

A new method for the rapid purification of both the membrane-bound and released forms of the variant surface glycoprotein from *Trypanosoma brucei*

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A simple new technique was developed for the rapid purification of either the membrane-bound or the released forms of the variant surface glycoprotein of *Trypanosoma brucei* in high yield. Whole cells were used as the source of the membrane-bound form, and the supernatant of benzyl alcohol-treated cells was used as the source of the released form. The technique was based on extraction of the acid-treated protein into chloroform/methanol, followed by selective re-partition into aqueous salt solution. The yield of purified protein was found to be dependent critically on a low pH during the extraction/re-partition stages. This finding and the ability to cycle the protein repeatedly through organic and aqueous phases in a strictly pH-dependent manner suggested that the protein could undergo fully reversible denaturation/renaturation only while in an extensively protonated form. The yield was independent of the polarity of the organic phase and the protein concentration over a wide range. After purification, both forms retain their ability to react with specific antibody raised against the authentic native protein purified by conventional means. The amino acid composition and the identity of the *N*-terminal amino acid was the same for both forms of the protein. In addition, both forms had blocked *C*-terminal residues. There were determined to be 1.13×10^7 copies of the variant surface glycoprotein per cell.

Most of the published methods for the purification of the variant surface glycoprotein (VSG) of trypanosomes yield only the released form (VSG_r) of the protein (Cross, 1975; Strickler *et al.*, 1978; Reinwald *et al.*, 1981). The observations by Almeida & Turner (1983), that the membrane-bound form of the protein (VSG_m) is preserved and solubilized by addition of detergents to whole cells, has allowed the development of a purification procedure for VSG_m (Ferguson & Cross, 1984). However, it would be of much greater use to have a simple, rapid and reliable purification procedure for both forms of the VSG that in both cases was not based on the use of detergents, since VSG_m contains partially characterized lipid that is not

found in VSG_r (Ferguson & Cross, 1984). Furthermore, the exact structure of the covalently linked lipid is important, because VSG_m, but not VSG_r, is attached to the outer leaflet of the plasma membrane by means of a phosphodiester linkage to this lipid (Jackson & Voorheis, 1985). In addition, the covalently linked lipid may have a somewhat unusual structure (Jackson & Voorheis, 1985). For all of these reasons it is obvious that purified VSG, completely free of detergent and non-covalently linked lipid, would facilitate the unequivocal determination of the exact structure of the covalently linked phospholipid.

This paper describes a simple purification technique for VSG_m and VSG_r that does not use detergents at any stage of the procedure and ensures that non-covalently associated lipid is removed. The procedure is based on the original observation by Owen (1976) that the radiolabelled VSG, after treatment with trichloroacetic acid, partitions along with several other proteins into chloroform/methanol as long as the mol fraction of water is low enough to give a single-phase system and that, when the organic extract is separated into

Abbreviations used: SDS, sodium dodecyl sulphate; VSG_m, variant surface glycoprotein (membrane-bound form); VSG_r, variant surface glycoprotein (released form).

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two phases by the addition of aqueous NaCl, the VSG alone re-partitions into the aqueous phase. When whole cells are used as the starting material, the procedure yields purified VSG_m; when the supernatant of cells treated with the local anaesthetic benzyl alcohol (Jackson, 1983) is used as the starting material, the procedure yields purified VSG_r. In addition to the utility of this method, the observed reversible protonation-dependent denaturation/renaturation behaviour of both forms of the VSG have important implications for models of its structural organization.

Materials and methods

Chemicals

Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. Solvents were of the highest grades available from BDH Chemicals, Poole, Dorset, U.K., and were redistilled before use. All other chemicals were from BDH or from Sigma (London), Poole, Dorset, U.K.

Source and preparation of cells

The source, history, cloning, storage, growth, isolation and quantification of bloodstream forms of *Trypanosoma brucei* 427-12-ICI-060 have been reported previously (Voorheis, 1980). This clone has also been named MITat 1.1 (Holder & Cross, 1981).

