. During this process, the trypanosome changes its surface coat to a new one composed of a VSG with a different amino acid sequence (reviewed in 2–5).

Early studies on VSG raised questions about the way this molecule is bound to the cell surface and the mechanisms by which it can be released. It was clear that the VSG binds tightly to the surface of the living trypanosome, yet after cell lysis it is released rapidly as a soluble protein which has no apparent affinity for membranes. Cardoso de Almeida and Turner (6) resolved this paradox when they discovered that boiling trypanosomes in SDS allows isolation of an amphiphilic or membrane form of VSG (mfVSG). In contrast, lysis under nondenaturing conditions causes release of a hydrophilic or soluble form of VSG (sVSG) which lacks a hydrophobic moiety present in the C-terminal region of mfVSG. Cardoso de Almeida and Turner presented evidence that conversion of mfVSG to sVSG is enzyme catalyzed.

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Trypanosomal VSG Lipase

its mVSG to sVSG in a few minutes (22).

To understand the biological role and possible regulation of this enzyme, which we designate VSG lipase, it is essential to obtain it in pure form. In this paper we describe the purification of VSG lipase and some preliminary characterization of its biochemical properties.

MATERIALS AND METHODS

Trypanosomes—T. brucei (ITAT 1.3; from R. O. Williams) were isolated from the blood of Swiss mice or Wistar rats (parasitemia about 10^9/ml) by DEAE-cellulose chromatography (23) using 50 mM NaCl, 5 mM KCl, 55 mM d-glucose, 50 mM Na-Bicine, pH 8.0 (BBS). (24). After centrifugation (10 min, 4°C) using a Sorvall HB-4 rotor (2500 rpm), the cells were washed in ice-cold BBS (about 3 x 10^9 cells/ml) and centrifuged again.

\[ 3^H \text{Myristate-labeled mVSG} \rightarrow 3^H \text{Myristate-labeled mVSG, for assaying of VSG lipase, was prepared by labeling trypanosomes (2 x 10^9/10 ml) in vitro with [9,10-^3H]myristic acid (12.9 Ci/mmol, 0.1 mCi/ml, New England Nuclear) (7, 24). After 1 h at 37°C, the cell suspension was cooled to 0°C and centrifuged (HB-4 rotor, 2500 rpm, 10 min, 4°C). The medium was saved as it could be reused without significant loss of labeling efficiency. The cell pellet was washed with 5 ml of BBS at 0°C and centrifuged (HB-4 rotor, 2500 rpm, 10 min, 4°C). The cells were lysed osmotically by resuspending in 10 ml of 10 mM NaF, pH 7.0, containing 0.1% n-octyl glucoside (Boehringer Mannheim), and TLCK, and sodium p-chloromercuriphenylsulfonate (added from a 100 mM stock solution in 0.1 M NaOH). Sodium p-chloromercuriphenylsulfonate inhibits VSG lipase (1, 18) and sulfhydryl reagents were used to maintain the aqueous phase. In that system, the release of radioactivity was about 2,800 cpmlpg)

Buffers throughout the purification included 1 μg/ml leupeptin and 0.1 mM TLCK, and operations were performed at 0–4°C unless specified otherwise. After obtaining trypanosomes, the entire purification procedure can be completed within 3 days. The typical purification described here is summarized in Table I.

Preparation of VSG-depleted Membranes—Trypanosomes (7.2 x 10^9) were lysed osmotically by suspending in 70 ml of 1 mM EDTA, 10 mM NaF, pH 8.0 (Buffer A) for 15 min. This lysate was designated Fraction I. Following centrifugation (Sorvall HB-4 rotor, 6000 rpm, 5 min), the pellet was resuspended in 70 ml of Buffer A at 37°C and maintained at this temperature for 5 min to promote the VSG lipase-catalyzed release of membrane-bound VSG. The VSG-depleted membranes were centrifuged (HB-4 rotor, 8500 rpm, 15 min), washed with 70 ml of Buffer A, and centrifuged again.

