Physical Studies of *Trypanosoma brucei* Variant Surface Glycoproteins and Their Antigenic Determinants†

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ABSTRACT: Secondary structure determinations have been carried out on two antigenically related variant surface glycoproteins (VSG's) from *Trypanosoma brucei*, WaTat 1.1 and WaTat 1.12. The two molecules, which bear highly homologous amino-terminal sequences, showed subtle differences in their circular dichroism (CD). Computer analysis revealed that the contribution of α helix to the secondary structure of the VSG's was 49% for WaTat 1.1 and 52% for WaTat 1.12. Unfolding studies using guanidine hydrochloride suggested that the WaTat 1.12 VSG was slightly more resistant than WaTat 1.1 VSG to the effect of this reagent. The membrane form of WaTat 1.1 VSG purified by reverse-phase high-performance liquid chromatography gave CD and fluorescence spectra indicative of a partially unfolded or denatured molecule. It was also shown that the antigenic differences between the VSG's were due to surface-oriented topographically assembled epitopes which were highly sensitive to structural perturbations. Monoclonal antibodies specific for these epitopes bound to four discreet determinants on WaTat 1.1, one of which was absent from WaTat 1.12.

Trypanosome variant surface glycoproteins (VSG's)[1] are a family of highly polymorphic membrane proteins which provide an important mechanism for the escape of African trypanosomes from immunological elimination by the infected vertebrate host (Vickerman, 1975). The structural polymorphism of VSG molecules is reflected in the great antigenic variability of these proteins, and this relates to their function in the process of antigenic variation. The surface of the trypanosome is covered with a homogeneous layer of VSG, and thus, the antigenic phenotype of the intact organism is determined by the VSG. Trypanosome populations in the peripheral blood of infected mammals are usually composed of a major antigenic phenotype and several minor antigenic types. As the major phenotype is eliminated by the immune response of the host, a minor variant increases in number to become the major one in a subsequent parasitic wave in the peripheral blood (LePage, 1967). The ability of trypanosomes to express only one of a large number of available VSG genes and to switch antigenic phenotype in the process is referred to as antigenic variation.

The antigenic polymorphism of VSG's suggests that there is significant structural variation among VSG's at the primary level. Amino acid and DNA sequence data from several antigenically distinct VSG's (Bridgen et al., 1976; Pays et al., 1983) showed a high degree of variability at the amino terminals and significant homology at the carboxyl termini (Rice-Ficht et al., 1981). Earlier work on the proteolytic cleavage patterns of VSG (Johnson & Cross, 1979) showed that VSG molecules were organized into two functionally distinct domains. Recently, it has been shown that the larger amino-terminal domain which possesses the surface-oriented variable epitopes (Miller et al., 1984a,b) exhibits secondary structure potential which is highly representative of the intact molecule (Gomes et al., 1986). Conservation of sequence at the carboxyl terminal is likely maintained for structural reasons including, for example, the attachment of VSG to the trypansome plasma membrane (Ferguson & Cross, 1984).

A more recent examination of the amino-terminal sequences of VSG's from trypanosomes of the WaTat serodeme of *Trypanosoma brucei* (and from other serodemes and even other species) showed that there were significant primary structure homologies (Olafson et al., 1984) in this region of the molecule as well. Conserved residues were found to include structurally important amino acids such as cysteine and tryptophan. These observations lead the authors to conclude that the tertiary structures of VSG's were also likely conserved.

Two VSG's (WaTat 1.1 and 1.12) isolated from the WaTat serodeme were found to possess common surface-oriented epitopes (Barbet et al., 1982). Sequence analysis of the amino terminals of these molecules showed that they were highly homologous with only 2 conservative replacements in the first 30 residues (Olafson et al., 1984). Since it was previously suggested that the N-terminal of VSG's was exposed on the

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[1] Abbreviations: CNBr, cyanogen bromide; CD, circular dichroism; HPLC, high-performance liquid chromatography; Gdn·HCl (GuHCl in figures), guanidine hydrochloride; GITC, guanidine isothiocyanate; 2-ME, 2-mercaptoethanol; NC, nitrocellulose; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline with 0.05% Tween-20; 2-POH, 2-propanol; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; VSG, variant surface glycoprotein; DTT, dithiothreitol; Tris-HCl, tris-(hydroxymethyl)aminomethane hydrochloride; kDa, kilodalton(s).

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surface of the trypanosome (Bridgen et al., 1976), it appeared that this region of the molecule contained variable surface-oriented epitopes. However, antisera raised against cyanogen bromide digested VSG, containing antibodies binding to the amino-terminal CNBr fragment, were shown not to bind to the surface of living homologous trypanosomes (Clarke et al., 1987). Thus, either the amino-terminal epitope(s) recognized by the antisera is (are) not accessible on the surface of the trypanosome or the amino-terminal sequence of the native molecule does not contain the epitope(s) found in the denatured CNBr fragment.

Topological mapping of antigenic determinants on one VSG has shown that the epitopes which conferred the variable antigenic specificity of individual VSG mapped into five distinct groups, only one of which, containing a single epitope, was surface oriented (Miller et al., 1984a). Further work showed that all monoclonal antibodies which bound to VSG determinants on the surface of living trypanosomes could not be shown to bind to CNBr fragments (Miller et al., 1984b). Similar observations were made by Clarke et al. (1987) using a larger number of monoclonal antibodies. This evidence supports the suggestion that the surface-oriented epitopes of VSG's are topographically assembled epitopes which are likely to be highly sensitive to denaturation.