Purification of the membrane-bound form of the variant surface glycoprotein (VSG_m)

A suspension of bloodstream forms of *T. brucei* (1×10^{10} – 5×10^{10} cells) in 10–20 ml of Krebs–Ringer phosphate buffer (Voorheis, 1980) was treated at 0°C with an equal volume of 10% (w/v) trichloroacetic acid and the precipitate centrifuged at 9000g for 10s. It was critical to obtain a loose pellet at this stage. The material in the pellet was resuspended in distilled deionized water (4 ml/10¹⁰ cells) with the aid of a Dounce homogenizer with a loosely fitting Teflon pestle. The suspension was extracted with 20 vol. of chloroform/methanol (2:1, v/v) with vigorous shaking for 5 min and then stored overnight at 5°C. This storage procedure was found to increase the final yield of purified VSG. The extract then was separated into two phases by the addition of 0.2 vol. of 0.9% NaCl solution, and was centrifuged at 12000g for 1 h. The upper aqueous phase contained pure VSG_m, and was removed by aspiration and dialysed against 3 × 20 litres of distilled deionized water at 4°C for a total of 36 h. The retained material was freeze-dried and the resulting VSG stored dry over P₂O₅ *in vacuo* until required.

Purification of the released form of the variant surface glycoprotein (VSG_r)

A suspension of trypanosomes (1×10^{10} – 5×10^{10} cells) in 10–20 ml of Krebs–Ringer phosphate buffer at 0°C (Voorheis, 1980) was added with stirring to a solution at 37°C containing Tes (20 mM), NaCl (140 mM), MgSO₄ (5 mM), EGTA (0.1 mM), phenylmethanesulphonyl fluoride (0.1 mM), glucose (10 mM) and benzyl alcohol (75 mM) adjusted to pH 7.5 so that the final concentration of cells was 5×10^8 cells/ml. The incubation at 37°C was carried out for 5 min in a large conical flask and was terminated by adding an equal volume of the above Tes buffer without benzyl alcohol at 0°C and centrifuging for 5 s at 12000g.

The supernatant was carefully removed and concentrated to one-quarter of its volume by ultrafiltration (350 kPa) with an Amicon PM-10 membrane at 4°C. The protein in the concentrate was almost pure VSG_r, and was further purified by treatment with 0.2 vol. of 50% trichloroacetic acid, centrifuging at 3000g for 10s and subjecting the precipitated protein to exactly the same solvent-extraction procedure as used for VSG_m.

Dissolving the purified freeze-dried variant surface glycoprotein

Dry purified VSG_r was found to dissolve rapidly and completely when added to the surface of distilled deionized water and stirred gently. Only about 50% of VSG_m dissolved under the same conditions. However, VSG_m was found to dissolve rapidly and completely if sufficient 1 M-NaOH was added to bring the suspension to pH 10.0, followed immediately by neutralization with 1 M-Tris/HCl. SDS/polyacrylamide-gel electrophoresis showed that this procedure did not degrade VSG_m. Acid conditions also aided the solubility of VSG_m in water, but did not result in complete solubility.

Radiolabelling of the variant surface glycoprotein

VSG was labelled *in situ* (Bowles & Voorheis, 1982; Voorheis *et al.*, 1982) for some experiments before purification of either form of the coat protein.

SDS/polyacrylamide-gel electrophoresis

This was carried out in slab gels by the method of Laemmli (1970) as modified by Studier (1972).

Immunoblotting

The procedure of Vaessen *et al.* (1981) was used for immunoblotting, with one modification. After transfer of the proteins from the gel (10% acrylamide) to the nitrocellulose sheet, the sheet was incubated in phosphate-buffered saline (10 mM-

$\text{Na}_2\text{HPO}_4/150\text{mM-NaCl}$, pH 7.2) containing bovine serum albumin (8%, w/v) for 60 min and then transferred to a freshly prepared urea solution (8M) for 60 min. The urea was removed by washing the sheet in distilled deionized water (3×50 ml). The sheet ($5\text{cm} \times 12\text{cm}$) then was immersed in the solution (5 ml) of primary antibody [1/50 dilution of crude rabbit anti-(MITat 1.1 VSG_r) in phosphate-buffered saline containing 8% bovine serum albumin]. The remainder of the procedure was exactly as described by Vaessen *et al.* (1981).

Amino acid analysis

Samples of purified VSG, dissolved in HCl (6M), were hydrolysed *in vacuo* at 110°C for 24 h and the hydrolysates analysed on a Locarte amino acid analyser.

End-group analyses

N-Terminal amino acid residues were identified after conversion into the dimethylaminonaphthalene-1-sulphonyl derivatives by the method described by Allen (1981).