Detergent Solubilization of VSG Lipase—The pellet was resuspended in 50 ml of 5 mM EDTA, 50 mM Tris-HCl, pH 8.0 (Buffer B), containing 0.1% n-octyl glucoside (Boehringer Mannheim). After 20 min, the suspension was centrifuged (HB-4 rotor, 10,000 rpm, 15 min). The supernatant contained less than 6% of the VSG lipase activity and was discarded. The pellet was extracted again, for 20 min, with 50 ml of Buffer B containing 1% n-octyl glucoside. The suspension was centrifuged (Beckman Ti-70 rotor, 31,000 rpm, 1 h), and the supernatant (48 ml) was designated Fraction II.

Ammonium Sulfate Fractionation—Fraction II was combined with an equal volume of saturated (NH₄)₂SO₄ solution (Bethesda Research Laboratories, ultrapure grade, saturated

**RESULTS**

Purification of VSG Lipase

**TABLE I**

| Fraction | Activity | Protein | Specific Activity | Relative Purity | Yield |
|----------|----------|---------|------------------|----------------|-------|
| I: total cell lysate | 1.5 x 10⁶ | 850 | 4.2 x 10⁴ | 1 | 100 |
| II: 1% NOG extract | 1.1 x 10⁷ | 9.0 x 10⁵ | 17 | 7 | 73 |
| III: ammonium sulfate supernatant | 9.6 x 10⁶ | 8.7 | 1.1 x 10⁶ | 26 | 67 |
| IV: phenyl-Sepharose pool | | | | | |
| V: CM-Sephadex pool | 2.6 x 10⁴ | n.d. | n.d. | n.d. | 19 |
| VI: Concentrated CM-Sephadex pool | 2.3 x 10⁶ | 0.022 x 1.1 x 10⁶ | 2580 | 17 |
| VII: S-200 pool | 1.0 x 10⁶ | n.d. | n.d. | n.d. | 9 |

* Yields are corrected for aliquots removed during purification.

**TABLE II**

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The identity of dimyristoyl glycerol as the product, as assayed by SDS-PAGE and Coomassie staining (not shown), was confirmed by chromatography using another solvent system (hexane/diethyl ether/glacial acetic acid, 70:30:1). In that system both the reaction product and a 1,2-dimyristoyl-sn-glycero-3-phosphorylcholine standard (Sigma) comigrated as a doublet, presumably because of some iso-merization to 1,3-dimyristoyl glycerol (not shown).

**Gel Electrophoresis and Protein Determinations**—SDS-PAGE (25) was performed using 7.5–15% linear gradient polyacrylamide gels. Gels were stained with Coomassie Blue and then, unless noted otherwise, with silver according to Morrissey (26). Using bovine serum albumin as a standard, protein was determined, after precipitation by 10% trichloroacetic acid, as described by Lowry et al. (27) unless noted otherwise. SDS (0.5%) was included to avoid interference by other detergents (28).

**DISCUSSION**

To understand the biological role and possible regulation of VSG lipase, it is essential to obtain it in pure form. In this paper we describe the purification of VSG lipase and some preliminary characterization of its biochemical properties. The identity of dimyristoyl glycerol as the product, as assayed by SDS-PAGE and Coomassie staining (not shown), was confirmed by chromatography using another solvent system (hexane/diethyl ether/glacial acetic acid, 70:30:1). In that system both the reaction product and a 1,2-dimyristoyl-sn-glycero-3-phosphorylcholine standard (Sigma) comigrated as a doublet, presumably because of some iso-merization to 1,3-dimyristoyl glycerol (not shown).

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Ammonium Sulfate Fractionation—Fraction II was combined with an equal volume of saturated (NH₄)₂SO₄ solution (Bethesda Research Laboratories, ultrapure grade, saturated
at 4 °C. The dropwise addition of (NH₄)₂SO₄ solution was made over 30 min with constant stirring. After another 60 min of stirring, the turbid solution was centrifuged (HB-4 rotor, 10,000 rpm, 15 min). The supernatant (96 ml) was designated Fraction III.

