Variant specific antigens of *Trypanosoma brucei* exist in solution as glycoprotein dimers

Catherine A. AUFFRET and Mervyn J. TURNER

M.R.C. Biochemical Parasitology Unit, The Molteno Institute, University of Cambridge, Downing Street, Cambridge CB2 3EE, U.K.

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Purified variant specific antigens of *Trypanosoma brucei* were shown to exist in solution as dimers, and occasionally as higher oligomers, as judged by gel filtration and by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after treatment with bifunctional cross-linking reagents.

The parasitic protozoan *Trypanosoma brucei* evades the host's immune system by a process of antigenic variation (reviewed by Cross, 1978). Infection is characterized by a relapsing parasitaemia, with each relapse representing elimination of parasites by antibody, and each recrudescence the appearance of a new antigenic type. Antigenic variation is a consequence of changes in the composition of a glycoprotein, which forms a dense covering 12–15 nm thick over the entire surface of the organism (Vickerman, 1969). Each new antigenic type is characterized by an immunologically distinct glycoprotein (Cross, 1975).

These variant specific antigens may be readily purified (Cross, 1975). We have purified a number of such specific antigens to homogeneity, and during their characterization we observed anomalies in the apparent molecular weight obtained on gel filtration. These results could be explained by the existence of the antigen as a dimer in solution, and this interpretation was tested by the use of bifunctional cross-linking reagents.

Materials and methods

The variant specific antigens used were prepared from clones of *Trypanosoma brucei* strains 427 and LUMP 227 by the method of Cross (1975). Purity of the glycoprotein preparations was assessed by SDS/polyacrylamide-gel electrophoresis, performed on polyacrylamide gradients (7–20%) in slab gels, with the buffer system of Laemmli (1970). Gel filtration of variant specific antigens was performed on Sephadex G-200 (Pharmacia, London W.5, U.K.) in 0.1 M-NH₄HCO₃ or in 0.1 M-NH₄HCO₃ supplemented with either 1% (w/v) SDS or 1%

(w/v) sodium cholate. Columns were calibrated with Blue Dextran (V_0), rabbit immunoglobulin (mol.wt. 160 000), bovine serum albumin (mol.wt. 68 000), ovalbumin (mol.wt. 45 000), pepsin (mol.wt. 34 500), carbonic anhydrase (mol.wt. 29 000), lysozyme (mol.wt. 14 400) and sodium chromate (V_1).

The C-terminal glycopeptide of antigen 121 was purified after CNBr treatment (Gross, 1967) of carboxymethylated antigen, which was prepared by the method of Kolb *et al.* (1974). After dialysis against 10 mM-sodium acetate buffer, pH 5.9, containing 0.6% NaCl, insoluble peptides were removed by centrifugation at 5000 g (r_{av} 12 cm) for 10 min at 23°C, and the supernatant was applied to a column of concanavalin A–Sephacrose (Pharmacia) equilibrated in the same buffer at 23°C. The glycopeptide was eluted from the column in the same buffer supplemented with 2% (w/v) α -methyl D-glucoside. On SDS/polyacrylamide-gel electrophoresis the glycopeptide had a molecular weight of 29 000, compared with 24 500 for the C-terminal tryptic glycopeptide prepared from the same antigen by the method of Cross & Johnson (1976). Tryptic-digest 'maps' of the two glycopeptides were identical with the exception of some additional spots in the CNBr-cleavage fragment (C. A. Auffret, unpublished work). Gel filtration of the C-terminal glycopeptide was performed on Ultrogel AcA-54 and AcA-34 (LKB Instruments, South Croydon, Surrey, U.K.) in 0.1 M-NH₄HCO₃ or 0.1 M-NH₄HCO₃ containing 1% (w/v) SDS.