C-Terminal amino acid residues were identified either by the selective ^3H -labelling procedure of Matsuo *et al.* (1966), or after hydrazinolysis (80°C, 24 h) as described by Fraenkel-Conrat & Tsung (1967).

Surface area of bloodstream forms of *T. brucei*.

The surface area of *T. brucei* was calculated to be $186.5\mu\text{m}^2$ from an idealized model of a trypanosome constructed from a composite series of cones and cylinders by using the appropriate mensuration formulae (Fig. 1). The dimensions of each cone or cylinder were taken from electron photomicrographs of whole negatively stained cells and thin sections of cells prepared as previously described (Voorheis *et al.*, 1979). The accuracy of this procedure was checked by comparing the volume of the cell predicted by the model ($17.3\mu\text{m}^3$) with that found experimentally by using a Coulter Counter equipped with a size-distribution analyser ($17.1\mu\text{m}^3$).

Other analytical techniques

Protein was measured by the method of Lowry *et al.* (1951), and radioactivity by liquid-scintillation counting with quench correction.

Results

Purification of VSG_m

Stage 1: chloroform/methanol extraction. Extraction of whole trichloroacetic acid-treated cells into a single-phase mixture of chloroform/methanol (2:1, v/v) resulted in the recovery of over 90% of VSG_m in the organic solvent (Table 1). The extent

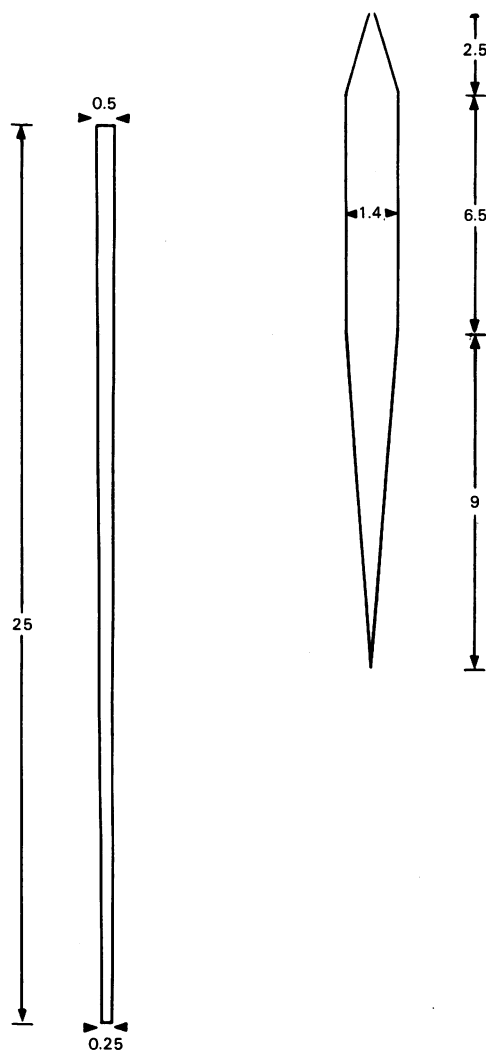


Fig. 1. Scale diagram of the geometric units used in calculating the total surface area of a single *T. brucei* bloodstream trypomastigote

The cell body was represented as a composite of two cones connected at their bases by a cylinder, and the flagellum as the frustum of an extended cone. The surface areas, calculated by using the appropriate mensuration formulae, were $107.3\mu\text{m}^2$ (cell body) and $79.18\mu\text{m}^2$ (flagellum). All values shown are in μm .

of extraction of VSG_m was not affected when the polarity of the extraction mixture was varied by changing its content of water or concentration of cell protein (Table 1). Analysis by SDS/polyacrylamide-gel electrophoresis (Fig. 2a) confirmed that the chloroform/methanol extract contained VSG_m (M_r 58000), free of contamination by VSG_r. Consequently, the initial treatment of cells with trichloroacetic acid must have completely inacti-

Table 1. Efficiency of extraction of [^3H]acetimido-labelled VSG into chloroform/methanol under different conditions. Trypanosomes ($100\ \mu\text{l}$ of a suspension containing 10^9 cells/ml) that had been surface-labelled with [^3H]isethionyl acetimidate were added to an equal volume of 10% trichloroacetic acid. After centrifugation ($9000g$, 5s) and removal of the supernatants, the pellets were resuspended in water ($20\text{--}100\ \mu\text{l}$) and extracted with different amounts ($20\text{--}60$ vol.) of chloroform/methanol (2:1, v/v). The extracts were filtered (Whatman no. 54) and the amounts of radioactivity in samples of filtrates were determined by liquid-scintillation spectrometry after evaporation to dryness. The results were corrected for the amount of residual binding of VSG to the filters. Each value is the mean \pm s.e.m. for three replicate determinations.