**Phenyl-Sepharose Chromatography—**Fraction III was applied to a column of phenyl-Sepharose CL-4B (Pharmacia, 1.1 cm² × 14 cm) equilibrated with 50% saturated (NH₄)₂SO₄. The column was then washed with 25 mM sodium succinate, pH 6.0, containing (NH₄)₂SO₄ which was decreased linearly from 50 to 0% of saturation over 100 ml. After further washing with 50 ml of 25 mM sodium succinate, pH 6.0, VSG lipase was eluted with 1% CHAPS (Sigma), 25 mM sodium succinate, pH 6.0 (Buffer C). Fractions (5.3 ml) were collected at a rate of 30 ml/h. As in the typical example shown in Fig. 1, most protein eluted during the wash or in a sharp peak immediately after elution with CHAPS. In contrast, VSG lipase eluted in a broad peak after introduction of CHAPS. The trailing fractions of the activity peak, which contained about 70% of the activity but relatively little protein, were pooled (Fraction IV, 333 ml).

**Carboxymethyl-Sephadex Chromatography—**Fraction IV was applied directly to a column of carboxymethyl-Sephadex (C-25, Pharmacia, 0.4 cm² × 12.5 cm) equilibrated with Buffer C. The column was washed with 25 ml of Buffer C and eluted with a 50-ml linear gradient of 0–100 mM NaCl in Buffer C. Fractions (1.5 ml) were collected at a rate of 15 ml/h. A typical CM-Sephadex fractionation is depicted in Fig. 2. VSG lipase was eluted as a single peak at about 30 mM NaCl. The most active fractions were pooled (Fraction V, 20 ml). To minimize contamination with other proteins, trailing fractions, containing about 30–35% of the recovered activity, were excluded from the pool.

Fraction V was concentrated by vacuum dialysis against 280 ml of 1% CHAPS, 50 mM Tris-HCl, pH 8.0, for 20 h at 0 °C. The concentrate (1.65 ml) was designated Fraction VI.

**Sephacryl Gel Filtration—**In an optional step, Fraction VI was applied to a Sephacryl S-200 column (0.72 cm² × 107 cm), equilibrated, and eluted with 1% CHAPS, 50 mM Tris-HCl, pH 8.0. Fractions of 1.2 ml were collected at a rate of 7 ml/h, and 76% of the applied activity was recovered. The most active fractions, containing 70% of the eluted activity, were pooled (9.7 ml) and designated Fraction VII.

**Purity of VSG Lipase**

VSG lipase was purified about 2600-fold through Fraction VI (Table I); an analysis of the purification by SDS-PAGE is...
shown in Fig. 3. Both Fraction VI and Fraction VII contain a single major 37-kDa polypeptide. No other Coomassie-stained proteins are detectable in either fraction, although trace 48- and 64-kDa contaminants of Fraction VI were revealed by silver staining (not shown). The 37-kDa protein cofractionated with activity during chromatography on phenyl-Sepharose (Fig. 1) and carboxymethyl-Sephadex (Fig. 2), sedimentation on a sucrose gradient (Fig. 4A), and gel filtration on Sephacryl S-200 (Fig. 4B). In SDS-PAGE analyses, the intensity of staining of the 37-kDa polypeptide and VSG lipase activity were approximately proportional in almost every case. Silver staining (Figs. 1-3 (lane IV), and 4A; note the amounts of each fraction analyzed) required about 10^9 units for detection. (In Fig. 4B, a different, perhaps less sensitive, staining method was used.) Coomassie Blue (Fig. 3 (lanes VI and VII) and other data not shown) was sensitive to about 10^6 units. We conclude that Fractions VI and VII (and also Fraction V which is an unconcentrated form of Fraction VI) are virtually homogeneous VSG lipase.

Stability of VSG Lipase

VSG lipase is very stable. We obtained nearly the same yield of enzyme activity when we used VSG-depleted membranes which had been stored 6 months at -70 °C as we did from freshly isolated trypanosomes. Fractions II and III could also be stored at -70 °C for several weeks without significant loss of activity. Fraction V could be stored for at least 2 months at -70 °C with little or no loss of activity. A 30% loss of activity occurred upon storage of this fraction for 5 days at 4 °C, but storage in 50% glycerol reduced this loss to 10%. Incubation of Fraction V for 10 min at 37 °C resulted in little loss of activity, but incubation at 50 °C caused 99% inactivation.