Cross-linking experiments were performed with dimethyl suberimidate (Sigma Chemical Co., Poole, Dorset, U.K.) in 0.2 M-N-methylmorpholine/HCl buffer, pH 8.5, for 16 h at 23°C, at a protein concentration of 1 mg/ml and 5 mg of dimethyl suberimidate/ml. Tartryl diazide and tartryl

Abbreviation used: SDS, sodium dodecyl sulphate.

di(glycylazide) were prepared and used as described (Lutter *et al.*, 1974). Cross-linking was also attempted with sodium metaperiodate and sodium borohydride (Svoboda & McConkey, 1978). The method essentially followed that described for linking ribosomal proteins to rRNA. Glycoprotein was used at a concentration of 1–2.5 mg/ml. Incubation times were increased to approx. 4 h at 4°C, the oxidation being terminated by making the mixture 2% (v/v) with respect to ethylene glycol. In all cases the molecular weights of products formed were assessed by SDS/polyacrylamide-gel electrophoresis.

Results and discussion

Table 1 shows the results obtained by gel filtration of antigen 121, carboxymethylated antigen 121 and the C-terminal glycopeptide of antigen 121 under a variety of conditions. In the absence of detergent, antigen 121 has an apparent molecular weight of 125 000, approximately twice the molecular weight (63 000) observed on SDS/polyacrylamide-gel electrophoresis under denaturing conditions. Carboxymethylated antigen 121, which is only sparingly soluble, forms high-molecular-weight aggregates, which are eluted in the void volume. Treatment of both native antigen 121 and carboxymethylated antigen 121 with SDS decreases the apparent molecular weight to 67 000. The same decrease is observed after treatment of native antigen 121 with the non-denaturing ionic detergent sodium cholate. The C-terminal glycopeptide, which, in contrast with carboxymethylated antigen from which it is prepared, is very soluble in aqueous buffers, also appears to form dimers. In the absence of SDS, it runs with a molecular weight of 50 000. This is decreased to 24 000 after treatment with SDS.

Table 2 summarizes the results of the cross-linking experiments. Five of the eight variant specific antigens tested gave at least 80% cross-linking. In all cases the products gave diffuse bands on SDS/polyacrylamide-gel electrophoresis, but the molecular-weight range observed is consistent with the formation of dimers. The large numbers of lysine residues present (approximately 70) could lead to a wide range in the molecular weights of the products formed. Alternatively, SDS binding and the configuration of the products in SDS would be affected by variations in the amount of inter- and intramolecular cross-linking, giving rise to different sieving properties in the polyacrylamide gel. In some cases, however, and notably with antigens 201 and 207, the existence of trimers and tetramers cannot be ruled out. These antigens have not been studied by gel filtration to date. After treatment with sodium cholate, which was shown to dissociate antigen 121 dimer to monomer, as judged by gel filtration, no cross-linking of antigen 121 by dimethyl suberimide was observed.

Carboxymethylated antigen 121 clearly produced oligomers after treatment with dimethyl suberimide. All oligomers up to pentamers were visible. In addition, some material, presumably representing higher multimers, failed to enter the gel. This is clearly compatible with the results obtained by gel filtration.

Cross-linking of antigen 121 could be obtained with three other reagents, at lower yields. Two of these, tartryl diazide and tartryl di(glycylazide), are similar to dimethyl suberimide, in that all three cross-link by coupling lysine residues. The reagents differ in the distance between the two functional groups. Thus tartryl diazide, in which the functional groups are approx. 0.6 nm apart, was found to be less efficient than tartryl di(glycylazide) (distance between functional groups 1.3 nm).

Table 1. *Gel filtration of antigen 121 and its derivatives in 0.1 M-NH₄HCO₃*

Chromatography was performed on a 1 cm × 60 cm column with a flow rate in all cases of 5 ml/h. Samples contained approx. 1 mg of protein. The column eluate was continuously monitored at 280 nm with an LKB Uvicord III recording absorption-meter. A single symmetrical peak was observed in all cases, with the exception of carboxymethylated antigen 121, which in the absence of SDS showed a trailing edge at the void volume. Regression analysis showed the likely error in each molecular-weight determination to be ±5%.