Composition of chloroform/methanol/water extraction medium (by vol.)	$10^{-7} \times$ Concn. of [^3H]acetimido-labelled trypanosomes during extraction (cells/ml)	Recovery of [^3H]acetimido-labelled VSG in filtered extract (%)
20/10/1.5	24.0	85 ± 3
20/10/1.5	12.0	97 ± 6
20/10/1.5	7.9	105 ± 5
20/10/1.5	5.95	82 ± 5
20/10/1.5	4.76	109 ± 2
20/10/1.5	8.1	80 ± 9
20/10/1.25	8.1	74 ± 9
20/10/1.0	8.1	77 ± 9
20/10/0.75	8.1	85 ± 9
20/10/0.5	8.1	105 ± 14

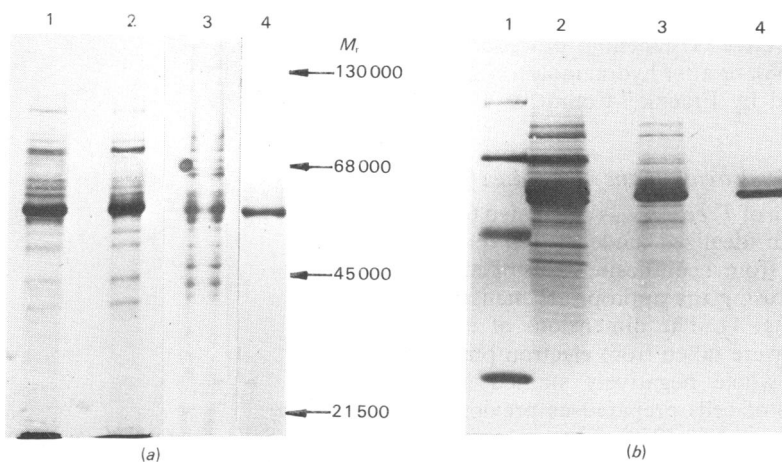


Fig. 2. SDS/polyacrylamide-gel electrophoresis of the proteins present at each stage during purification of VSG_m and VSG_r from *T. brucei*

(a) SDS/polyacrylamide-gel electrophoresis (10%-acrylamide gels) of proteins at each stage during the purification of VSG_m. Track 1, whole cells; track 2, filtered chloroform/methanol (2:1, v/v) extract of trichloroacetic acid-treated whole cells; track 3, interfacial material formed after separation of extract into two phases; track 4, aqueous phase. The positions of M_r marker proteins are indicated by arrows. (b) SDS/polyacrylamide-gel electrophoresis (10%-acrylamide gels) of proteins at each stage during the purification of VSG_r. Track 1, M_r marker proteins; β -galactosidase (M_r , 130000), bovine serum albumin (M_r , 68000), ovalbumin (M_r , 45000) and soya-bean trypsin inhibitor (M_r , 21500); track 2, trichloroacetic acid-treated whole cells; track 3, supernatant ($12000g$) from whole cells treated with 75 mM-benzyl alcohol; track 4, aqueous phase from two-phase solvent extract of trichloroacetic acid-treated supernatant.

vated the coat-releasing enzyme. In addition, the amount of VSG recovered during solvent extraction fell by a factor of 2–4-fold when the initial treatment of cells with trichloroacetic acid was omitted, or when the initial treatment was instead either urea (8M) or NaOH (50mM).

Stage 2: partition from chloroform/methanol into aqueous NaCl. Crude VSG_m present in a single-phase chloroform/methanol extract was re-partitioned into an aqueous phase by vigorous shaking with aq. 0.9% NaCl, to produce a two-phase solvent system. A dense interfacial precipitate also

was formed, comprising those cellular proteins that were completely insoluble in either the aqueous or the organic phases. Analysis of the proteins present in each of the phases of this latter extract by SDS/polyacrylamide-gel electrophoresis (Fig. 2a) showed that the upper aqueous phase contained only VSG_m in a highly purified state (recovery $68 \pm 9\%$; $n = 9$). The remaining cellular protein, together with a small amount of trapped VSG_m (recovery $12 \pm 1\%$; $n = 6$), was present in the interfacial precipitate. Only trace amounts of VSG_m ($4 \pm 1\%$; $n = 6$) were recovered in the lower organic phase. VSG_m that had been extracted once into chloroform/methanol and re-partitioned into aqueous NaCl could be re-extracted/re-partitioned during a second cycle of purification only after re-treatment of the VSG with trichloroacetic acid.