Low-resolution X-ray crystallographic data are available for two VSG molecules (Freyman et al., 1984; Metcalf et al., 1987). These data showed that the VSG's contained a high percentage of α-helical structure which gave the molecule on overall elongated shape. Electron microscopic analysis of two rotary-shadowed VSG molecules (Cohen et al., 1984) indicated that the three-dimensional structures were distinct from one another. It is therefore clear that the study of the structure of several VSG's is necessary to determine the extent of structural conservation within the entire VSG family. Recent advances in the generation of synthetic epitopes (Geysen et al., 1985) may make possible the development of vaccines based on conserved structural features of VSG's that are not immunogenic on the native molecule. As part of our investigations into the immunochemical and physical structures of VSG's from large *Trypanosoma brucei* serodeme, the WaTat serodeme (Barbet et al., 1982), we report here studies in which the physical characteristics of two cross-reactive VSG's and their antigenic determinants were examined. Two cross-reactive VSG's from the WaTat serodeme, WaTat 1.1 and WaTat 1.12, were examined for the extent of unfolding in the presence of Gdn·HCl by CD. In parallel experiments, the loss of binding by monoclonal antibodies to the unfolded VSG was followed in a dot blot assay. The data presented here show that the secondary structure potentials of the two related VSG's show subtle but significant differences. Additionally, the data showed that surface-oriented variant specific epitopes are extremely sensitive to structural perturbations and that relative sensitivity can be used to distinguish individual epitopes.

Materials and Methods

Trypanosomes. The origin and derivation of the WaTat serodeme of *T. brucei* have been previously described (Barbet et al., 1982). Trypanosomes were purified from the blood of infected rats by DEAE-cellulose chromatography as described (Lanham & Godfrey, 1970).

VSG Purification. The soluble form of VSG was purified by the method of Cross (1975), and the membrane form was purified by reversed-phase HPLC (Clarke et al., 1985).

Antigen Modification. WaTat 1.1 VSG (final concentration 50 μg/mL) was treated with the following reagents and procedures having a variety of effects on secondary structures: (i) PBS; (ii) aqueous 0.1% TFA; (iii) 65% aqueous 0.1% TFA plus 35% 2-POH (TFA/2-POH); (iv) 0.1% (w/v) SDS in PBS; (v) 0.5% (w/v) SDS in PBS; (vi) 10 mM 2-Me in PBS; (vii) 8 M urea in 10 mM Tris, pH 8.0, for 1 h at room temperature; (viii) 6 M Gdn·HCl in 10 mM Tris, pH 8.0, for 1 h at room temperature; (ix) 6 M GITC in 10 mM Tris, pH 8.0, for 1 h at room temperature; (x) 100 °C for 10 min in PBS.

Binding Assay. The binding of monoclonal antibodies to treated antigen (see below) was assayed in a nitrocellulose (NC) dot-blot assay. One-microliter volumes (50 μg/mL in 10 mM ammonium bicarbonate) of treated and untreated antigen were dotted onto NC paper in a 96-well configuration. The paper was dried at room temperature for 30 min, and remaining protein binding sites were blocked with 3% fish gelatin (Saravis, 1984) in phosphate-buffered saline (PBS), pH 7.4, and placed in a 96-well dot-blot manifold (BRL, Bethesda, MD). Monoclonal antibodies were diluted in PBS containing 0.05% Tween-20 (PBS-T) at a concentration which gave 50% maximal precipitation in an RIA assay with native, radiolabeled antigen (data not shown) and placed in the wells of the manifold. Binding was allowed to proceed for 2 h at room temperature, and the NC paper was removed from the manifold and washed for 30 min in three changes of PBS-T. The NC paper was incubated with an appropriate dilution of alkaline phosphatase labeled goat anti-mouse IgG (TAGO, Burlingame, CA). After 2 h at room temperature, the paper was washed as above, and bound antibody was visualized as previously described (Clarke et al., 1984). Results were expressed qualitatively as positive or negative based on comparison with appropriate controls, i.e., positive (untreated) antigen control, supernatants from myeloma cultures, and known positive rabbit anti-VSG antisera.

Monoclonal Antibodies. The monoclonal antibodies TRYP 1E1, TRYP 2B1, TRYP 4C1, TRYP 22A1, WAT 8A1, WAT 20A1, and WAT 24A1 have been described previously (Barbet et al., 1982). Monoclonal antibodies in mouse ascites fluids were precipitated with 50% ammonium sulfate and dialyzed overnight against 20 mM Tris-HCl pH 8.5. The dialysate was injected onto an Altex DEAE-5PW ion-exchange HPLC column. Antibody was eluted with a linear gradient from 20 mM Tris-HCl, pH 8.5, to 20 mM Tris, pH 7.0, containing 0.5 M NaCl. The major peak was collected and dialyzed against PBS.

CD Measurements and Epitope Denaturation Studies. These measurements were made on a Jasco 500C spectropolarimeter interfaced with a DP 500N data processor under constant nitrogen flushing. The cell was maintained at 25 °C using a Lauda K-2/R circulating water bath. Near-UV (320–250 nm) scans were performed in a microcell with a path length of 1 cm which required only 90 μL of solution. VSG concentrations were in the range of 0.7–1.0 mg/mL. Far-UV (250–190 nm) runs were done either in a 0.0103-cm pathlength cell, protein concentration ~0.7 mg/mL, or in a 0.0503-cm cell, protein concentration ~0.4 mg/mL. The computer-averaged trace of either four or eight scans was employed in all calculations. Signal due to solvent (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 0.5 mM DTT) was subtracted. The instrument was routinely standardized with *d*-10-camphorsulfonic and pantoyl lactone according to procedures outlined by the manufacturers. The data were normally plotted as mean residue weight ellipticity (units of degrees centimeter squared per decimole) vs wavelength in nanometers. The mean residue weight was taken to be 115. In