FIG. 3. SDS-PAGE of fractions from VSG lipase purification. With the exception of Fraction VII, aliquots of fractions were precipitated with trichloroacetic acid (10% final concentration), washed with 90% acetone, dissolved in SDS-PAGE sample buffer, and electrophoresed. Fraction VII, concentrated by lyophilization, was dissolved in 2% SDS, precipitated with 8 volumes of acetone (~20 °C overnight), and centrifuged to recover the precipitate. These steps were repeated twice more to eliminate an oily CHAPS residue. Fractions are indicated above the gel lanes. Fractions I, II, III, VI, and VII were from the purification summarized in Table I; this gel was stained with Coomassie Blue. Fraction IV was from a different purification; this gel was silver-stained. The samples were: Fraction I, 500 units VSG lipase; 120 μg of protein; Fraction II, 5,000 units, 75 μg; Fraction III, 5,000 units, 45 μg; Fraction IV, 4,500 units; Fraction VI, 20,000 units, 2 μg; Fraction VII 100,000 units. The scale shows molecular weights of marker proteins; their positions are marked by the horizontal lines to the left of lanes I, IV, VI, and VII.

FIG. 4. Hydrodynamic properties of VSG lipase. Panel A, sedimentation analysis. VSG lipase (Fraction V, 7,000 units/0.5 ml) was sedimented through a 5-20% linear sucrose gradient (11 ml) containing 50 mM Tris-HCl, pH 8.0, and 1% CHAPS (Beckman SW-41 rotor, 41,000 rpm, 20 h, 4 °C). VSG lipase activities refer to entire fractions (0.24 ml); recovery of activity was 36%. Fractions were assayed by SDS-PAGE as described in the legend of Fig. 1; the inset shows a portion of the silver-stained gel. Lane numbers refer to gradient fractions; each lane represents a pool of the entire contents of a fraction and the subsequent fraction. The smear in lane 17 is due to accidental contamination of the sample. The horizontal arrow indicates the 37-kDa VSG lipase band. Reference proteins—horse myoglobin (2.0 S, 0.741 cm³/g), chicken ovalbumin (3.6 S, 0.749 cm³/g), bovine serum albumin (4.3 S, 0.734 cm³/g), and bovine γ-globulin (7 S, 0.722 cm³/g)—were centrifuged simultaneously in a separate gradient. Their peak positions, as determined by SDS-PAGE, are indicated by vertical arrows with their s_{osm} values (Svedberg units). Panel B, gel filtration analysis. VSG lipase (Fraction V, 3.2 × 10^6 units/2 ml) was applied to a column of Sephacryl S-200 (Pharmacia, 0.72 cm × 112 cm) equilibrated with 1% CHAPS, 0.1 mM TLCK, 1 μg/ml leupeptin, 50 mM Tris-HCl, pH 8.0, and eluted at 4 °C with the same buffer. Fractions (1.35 ml) were collected at a rate of 7 ml/h. VSG lipase activities refer to entire fractions; recovery of activity was 73%. For SDS-PAGE, odd-numbered fractions were combined with 10 ml of acetonitrile and stored at -20 °C overnight. Precipitates were collected by centrifugation (Sorvall HS-4 rotor, 4,000 rpm, 20 min, 4 °C), dissolved (100 °C, 1 min) in SDS-PAGE sample buffer, and electrophoresed. The inset shows a portion of the gel, silver-stained according to Wray et al. (51). Lane numbers refer to column fractions; the horizontal arrow indicates the 37-kDa VSG lipase band. Reference proteins were analyzed in a separate run; their elution positions, as detected by SDS-PAGE, are indicated by vertical arrows with their Stokes radii (nm) which were calculated from diffusion data (52). The standards are bovine serum albumin (3.6 nm), chicken ovalbumin (2.8 nm), soybean trypsin inhibitor (2.4 nm), bovine lactalbumin (2.0 nm), and horse myoglobin (1.9 nm). Other elution positions indicated, V₀, Blue Dextran 2000 (Pharmacia), detected by absorption at 260 nm; two other peaks were not detected.

Hydrodynamic Properties of VSG Lipase

Hydrodynamic measurements were taken in the presence of CHAPS which may bind to VSG lipase. Since CHAPS has a partial specific volume (v) of 0.81 cm³/g (29), a VSG lipase-CHAPS complex could have a v that is higher than that of most soluble proteins (usually 0.73–0.74 cm³/g). Therefore,
of Trypanosoma VSG lipase.