Purification of VSG_r

When the solvent-extraction/re-partition procedure described above was applied to the trichloroacetic acid-treated supernatant derived from benzyl alcohol-treated trypanosomes, VSG_r (M_r 60000) was recovered in highly purified form (final yield $51 \pm 9\%$; $n = 9$) in the final aqueous extract (Fig. 2b).

The faint bands that always co-purified with both forms of the variant surface glycoprotein (Figs. 2a and 2b) represented micro-heterogeneity in the *N*-linked carbohydrate (Cross, 1975; Strickler *et al.*, 1978; Strickler & Patton, 1980; Reinwald *et al.*, 1981; Holder & Cross, 1981; McConnell *et al.*, 1983).

Source of the major losses during purification

The 15–25% loss of VSG during purification was probably due to binding of the denatured VSG while in the chloroform/methanol phase to the sides of the glass extraction vessel. This conclusion was based on the observation that $75 \pm 18\%$ ($n = 3$) of [³H]acetimido-labelled VSG bound to the large surface area of glass-fibre filters. Even cellulose filters bound 30–38% of the denatured VSG when dissolved in chloroform/methanol.

Immunoblotting of VSG_m and VSG_r

Confirmation that the two forms of the VSG purified by the method described in this report constituted M_r variants of the true VSG was obtained by means of an immunoblot using an antibody (kindly given by Dr. M. J. Turner, Molteno Institute, Cambridge, U.K.) raised in rabbits against authentic VSG (MITat 1.1) that had been purified by the method of Cross (1975). The resulting immunoblot (Fig. 3) shows that both VSG_m and VSG_r bound antibody with high affinity. Confirmation that both forms of the VSG

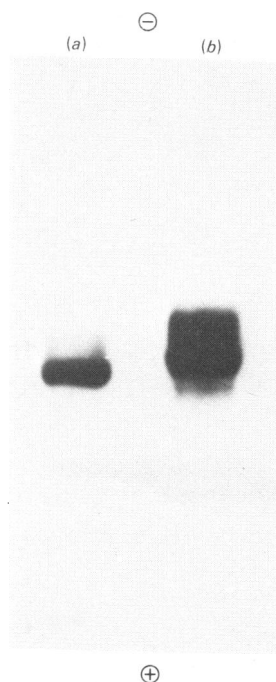


Fig. 3. Immunological identification of purified VSG_m and VSG_r.

Immunoblots of purified VSG_m (track a) and VSG_r (track b) detected with rabbit anti-(MITat 1.1 VSG_r) antiserum (see the Materials and methods section) and ¹²⁵I-Protein A.

carrying the same antigenic determinants were distinct entities was obtained by showing that they migrated with markedly different electrophoretic mobilities either separately or when mixed together before SDS/polyacrylamide-gel electrophoresis (Fig. 4).

Characterization of the two forms of purified VSG

It was clear from the amino acid analyses of VSG_m and VSG_r (Table 2) that there was no significant difference in amino acid composition between the two forms. Furthermore, these data are in close agreement with those reported originally by Cross (1975) for VSG (released form) of the same variant (MITat 1.1) when purified by conventional procedures, and that found by Owen (1976) for the VSG purified from trichloroacetic acid-treated cells (membrane-bound form).