addition to these plots, the secondary structures of VSG's were determined by using a computer program, CONTIN, developed by Provencher and Glöckner (1981) which analyzes CD spectra as a sum of the spectra of 16 proteins whose structures are known from X-ray crystallography. Program input was mean residue weight ellipticites (1-nm intervals) from the minimum value measured to 240 nm. The unfolding effect of Gdn·HCl on the VSG's was monitored by CD as follows. A known volume of a solution of VSG was introduced into the cell and the appropriate CD spectrum measured. An aliquot of 6 M Gdn·HCl (Schwarz/Mann Ultra Pure) in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 0.5 mM DTT was added to the protein in the cell and the solution carefully mixed by gentle shaking. The spectrum was recorded. This procedure was repeated for additional higher concentrations of denaturant. An aliquot of protein prepared in 5.0 M Gdn·HCl was used as the final fully denatured form. Appropriate solvent base-line readings were subtracted in the usual way at each point on the denaturation curve. Over the time interval 2–30 min, no time-dependent changes were noted in the ellipticity values.

WaTat 1.1 and WaTat 1.12 VSG's were also exposed to a range of Gdn·HCl concentrations in 20 mM Tris, pH 7.4, containing 0.15 M NaCl for 24 h and then lightly fixed with 0.01% glutaraldehyde. This concentration of glutaraldehyde was shown in preliminary experiments to be the maximum which did not interfere with antibody binding to VSG before treatment with Gdn·HCl. The treated VSG's were then applied to NC paper and assayed in the dot-blot assay described above with the panel of monoclonal antibodies.

Fluorescence Studies. Fluorescence emission and excitation spectra were measured on a Perkin-Elmer MPF 44B recording spectrofluorometer equipped with the DCSU-2 corrected spectra accessory which allows for automatic subtraction of fluorescence due to solvent. The instrument was operated in the ratio mode, and thermostated cells were maintained at 20 °C. The emission and excitation slits were usually set at 5 nm.

Estimation of Protein Concentration. Protein concentrations were measured by the absorbance at 280 nm with extinction coefficients established using the refractometric method of Babul and Stellwagen (1969) in which the absorbance of a protein sample is correlated with its weight concentration, the latter determined from synthetic boundary experiments in the analytical ultracentrifuge. An average refractive increment of 4.1 fringes mg^{-1} mL^{-1} was used in these calculations.

Sedimentation Equilibrium and Comeasurements. A Beckman Spinco Model E analytical ultracentrifuge equipped with an RTIC temperature control unit and electronic speed control was operated at 20 °C. Cells with charcoal-filled Epon centerpieces and sapphire windows were used. Photographs from the Rayleigh interference optical system were analyzed on a Nikon Model 6C microcomparator. Weight-average molecular weights of VSG's were measured by conventional low-speed sedimentation equilibrium techniques (Richards et al., 1968). Equilibrium photos were taken at intervals after 20 h until no further change was apparent. Prior to these measurements, VSG samples were dialyzed for 48 h at 4 °C against 1000 volumes of 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 0.5 mM DTT to ensure complete equilibration. A value of 0.723 was used for the partial specific volume; this takes into consideration the 7% or so carbohydrate present.

RESULTS

Sedimentation Equilibrium Studies. Satisfactory sedimentation equilibrium experiments were performed for the soluble forms of WaTat 1.1 and WaTat 1.12. Essentially linear plots were obtained for the natural logarithm of the protein concentration versus the square of the distance from the center of the rotation; some deviation was noted near the meniscus, indicating a few percent of lighter material. For WaTat 1.12, the molecular weight ranged across the solution column from 107 400 at the meniscus to 113 000 at the cell bottom. In the case of WaTat 1.1, these figures ranged from 91 600 to 113 100. These data suggest extensive dimer formation in solution for both forms as has been noted for other soluble form VSG's (Auffret & Turner, 1981). Since the molecular weights for WaTat 1.1 and 1.12 are virtually identical at 58 000 (M. W. Clarke, unpublished observations) as determined by SDS gel electrophoresis, the differences noted in the range of molecular weights are probably a reflection of differences in the association constants for monomer to dimer conversion. More extensive studies would be required to answer this question. It has not proved possible to obtain an estimate of the molecular weight in solution for the membrane form of WaTat 1.1 purified by HPLC. Attempts to obtain redistribution of this material in the ultracentrifuge cell under a variety of conditions for sedimentation equilibrium have all been unsuccessful. Whether extensive aggregation or protein degradation is responsible for this problem is unclear at this time.

UV Absorption Spectra and Concentration Determination. The UV absorption spectra have been measured for the soluble forms of WaTat 1.1 and WaTat 1.12 and for the HPLC-purified membrane form of WaTat 1.1. The spectra of the soluble forms are both characterized by maxima near 280 nm and pronounced "notch-effects" near 291 nm. For soluble form WaTat 1.1, the 280/260-nm absorption ratio is 1.57, and the width of the spectrum at half-height is 40.0 nm. In the case of soluble form WaTat 1.12, these figures are 1.67 and 32.0 nm, respectively. There was no abnormal contribution due to light scattering in either of these spectra. The spectrum of HPLC-purified membrane form WaTat 1.1 VSG had a broad maximum centered near 277 nm. The notch-effect was much less pronounced. The 280/260-nm absorption ratio was 1.45, and the width at half-height was ~43 nm. There was a more pronounced contribution from light scattering. Second-derivative spectroscopy confirmed the presence of tryptophan in all three VSG's (a large minimum noted at 291 nm).

Synthetic boundary, fringe counting (C_0) experiments in the ultracentrifuge established the following coefficients: soluble WaTat 1.1 $A_{280nm}^{1mg/mL} = 0.842$; soluble WaTat 1.12 $A_{280nm}^{1mg/mL} = 0.787$; membrane form WaTat 1.1 $A_{280nm}^{1mg/mL} = 0.580$.