Sample	Support	Supplement to buffer	Sample preparation	Mol.wt.
Native antigen 121	Sephadex G-200	None	Dissolved in buffer	125 000
Native antigen 121	Sephadex G-200	1% SDS	Boiled for 2 min in buffer	67 000
Native antigen 121	Sephadex G-200	1% sodium cholate	Boiled for 2 min in buffer	67 000
Native antigen 121	Sephadex G-200	1% sodium cholate	Dialysed in buffer	65 000
Carboxymethylated antigen 121	Sephadex G-200	None	Dialysed in buffer	Aggregate at void volume
Carboxymethylated antigen 121	Sephadex G-200	1% SDS	Boiled for 2 min in buffer	67 000
C-Terminal glycopeptide	Ultrogel AcA-54	None	Dissolved in buffer	50 000
C-Terminal glycopeptide	Ultrogel AcA-34	1% SDS	Boiled for 2 min in buffer	24 000

Table 2. *Cross-linking of variant specific antigens in solution with bifunctional reagents*

Percentage cross-linking was estimated by scanning negatives of photographs of Coomassie Brilliant Blue-stained gels on a Joyce-Loebl microdensitometer, and integrating the areas under the peaks. Conditions for cross-linking are described in the text. (a) Molecular weights were obtained from SDS/polyacrylamide-gel electrophoresis under reducing conditions. Omission of 2-mercaptoethanol does not result in any significant differences in observed values. (b) Each cross-linked product gave a diffuse band on SDS/polyacrylamide-gel electrophoresis (see Fig. 1). In each case the molecular weight quoted represents the most abundant product (Fig. 1). (c) Multiple diffuse bands were observed. Molecular weights quoted represent the most abundant products. (d) Antigen was denatured by incubation at 20°C in 6 M-guanidinium chloride/1 mM-dithiothreitol for 1 h before dialysis into cross-linking buffer. Abbreviations used for derivatives of antigens: CM, carboxymethylated derivative; CT, C-terminal glycopeptide derivative. Abbreviations used for cross-linking reagents: DMS, dimethyl suberimide; TDA, tartryl diazide; TDGA, tartryl di(glycylazide); IO₄/BH₄, periodate/borohydride.

Substrate	Reagent	Cross-linking (%)	10 ⁻³ × Mol.wt. of monomer (a)	10 ⁻³ × Mol.wt. of cross-linked product (a)
Antigen 121	DMS	95	63	150; 120 (c)
Antigen 060	DMS	80	56	115 (b)
Antigen 116	DMS	94	61	120 (b)
Antigen 119	DMS	78	59	120 (b)
Antigen 221	DMS	90	58	118 (b)
Antigen 201	DMS	49	62	130; 200 (c)
Antigen 207	DMS	20	65	140; 200 (c)
Antigen 211	DMS	10	63	135 (b)
Antigen 121	TDA	75	63	150 (b)
Antigen 121	TDGA	85	63	150 (b)
Antigen 121	IO ₄ /BH ₄	25	63	130 (b)
Antigen 121 in sodium cholate	DMS	0	63	
CM antigen 121	DMS	73	67	130; 200; >300 (c)
Denatured antigen 121 (d)	DMS	63	70	130; 200; >300 (c)
CT antigen 121	DMS	10	29	
CT antigen 121	TDA	50	66	130; 200; >300 (c)
Denatured antigen 121 (d)	TDA	52	66	130; 200; >300 (c)
CT antigen 121	TDA	0	35	
CM antigen 121	TDGA	64	68	130; 200; >300 (c)
Denatured antigen 121 (d)	TDGA	86	64	130; 200; >300 (c)
CT antigen 121	TDGA	0	35	
CM antigen 121	IO ₄ /BH ₄	0	68	
CT antigen 121	IO ₄ /BH ₄	0	29	

The third reagent (periodate/borohydride) cross-links carbohydrate to lysine. Treatment with sodium metaperiodate cleaves *cis*-diols in the carbohydrate to generate aldehyde groups, which can form a Schiff base with the ϵ -amino group of lysine if the two groups come within about 0.2 nm of each other. The Schiff base can then be reduced with borohydride. Antigen 121 produced dimers in low yield (25%) after this treatment, but neither carboxymethylated antigen 121 nor the C-terminal glycopeptide gave detectable cross-linking. Fig. 1 shows a microdensitometer tracing from an SDS/polyacrylamide-gel electrophoretogram of antigen 121 and of antigen 121 cross-linked with periodate/borohydride, with dimethyl suberimide and with tartryl di(glycylazide).