The enzyme (Fraction V) was optimally active between pH 7.5 and 8.5 using Tris-HCl, NaCl, or Na-Bicine. Half of the maximum activity was obtained at pH 6.5 using either NaCl; or sodium succinate and at pH 9.5 using sodium glycinate. There was little or no activity between pH 4.5 and 6.0 using either sodium citrate or sodium succinate buffers. All buffers used in studies on the effect of pH were 50 mM. These results agree with those obtained by Turner et al. (1) with impure VSG lipase.

### Substrate Specificity of VSG Lipase

VSG lipase is a phospholipase C. To examine its specificity in more detail, we tested several different substrates and analyzed the reaction products by thin layer chromatography (Fig. 5). Of all of the [3H]myristic acid-labeled lipids in a CHCl₃/MoOH (2:1) extract of [3H]myristate-labeled trypanosomes, only one, lipid A, was cleaved by VSG lipase to yield a compound which comigrated with dimyristoyl glycerol (lanes 1 and 2). Lipid A is a biosynthetic precursor of the VSG glycolipid, and it has a structure closely related to that of the VSG glycolipid. Although the identities of the other labeled compounds in the extract are not known, this result suggests that VSG lipase has a narrowly defined specificity. Furthermore, only a small amount of cleavage of 1,2-dimyristoyl-sn-phosphatidylglycerol to produce dimyristoyl glycerol was detected (lanes 3 and 4); the amount produced was proportional to the amount of enzyme added (not shown). We detected no cleavage of 1-stearoyl-2-araehidonyl-sn-phosphatidylinositol (lanes 5 and 6). In reactions containing mixtures of mVSG and either of the phosphatidylinositols, mVSG was completely hydrolyzed while neither phosphatidylinositol was significantly hydrolyzed (compare lanes 7 and 8 with lanes 3 and 5, respectively). Identical results (not shown) were obtained when reaction mixtures were transferred to new tubes prior to n-butyl alcohol extraction, indicating that the substrates were in solution during the incubation with the enzyme. The tentative conclusion from these experiments is that VSG lipase is highly specific. It cleaves the VSG glycolipid and structurally related compounds including its glycolipid precursor (lipid A) and, to a lesser extent, dimyristoyl phosphatidylglycerol. Nevertheless, this apparent specificity of VSG lipase could be dependent on the conditions we employed and could be affected by other factors such as the presence of detergents or other lipids (32). The possibility that other coextracted lipids stimulated lipid A cleavage (Fig. 5, lanes 1 and 2) is unlikely since lipid A purified by thin layer chromatography was also cleaved efficiently (not shown).

### DISCUSSION

VSG lipase is a phospholipase C of the bloodstream form of T. brucei which catalyzes the hydrolysis of the VSG glycolipid. It converts mVSG to sVSG and releases dimyristoyl glycerol. Based on SDS-PAGE, the nearly homogeneous enzyme has an apparent subunit molecular mass of 37 kDa. Its Stokes radius of about 2.6 nm and apparent s₂₀,₆ of 3.7 S indicate a molecular mass for the native enzyme of about 47 kDa (this value could include bound CHAPS). Therefore, the purified VSG lipase appears to be monomeric.

The detergent CHAPS, a zwitterionic derivative of cholic acid, plays an important role in the purification. In early trials with Nonidet P-40, n-octyl glucoside, or no detergent, we were unable to bind VSG lipase to ion exchange resins over a broad pH range. Only CHAPS permitted binding to carboxymethyl-
Trypanosomal VSG Lipase