CD Spectra. Far-UV CD data for the soluble form of WaTat 1.1 and WaTat 1.12 are shown in Figures 1 and 2, respectively, in their native conformations and in several denatured states in increasing concentrations of Gdn·HCl. The spectra for the native proteins are characterized by the bimodal patterns with minima near 220 and 208 nm typical of proteins containing considerable amounts of α-helix. That these two VSG's do not have identical secondary structures is shown by the difference in $[\theta]_{220nm}$, −13 300° versus −15 000° for WaTat 1.1 and WaTat 1.12, respectively.

The far-UV CD spectrum for the HPLC-purified membrane form of WaTat 1.1 is shown in Figure 3. This spectrum, which shows a single minimum around 206 nm with a shoulder near 215 nm, is typical of a protein with extensive amounts of aperiodic and β-sheet in its structure. It bears little resemblance to the spectra of the soluble form of either WaTat 1.1 or WaTat 1.12.

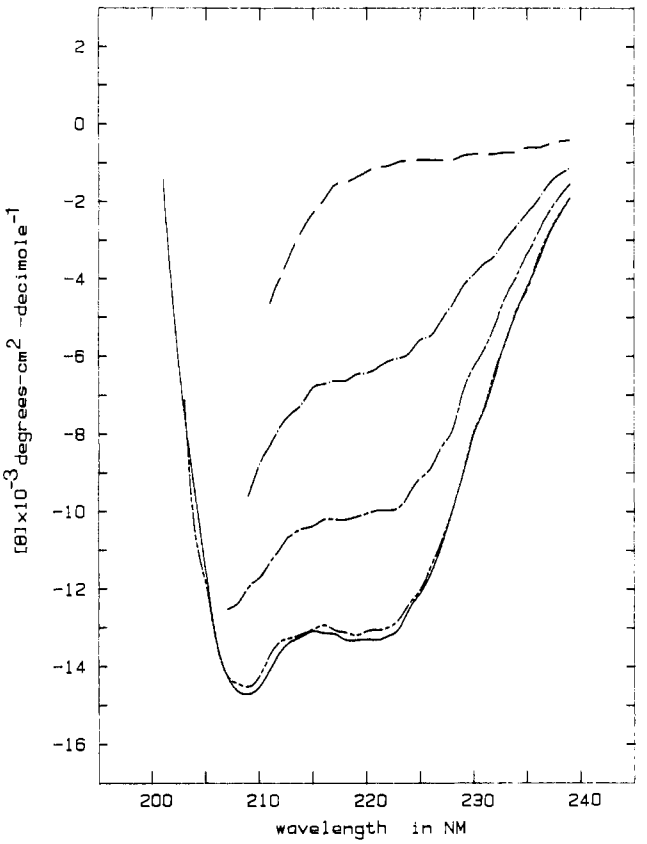

FIGURE 1: Far-UV CD spectra for the soluble form of WaTat 1.1 VSG. The solvent was 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 0.5 mM DTT. Aliquots of Gdn·HCl (6 M in the above buffer) were added. The initial protein concentration was 0.4 mg/mL, and the spectra were recorded at 25 °C. (—) Native protein; (-·--) 0.375 M; (-·-) 1.0 M; (-·-) 1.26 M; and (--) 5.0 M Gdn·HCl, respectively.

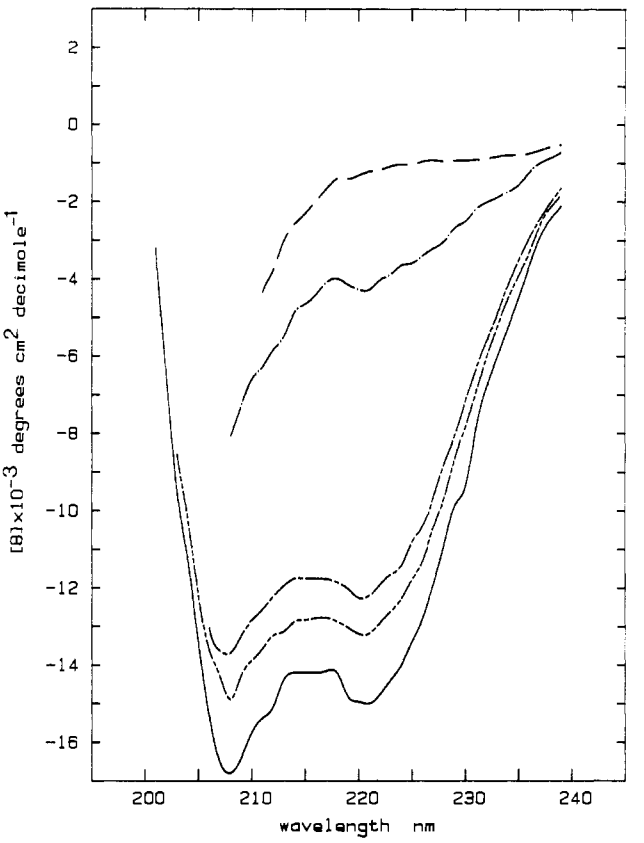

FIGURE 2: Far-UV CD spectra for the soluble form of WaTat 1.12 VSG. Solvent as in Figure 1. The initial protein concentration was 0.42 mg/mL, and the temperature was 25 °C. (—) Native protein; (-·--) 0.375 M; (-·-) 0.706 M; (-·-) 1.5 M; and (--) 4.8 M Gdn·HCl, respectively.