End-group analyses of purified VSG species resulted in the identification of a single unmodified alanine residue at the *N*-terminus of both VSG_m and VSG_r, consistent with the absence of any covalent modification of the *N*-terminus during release of VSG from the cell surface. However, internal cleavage of the VSG to expose a new *N*-terminal alanine, although unlikely, cannot be

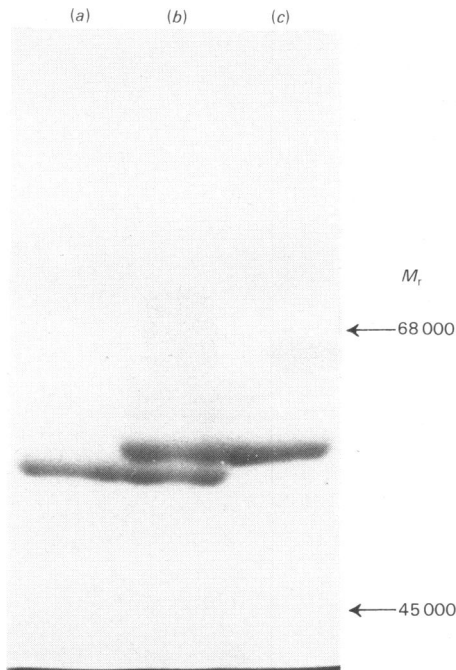


Fig. 4. SDS/polyacrylamide-gel electrophoresis of VSG_m and VSG_r from *T. brucei*

Samples of VSG_m and VSG_r purified by the solvent-extraction procedure were separated on a 7.5% acrylamide gel. The gel was stained with Coomassie Blue. The samples electrophoresed were the membrane-bound form after purification (track *a*), the released form after purification (track *c*) and equal amounts of both forms after purification, mixed and electrophoresed together (track *b*). The positions of the M_r markers bovine serum albumin (M_r 68000) and ovalbumin (M_r 45000) are indicated with arrows.

eliminated unequivocally. Both the selective ^3H -labelling procedure of Matsuo *et al.* (1966) and hydrazinolysis (Fraenkel-Conrat & Tsung, 1967) failed to identify a free C-terminus in either form of the VSG. This latter result demonstrated that both forms of the VSG contained blocked C-termini and suggested that the cross-reacting carbohydrate determinant, which is known to be linked to the C-terminal carboxy group of VSG_r via a molecule of ethanolamine (Holder, 1983), most probably was linked also to the C-terminus of VSG_m .

Number of copies of the variant surface glycoprotein attached to the plasma membrane of a single cell and its packing density

Each cell was found to contain $1.13 (\pm 0.02) \times 10^7$ copies of VSG attached to the outer surface of the plasma membrane (Table 3). In addition, the surface area of an individual trypanosome was

found to be $1.865 \times 10^8 \text{ nm}^2$ (Fig. 1). These values allow the packing density of the VSG to be calculated as 1 copy/ 16.5 nm^2 .

Discussion

The solvent-extraction/partition technique is a new method for purifying either form of the variant surface glycoprotein that should facilitate the determination of the covalent difference(s) between both forms of the VSG. When the technique is applied to trichloroacetic acid-treated trypanosomes, the membrane-bound form of the VSG is purified. Alternatively, when the technique is applied to the trichloroacetic acid-treated supernatants of cells, previously incubated with either benzyl alcohol (Jackson, 1983) or Ca^{2+} plus ionophore A23187 (Bowles & Voorheis, 1982; Voorheis *et al.*, 1982) to release VSG_r specifically, or with sufficiently hypo-osmotic medium at 37°C to cause cell rupture (Cross, 1975; Bowles & Voorheis, 1982), the released form of the VSG is purified. The released form of the VSG from the variant used in this study has the higher apparent M_r of the two forms as assessed by SDS/polyacrylamide-gel electrophoresis. However, this change in electrophoretic mobility is also observed in non-equilibrium pH-gradient electrophoresis and has been shown to result from the appearance of an additional negative charge on VSG_r , created by cleavage of a phosphodiester bond on VSG_m during its release from the surface of the cell (Jackson & Voorheis, 1985).

Treatment of trypanosomes with trichloroacetic acid must inactivate the enzyme responsible for VSG release *in vivo* as well as leading to protonation of many functional groups on the VSG and unfolding of the tertiary and possibly the secondary structure of the VSG, facilitating its solubility in chloroform/methanol. In fact, acid treatment of this protein promoted its re-partition into the aqueous phase to an extent that was 10-fold greater than the extent of its initial solubility in the organic phase. Furthermore, protonation of VSG_m was required in its own right for the observed partition behaviour, since denaturation with either urea or NaOH was insufficient for sequential extraction/re-partition. The observation that the acid-treated denatured VSG that has dissolved in chloroform/methanol can be partitioned subsequently into an aqueous phase suggests that under appropriate conditions the VSG may undergo reversible denaturation/renaturation. This suggestion also is strengthened by the further observation that VSG that has been through the complete purification procedure and freeze-dried can then once again, only if re-treated with trichloroacetic acid, be extracted into chloroform/

Table 2. *Amino acid compositions of the membrane-bound and released forms of the variant surface glycoprotein (MITat 1.1)*
Purified variant surface glycoproteins (MITat 1.1) were hydrolysed in HCl (6M) at 110°C *in vacuo* for 24h before amino acid analysis with a Locarte single-column automated amino acid analyser. Results are expressed as means \pm S.E.M. for three separate determinations. The contents of cysteine, methionine and tryptophan were not determined.