The significance of the existence of solubilized variant specific antigens as dimers is not yet clear. We have shown that purified antigen, whether in the form of monomer, dimer or cross-linked by dimethyl

suberimide, binds to variant-specific antibody (results not shown), demonstrating that no readily detectable immunochemical differences exist. The variant specific antigens are readily solubilized from intact trypanosomes: about 80% of the antigen is released simply by rupturing the cells. As yet, we have been unable to detect any differences in the biochemistry of the remaining 20% of the antigen that remains bound to the plasma membrane, and which may be solubilized by the addition of detergents (M. J. Turner, unpublished work). Nor have we been able to demonstrate any form of the antigen giving a molecular weight greater than that of the water-soluble form. The presence of inhibitors of proteinases (phenylmethanesulphonyl fluoride, EDTA, aprotinin) has no effect on the release of antigen (M. J. Turner, unpublished work). At present, it must be concluded that antigen is released in the form in which it is bound to the membrane. The dimers observed may be generated during the

Fig. 1. Microdensitometer scan of SDS/polyacrylamide-gel electrophoretograms of antigen 121 and cross-linked derivatives

(a) Antigen 121; (b) periodate/borohydride-treated antigen 121; (c) dimethyl suberimide-treated antigen 121; (d) tartryl di(glycylazide)-treated antigen 121. The small peak at mol.wt. approx. 59 000 represents a proteolysis product of antigen 121, present to some extent in all preparations. Multiple molecular-weight determinations gave mean values $\pm$ S.E.M. of $64\,000 \pm 780$ for antigen 121 and of $150\,500 \pm 2300$ for the principal product of dimethyl suberimide cross-linkage.

release of antigen, which occurs by a mechanism as yet not understood, with the exposure of a hydrophobic membrane-binding site on the glycoprotein. Two antigen molecules could then come together to form a dimer to minimize interaction of these sites with water. To date we do not know if antigen exists as dimers on the cell surface because we have been unable to find conditions compatible with both the requirements for cross-linking and with trypanosome viability. Other workers (Grundy, 1979) have succeeded in detecting dimers on the cell surface. Whether cross-linking occurs at the same site in solution and on the cell surface is not clear.

The membrane-attachment site in the variant specific antigens of *T. brucei* appear to be within the C-terminal third of the molecule (Cross & Johnson, 1976). In this context, then, it is worthy of note that

the C-terminal glycopeptide of antigen 121 behaves as a dimer on gel filtration. However, only very small extents of chemical cross-linking of the glycopeptide have been observed. This portion of the molecule, representing about one-third of the polypeptide chain, contains about half the total lysine content (30 residues), and high concentrations of lysine could preclude the formation of intermolecular cross-links in favour of intramolecular bonds.

Alternatively, the denaturation of the antigen required for preparation of the glycopeptide may remove reactive lysine residues from the region of interchain contact. This may also explain why no cross-linking of the glycopeptide is observed on treatment with periodate/borohydride, whereas about 25% cross-linking can be obtained with the native antigen, suggesting that the carbohydrate side chain is close to the site of dimerization.

The dimers appear to be held together by hydrophobic forces, since treatment with detergents, and in particular the non-denaturing ionic detergent sodium cholate, disaggregates the molecules. We have demonstrated that these apparently water-soluble antigen preparations interact with lipid monolayers in a cholesterol-dependent fashion (Klein *et al.*, 1980). The relationship between oligomer formation, lipid binding and membrane interaction requires further investigation.

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