Near-UV CD data for the soluble forms of WaTat 1.1 and WaTat 1.12 are shown in Figures 4 and 5, respectively, for the native forms and several denatured states in increasing concentrations of Gdn·HCl. The native spectra for these two VSG's are very similar and are characterized by troughs near 295, 288, and 281 nm with peaks near 290, 283, and 260 nm. Apparently, representatives of all three types of aromatic residue, tryptophan, tyrosine, and phenylalanine, are located in asymmetric centers and contribute to the observed optical activity. The effect of Gdn·HCl is to progressively reduce the ellipticity in this spectral region. At high denaturant concentration, the ellipticity over the range 270–290 nm was observed to flip over from negative to positive.

Figure 6 shows the corresponding spectra for the HPLC-purified membrane form of WaTat 1.1. The spectrum of the native protein is of the same general shape as those of the soluble forms of WaTat 1.1 and WaTat 1.12, but a considerable diminution has occurred in the overall ellipticity. The denaturative effect of Gdn·HCl is similar in that the spectrum in the region 270–290 nm changes from negative to positive ellipticity.

Fluorescence. Upon excitation at 280 nm (the maximum in the excitation spectrum), the soluble forms of WaTat 1.1 and WaTat 1.12 display emission maxima at 305 ± 2 nm. This value corresponds to the emission wavelength noted for proteins which lack tryptophan and contain only tyrosine. In light of the fact that second-derivative spectroscopy strongly suggested the presence of tryptophan in all three VSG's, these tryptophan residues must be buried in hydrophobic regions of the molecule such that their fluorescence is completely quenched. Determination of the amino acid compositions of

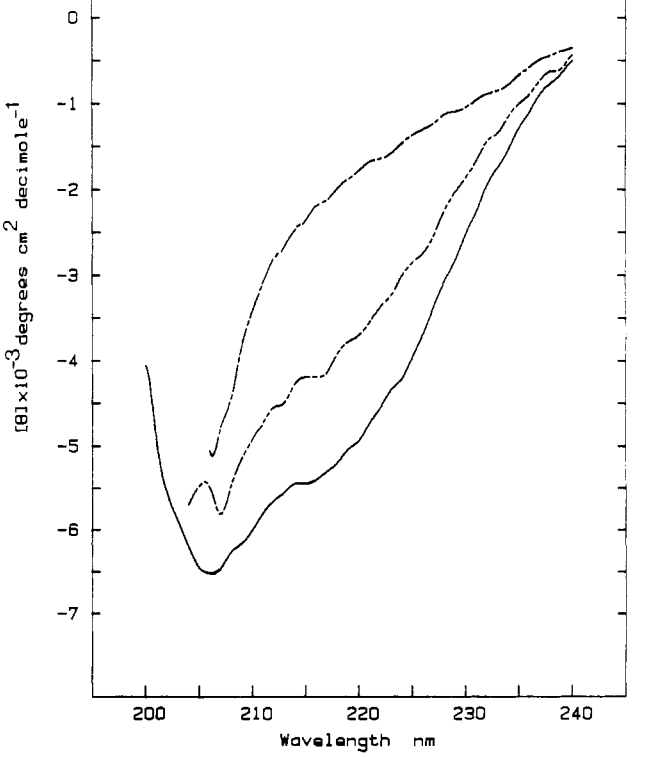

FIGURE 3: Far-UV CD spectra for HPLC-purified membrane form of WaTat 1.1. Solvent as in Figure 1. Initial protein concentration was 0.45 mg/mL. Temperature was 25 °C. (—) Native protein; (-·--) 0.375 M; and (-·-) 1.5 M Gdn·HCl, respectively.

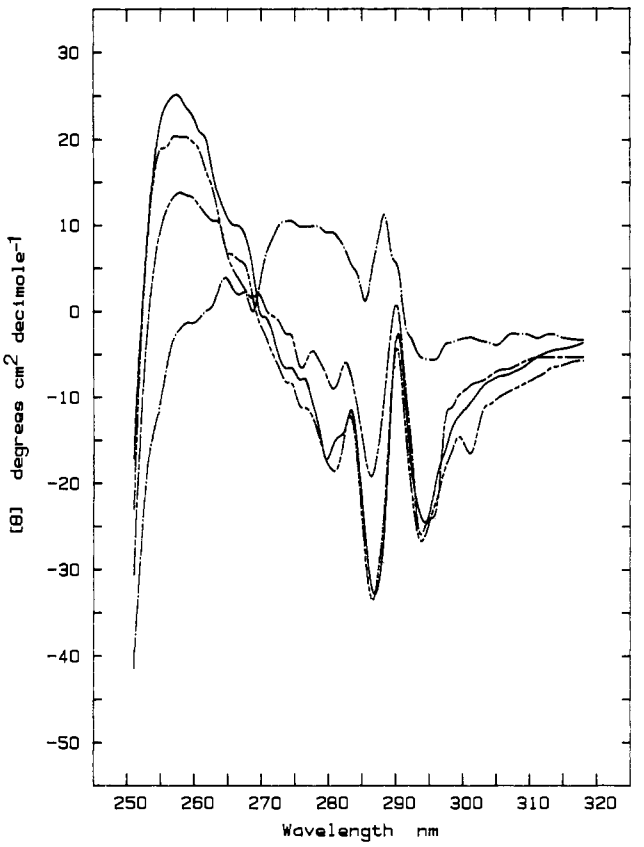

FIGURE 4: Near-UV CD spectra for the soluble form of WaTat 1.1 VSG. The solvent was 150 nm NaCl, 50 mM Tris-HCl, pH 7.5, and 0.5 mM DTT. Aliquots of Gdn·HCl (6 M in above buffer) were added. Path length 1 cm, temperature 25 °C. Protein concentration was 0.68 mg/mL. (—) Native protein; (----) 0.34 M; (-·—) 0.706 M; and (-·-) 1.55 M Gdn·HCl, respectively.