FIG. 5. Specificity of VSG lipase. Potential substrates were treated with VSG lipase (80 units, Fraction V) for 2 h at 37 °C in 50 μl containing 1% Nonidet P-40, 5 mM EDTA, 50 mM Tris-Cl, pH 8.0. Reaction mixtures were then extracted into H₂O-saturated n-butyl alcohol. After evaporation of the solvent, the extracted material was redissolved in CHCl₃/CH₃OH/H₂O (10:10:3) and chromato-

gated on thin layer of Silica Gel 60 (Merck; heated 1 h at 100 °C prior to use) using CHCl₃/CH₃OH/0.25% aqueous KCl (55:45:10) as solvent. The plate was sprayed with EN3HANCE (New England Nuclear) and fluorographed. Reactions contained 1,000 cpm of each substrate unless otherwise indicated. Lanes 1 and 2, CHCl₃/MeOH (2:1) extracted lipids (10,000 cpm) from 3 × 10⁵ [³H]myristate-labeled trypanosomes (a by-product of [³H]-labeled mVSG preparation; see "Materials and Methods"). Lanes 3 and 4, 1,2-[³H]dimyristoyl-sn-phosphatidylinositol. Lanes 5 and 6, 1-stearyl-2-[³H]arachidonoyl-

sn-phosphatidylinositol (10 Ci/mmol, New England Nuclear). Lane 7, mixture of 1,2-[³H]dimyristoyl-sn-phosphatidylinositol and [³H] myristate-labeled mVSG (2100 cpm/μg). Lane 8, mixture of 1-stea-

royl-2-[³H]arachidonoyl-sn-phosphatidylinositol and [³H]myristate-
labeled mVSG. Lanes 9 and 10, [³H]myristate-labeled mVSG. VSG lipase was added to the reactions analyzed in lanes 2, 4, 6, 7, 8, and 10; control reactions were analyzed in lanes 1, 3, 5, and 9. mVSG is not extracted into n-butyl alcohol and, thus, only the dimyristoyl glycerol product is apparent (lanes 7-10). The amount of VSG lipase used completely hydrolyzed the mVSG (lanes 7, 8, and 10) as confirmed by counting the aqueous phase after n-butyl alcohol extraction. 1,2-[³H]Dimyristoyl-sn-phosphatidylinositol was prepared from [³H] myristate-labeled mVSG (2100 cpm/μg) by nitrous acid treatment and isolated as previously described (11). In control experiments, both 1,2-[³H]dimyristoyl-sn-phosphatidylinositol and 1-stearyl-2-

[³H]arachidonoyl-sn-phosphatidylinositol were quantitatively hydro-

lyzed by S. aureus PI-PLC under previously described conditions (11). The molar specific radioactivity of the stearyl arachidonoyl phosphatidylinositol was about 100 times that of the mVSG and dimyristoyl phosphatidylinositol; increasing the concentration of the stearyl arachidonoyl phosphatidylinositol substrate 100-fold did not result in VSG lipase-catalyzed cleavage (not shown). The position of the labeled compound marked by the arrow (lanes 1 and 2) was slightly affected by CHAPS which was introduced with VSG lipase (lane 2) and chromatographed to this position. Also indicated: O, origin; A, lipid A (see "Results"); PI, soybean phosphatidylinositol (Sigma); M, myristic acid; D, 1,2-dimyristoyl-rac-glycerol (Sigma); F, solvent front.

Sephadex at pH 6.0. In preparation for carboxymethyl-Sephadex chromatography, we therefore eluted VSG lipase from phenyl-Sepharose with 1% CHAPS at pH 6.0. To our surprise, VSG lipase was among only a small number of proteins which eluted in a broad peak after the major protein peak (Fig. 1). Perhaps these proteins, including VSG lipase, are integral membrane proteins. Some VSG lipase activity (25–35%) typically elutes with the majority of proteins as a sharp peak immediately following introduction of CHAPS (Fig. 1). This activity is probably the same as that which elutes in the broad main activity peak because it behaves identically on CM-

Sephadex, coeluting precisely with appropriate amounts of the 37-kDa polypeptide.

Dithiothreitol had a surprisingly large stimulatory effect on VSG lipase. At 1 mM it stimulated 2-fold and at 25 mM it stimulated more than 6-fold (Table II). Dithiothreitol had the same effect on freshly prepared Fraction I and other fractions stored for prolonged periods at ~70 °C (not shown). Thus, this stimulatory effect does not seem to reflect progressive reversible inactivation of VSG lipase during purification. We do not know if dithiothreitol acts directly on the enzyme; instead, it may convert mVSG to a more effective substrate by reducing disulfide bonds likely to be near its C terminus (8, 33).