Amino acid	Composition of VSG (residues/100 amino acids)	
	Membrane-bound form	Released form
Lys	10 \pm 0.9	10 \pm 0.5
His	1.6 \pm 0.1	1.9 \pm 0.2
Arg	5.2 \pm 1.2	3.3 \pm 0.3
Asx	10.9 \pm 1.2	10.2 \pm 2
Thr	10.3 \pm 1.2	10 \pm 2
Ser	4.8 \pm 0.2	4.9 \pm 0.3
Glx	13.5 \pm 1.4	14 \pm 1
Pro	3.4 \pm 0.4	3.5 \pm 0.3
Gly	7.1 \pm 0.5	8.2 \pm 0.8
Ala	12.8 \pm 1.2	12.6 \pm 0.8
Val	4 \pm 0.2	4 \pm 0.2
Ile	3.5 \pm 0.5	3.9 \pm 0.2
Leu	8.7 \pm 0.9	9.6 \pm 1
Tyr	2 \pm 0.2	2.2 \pm 0.3
Phe	1.9 \pm 0.3	1.7 \pm 0.2

Table 3. *Estimation of the number of copies of variant surface glycoprotein in the plasma membrane of each cell of T. brucei*
Radiolabelled VSG_m was purified from surface-labelled trypanosomes by the solvent-extraction procedure, and the amount of radioactivity both in whole cells and in the purified VSG_m was determined by liquid-scintillation spectrometry. Protein was measured by the method of Lowry *et al.* (1951). For Expts. 1 and 2, each value is the mean \pm S.E.M. for three determinations. The S.E.M. for the average of both experiments was computed by Fieller's theorem (Finney, 1978).

	$10^3 \times$ Amount of [³ H]acetimido-labelled VSG in whole trypanosomes (d.p.m./cell)	Specific radioactivity of purified [³ H]acetimido- labelled VSG (d.p.m./ng)	Amount of VSG in whole trypanosomes	
			(pg/cell)	($10^{-7} \times$ No. of copies/cell)
Expt. 1	4.68 \pm 0.12	3.94 \pm 0.22	1.19 \pm 0.07	1.23 \pm 0.08
Expt. 2	4.55 \pm 0.18	4.52 \pm 0.15	1.00 \pm 0.05	1.02 \pm 0.05
Mean	4.62 \pm 0.11	4.23 \pm 0.13	1.10 \pm 0.04	1.13 \pm 0.04

methanol and subsequently re-partitioned into aqueous NaCl. No attempt has been made to discover how many times the VSG can be cycled in turn through organic and aqueous phases. However, this property was not shared by any other protein in *T. brucei*, but was demonstrated by both forms of the surface antigen. Reversible denaturation/renaturation most likely is a consequence of the large α -helical content of VSG, as reported previously (Cohen *et al.*, 1984; Freymann *et al.*, 1984). Such behaviour is commonly associated with proteins such as the keratins, which are known to have a large helical content.

The solvent-extraction/partition procedure has the advantage over conventional techniques for purifying the VSG by being both very rapid and simple as well as providing a high yield. In

addition, the procedure allows simultaneous purification of both apparent- M_r forms of the VSG from the same batch of cells without the use of detergent and in the complete absence of proteolytic degradation, which has been a problem for workers in the past (Cross, 1975; Barbet & McGuire, 1982).

The number of molecules of VSG per cell determined in these studies is about double the estimate made by Cross (1975). However, the density of packing found in the present study and that by Cross (1975) is about the same, since we also find a much larger surface area, probably owing to a somewhat closer approximation to the true surface geometry. This larger number of molecules of VSG is also found in other variants (M. J. Turner, unpublished work). It is also noteworthy that 42% of the VSG is predicted from

our model to be attached to that portion of the plasma membrane covering the flagellum.

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