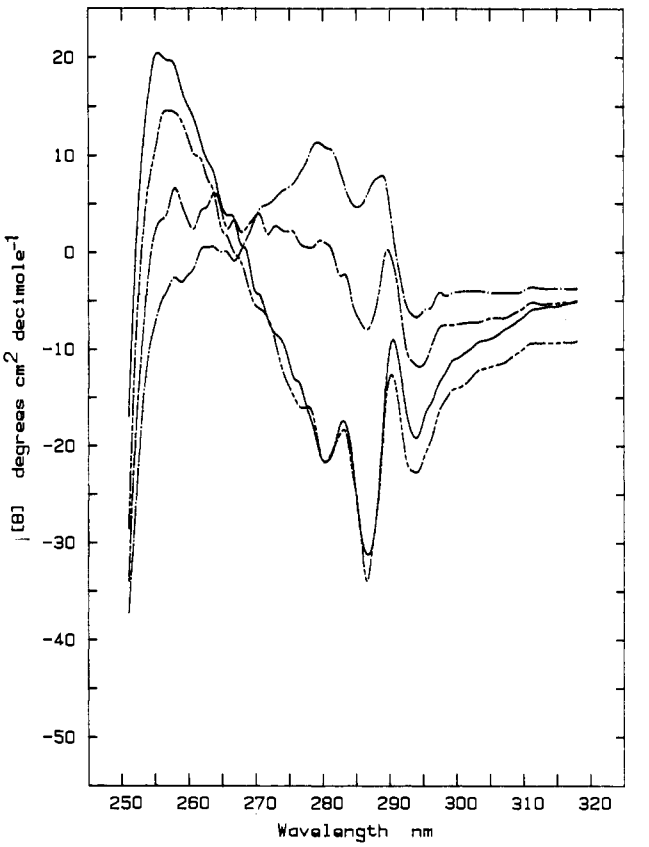

FIGURE 5: Near-UV CD spectra for the soluble form of WaTat 1.12 VSG. Solvent conditions as in Figure 4. Protein concentration was 0.83 mg/mL, 1-cm path-length cell, 25 °C. (—) Native protein; (-·--) 0.64 M; (---) 1.0 M; and (-·-) 1.5 M Gdn·HCl, respectively.

WaTat 1.1 and WaTat 1.12 (Olafson et al., 1984) did not include an analysis for tryptophan content. Information as to the mole percent concentration of tryptophan in these VSG's is therefore not available. It is noteworthy that the HPLC-purified membrane form of WaTat 1.1, shown to be partially unfolded by CD, displays an emission maximum at 335 ± 2 nm, a position indicative of partially buried tryptophan residues.

Unfolding in Gdn·HCl. The complete unfolding titrations of the soluble forms of WaTat 1.1 and WaTat 1.12 as measured by far-UV CD are presented in Figure 7 where the ellipticity values at 220 nm ($[\theta]_{220nm}$) are plotted as a function of denaturant concentration. The 50% transition point for soluble WaTat 1.1 occurs at 1.2 M Gdn·HCl while that for soluble WaTat 1.12 is at 1.28 M Gdn·HCl. It would also appear that WaTat 1.12, although it shows the higher initial ellipticity, is very sensitive to low concentrations (up to 0.4 M) of Gdn·HCl.

These data have been analyzed by the computer program CONTIN of Provencher and Glöckner (1981) to estimate the amount of α-helix, β-sheet, β-turn, and aperiodic structure present in these proteins. The results are presented in Table I. Data for the membrane form of WaTat 1.1 are also included here. A word of caution is needed before examining these data in depth. As the concentration of Gdn-HCl is increased, the CD spectrum cannot be measured as far into the deep ultraviolet, and, hence, the quality of fit is reduced, and some anomalous values for α-helix are obtained. It should be noted that native soluble WaTat 1.1 has 49% α-helix while native soluble WaTat 1.12 has 52%. This is a significant difference when one considers that the difference in $[\theta]_{220nm}$

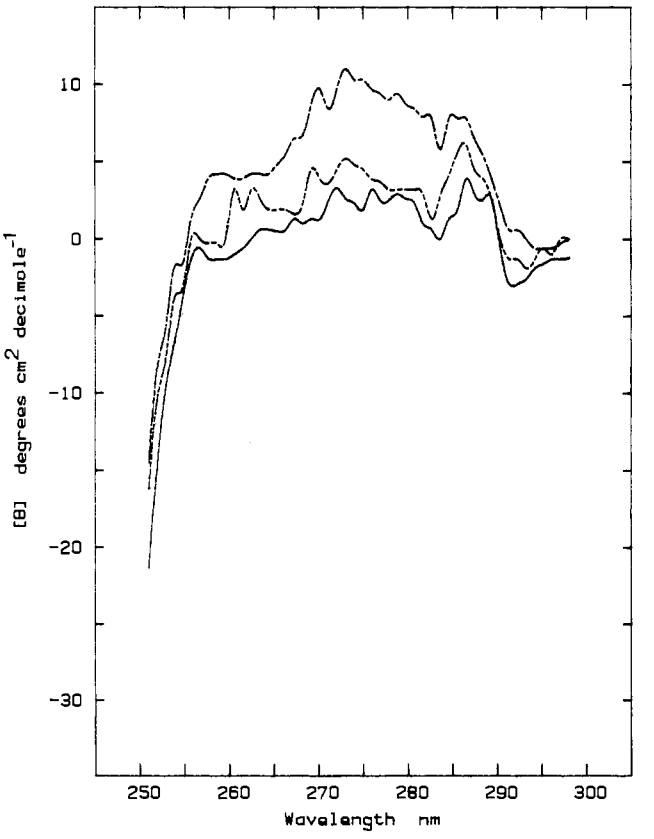

FIGURE 6: Near-UV CD spectra for HPLC-purified membrane form of WaTat 1.1 VSG. Solvent and temperature conditions as in Figure 4. Protein concentration was 0.95 mg/mL. (—) Native protein; (----) 0.29 M; and (---) 0.89 M Gdn·HCl, respectively.