From the specific activity of the homogeneous enzyme (Table I), we estimate that each trypanosome contains about 30,000 VSG lipase molecules and that this enzyme represents about 0.04% of the total cellular protein. Therefore, there are about 400 VSG molecules for every molecule of VSG lipase. Under the conditions of our assay, each VSG lipase molecule could hydrolyze about 100 molecules of mVSG each minute. Thus, this enzyme can account for the rapid release of VSG from lysed trypanosomes.

The cellular location of VSG lipase is not yet known. Its appearance in the particulate fraction of lysed trypanosomes and extractability by non-ionic detergent suggests that VSG lipase is associated with a membrane. Since VSG is found on the cell surface, VSG lipase could be associated with the plasma membrane. Support for this location came from recent studies in our laboratory on the biosynthesis of VSG (34). In pulse-chase experiments, we found that the transport of VSG to the cell surface and to a cellular compartment containing VSG lipase had comparable kinetics. Nevertheless, it remains possible that VSG lipase resides in an internal membrane which fuses with the plasma membrane only after a triggering event such as cell lysis. Immunoelectron microscopy, using VSG lipase-specific antibodies, should permit localization of the enzyme.

Phospholipase Cs with different specificities have been described in many cell types. VSG lipase is potentially related to those phospholipase Cs which specifically hydrolyze phosphatidylinositol (reviewed in 35) since this moiety is part of the VSG glycolipid. It is of great interest that PI-PLCs are believed to generate second messengers within many cells in response to a wide variety of extracellular stimuli (36). Eukaryotic PI-PLCs have been found mainly in cytosolic fractions, although there are lower levels in particulate fractions. Bacterial PI-PLCs have been purified from culture filtrates. VSG lipase differs from most of these other enzymes in that it is found exclusively in the particulate fraction. In addition, VSG lipase has a pH optimum of 7.5–8.5; most PI-PLCs are optimally active at neutral and acidic pH. Similar to the bacterial PI-PLCs but unlike the majority of mammalian enzymes, VSG lipase does not require Ca²⁺ for activity.

Is VSG the true substrate of the enzyme which we have purified, or is its hydrolysis in vitro without biological significance? We must consider the latter possibility since the action of VSG lipase in trypanosomes has been detected only under nonphysiological circumstances (e.g. cell lysis). However, our preliminary investigation of substrate specificity suggests that VSG lipase could be highly specific. VSG and its biosynthetic precursor, lipid A, are hydrolyzed; 1,2-dimyristoyl-sn-phosphatidylinositol is also hydrolyzed but much less efficiently. We could detect no cleavage of 1-stearyl-2-

arachidonoyl-sn-phosphatidylinositol or any of the [³H]my-

ristate-labeled lipids (other than lipid A) in a CHCl₃/MeOH

vitro reaction mixture.
prove our tentative conclusion that VSG lipase is highly specific for VSG. If this conclusion is true, it implies that this parasite, these include a 195-kDa surface antigen of Trypanosoma brucei that may be cleaved by VSG lipase. Remarkably, VSG lipase treatment to membranes by structures containing phosphatidylinositol 4,5-bisphosphate (PI-PLC) can extract of trypanosomes. It seems possible that VSG lipase may be the system of choice for revealing the structure, and, thus, can be released by exogenously added PI-PLCs.

Several membrane proteins in other eukaryotes are probably anchored to membranes by phospholipid moieties. Among parasitic protozoa, these include a 195-kDa surface antigen of Trypanosoma brucei that may be cleaved by VSG lipase. Remarkably, VSG lipase treatment to membranes by structures containing phosphatidylinositol 4,5-bisphosphate (PI-PLC) can extract of trypanosomes. It seems possible that VSG lipase may be the system of choice for revealing the structure, and, thus, can be released by exogenously added PI-PLCs.

The biological significance of phosphatidylinositol-containing glycolipids on these diverse proteins is not yet understood, and in some cases investigation will be hindered by limiting amounts of material. Because massive amounts of VSG can be purified from trypanosomes, and because VSG lipase can now be readily obtained in homogeneous form, this parasite may be the system of choice for revealing the structure, function, and significance of the enzymatic cleavage of these protein-bound glycolipids.

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