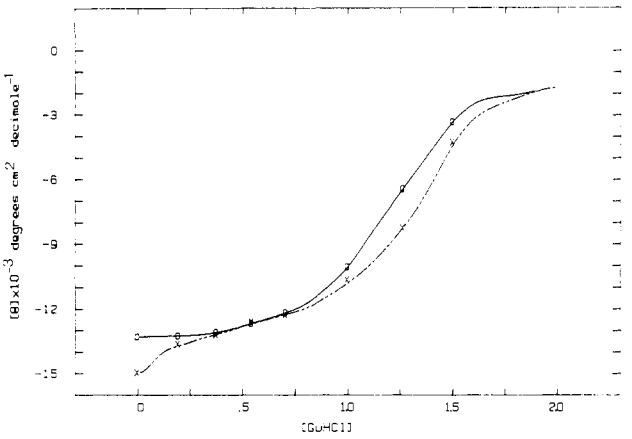

FIGURE 7: Gdn·HCl titration of the two soluble form VSG's as followed by the decrease in $[\theta]_{220nm}$ as a function of denaturant concentration. Since the changes in ellipticity were essentially complete for both VSG's by 2.0 M Gdn·HCl, this point has been taken as the end of the titration. Measurements were indeed extended to 5.0 M Gdn·HCl where $[\theta]_{220nm}$ was found to be −1260° and −1320° for WaTat 1.1 and WaTat 1.12, respectively. (×) Data for WaTat 1.1; (○) data for WaTat 1.12.

Table I: Computed Secondary Structure Contents of VSG's in Different Concentrations of Guanidine Hydrochloride

[Gdn·HCl] (M)	α-helix	β-sheet	β-turn	remainder
	(A) Soluble Form WaTat 1.1			
0 (native)	49	22	6	23
0.194	46	17	12	24
0.375	53	23	7	17
0.545	43	22	10	26
0.706	46	18	11	26
1.0	2.8	35	8	30
1.263	25	36	10	23
1.5	9	56	14	20
5.0	13	37	21	29
	(B) Soluble Form WaTat 1.12			
0 (native)	52	13	9	26
0.194	45	28	2	26
0.375	44	19	5	31
0.545	48	16	2	35
0.706	49	26	0	25
1.0	51	22	0	27
1.3	38	23	16	23
1.5	14	41	18	27
4.8	11	39	27	22
5.0	9	56	14	20
	(C) Membrane Form WaTat 1.1			
0 (native)	12	43	17	28
0 (native)	14	36	20	30
0 (native)	12	41	15	31
0.194	11	46	17	26
0.375	5	47	19	29
0.375	3	45	19	33
0.545	13	42	19	26
0.706	4	46	21	29
0.857	12	40	26	23
1.5	10	45	24	21
1.5	2	45	25	29

for these forms is some 1700° and that the uncertainty in measuring ellipticity at this wavelength is no more than ±300°. It is recognized that while these difference are beyond experimental error, since CD analysis is an empirical technique, one should not attach too literal a meaning to their translation in terms of small percentage changes in computed conformational forms. The partially unfolded nature of the HPLC-purified membrane form of WaTat 1.1 is quite evident as it only contains some 12% α-helix. This form is very sensitive to the presence of denaturant; structural changes are essentially complete by 1.5 M Gdn·HCl.

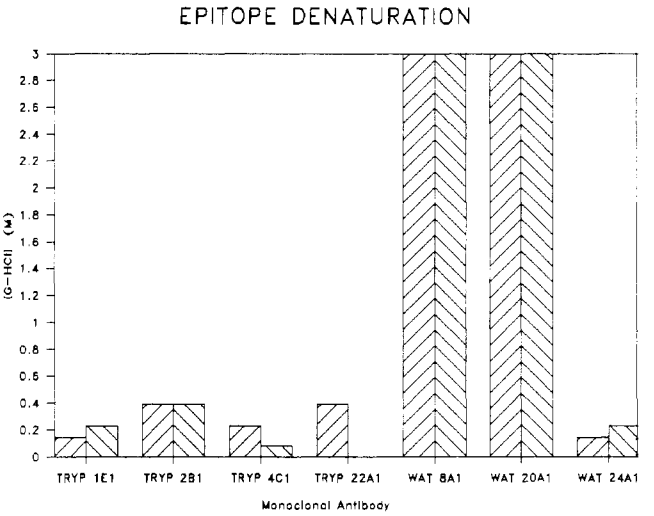

FIGURE 8: Partial denaturation of epitopes on WaTat 1.1 (▨) and WaTat 1.12 (▩) VSG's by exposure to Gdn·HCl. VSG's were exposed to varying concentrations of Gdn·HCl (*y* axis) for 24 h and then fixed with 0.01% glutaraldehyde and assayed in a NC dot-blot assay. The *y* axis represents the last concentration of Gdn·HCl at which binding was greater than a negative control (supernatants from SP2/0 myeloma cell cultures).

The general effect of Gdn·HCl on these systems is to reduce the amount of α-helix. In the case of the soluble forms of the VSG's, there is evidence that the amount of β-sheet and β-turns increases somewhat.

To assess the effect of unfolding on the integrity of antigenic determinants, the soluble forms of WaTat 1.1 and WaTat 1.12 were exposed to varying concentrations of Gdn·HCl for 24 h, lightly fixed with glutaraldehyde, and then assayed in the NC paper dot-blot assay. Results shown in Figure 8 are expressed as the highest concentration of Gdn·HCl in which antibody binding was detected by the individual members of the monoclonal antibody panel. Antibodies which bound to the surface of living WaTat 1.1 or WaTat 1.12 trypanosomes were not able to bind VSG which had been exposed to low concentrations of denaturant, suggesting that these determinants were composed of ordered structure. Two monoclonal antibodies, WaTat 8A1 and WAT 20A1, bound to both VSG's denatured by treatment with 3 M Gdn·HCl.

Antigen Modification. In these studies, WaTat 1.1 VSG was exposed to reagents having a variety of effects on polypeptide structure. Following the treatments, VSG was immobilized on NC paper and probed with the panel of monoclonal antibodies. The results in Table II show that surface-oriented epitopes are lost after relatively mild treatments. For example, exposure of WaTat 1.1 VSG to 0.1% SDS destroys the integrity of all surface-oriented epitopes bound by the monoclonal antibody panel. Treatment of VSG with 10 mM 2-ME does not result in the loss of integrity of any epitope bound by the panel of antibodies. The epitope on WaTat 1.1 bound by TRYP 4C1 was the only site sensitive to the effects of 8 M urea, and it was also affected by treatment with 6 M Gdn·HCl and 6 M GITC. The epitope bound by TRYP 1E1 was resistant to the effects of all three denaturants under the conditions employed in the assay. Further work has shown that this epitope is indeed sensitive to 6 M Gdn·HCl if the incubation period is 8 h or longer (see above, Antigen Modification). Monoclonal antibodies WAT 8A1 and WAT 20A1 are able to bind to VSG after treatment with all three denaturants as well as after boiling for 10 min. The epitope bound by TRYP 1E1 was resistant to treatment with 8 M urea and 6 M Gdn·HCl but sensitive to 6 M GITC. All sites were

Table II: Binding Activity of Anti-VSG Monoclonal Antibodies following Treatment of VSG with Various Reagents[a]

monoclonal antibody	reagent or treatment									
				SDS						
	PBS	0.1% TFA	TFA/2-POH	0.1%	0.5%	2-ME	8 M urea	6 M Gdn·HCl	6 M GITC	100 °C
TRYP 1E1[c]	+	+	+	−	−	+	+	+	+	−
TRYP 2B1[c]	+	+	+	−	−	+	+	−	−	−
TRYP 4C1[c]	+	+	+	−	−	+	−	−	−	−
TRYP 22A1[c]	+	+	+	−	−	+	+	+	−	−
WAT 8A1	+	+	+	+	−	+	+	+	+	+
WAT 20A1	+	+	+	+	−	+	+	+	+	+
WAT 24A1[c]	+	+	+	−	−	+	+	+	+	−

[a] Results are shown as positive (+) or negative (−) binding to WaTat 1.1 VSG in a visually assessed nitrocellulose dot-blot assay. [b] See text for explanation of reagents and treatments. [c] These monoclonal antibodies bind to the surface of living WaTat 1.1 trypanosomes.

resistant to treatment with TFA/2-POH at the relative concentrations in which VSG elutes from a reverse-phase HPLC column (Clarke et al., 1984).

Discussion

We have shown that there are subtle but significant secondary structure differences between two antigenically related VSG's. These differences may be related to the antigenic polymorphism of these molecules, a property which is central to their apparent biological function in antigenic variation. The antigenic polymorphism of trypanosome VSG's is reflected in highly variable amino acid sequences (Bridgen et al., 1976), with some notable exceptions at the amino terminal (Olafson et al., 1984) and the carboxyl terminal (Donelson & Rice-Ficht, 1985). It does not appear that the carbohydrate groups of VSG are involved in the antigenic specificity of the molecule (Turner, 1985). Therefore, the epitopes exposed to the immune response of the infected host are comprised of discrete regions of the polypeptide backbone. Evidence to date suggests that the exposed epitopes are topographically assembled structures and that no linear antigenic determinants are found on the surface of the molecule (Clarke et al., 1987; this paper). Thus, it is very likely that, in addition to the variability at the primary level, slight alterations of ordered structure at the surface of the molecule may result in the appearance of novel antigenic variants.

We have previously described the primary structure of the amino terminals of several VSG's (Olafson et al., 1984), two of which are antigenically cross-reactive (Barbet et al., 1982). Among all VSG's examined, there were significant conserved features including a Cys at position 17, Ala or amides at positions 2 and 4, and Arg or a hydrophobe at position 8. These observations led to the conclusion that the overall tertiary structure would reflect these common features and itself would be conserved. This has been subsequently supported by the preliminary data from X-ray crystallographic studies of two nonrelated VSG's (Metcalf et al., 1987). Together with the earlier description of the low-resolution X-ray data from MiTat 1.2 VSG (Freyman et al., 1984) and of electron microscopic studies of two VSG's (Cohen et al., 1984), this work has shown that the most prominent feature of the VSG is its overall α-helical structure. Duvillier et al. (1983) confirmed these findings using CD spectra from nine VSG's isolated from *Trypanosoma equiperdum*. α-Helix accounted for 28–49% of the ordered secondary structure of the molecules. Our results show that the α-helix content of the soluble forms of WaTat 1.1 is 49% and of WaTat 1.12 is 52% in their native conformations.

As was stated under Results, it is felt that these differences in α-helix, β-sheet, and β-turn are significant since the ellipticity figures from which these parameters are calculated are considerably beyond experimental error. The two variants also show different sensitivity to the presence of low concentrations of Gdn·HCl (<0.4 M). These are probably reflections of alterations in structure between the two VSG's, perhaps at the level of the degree of surface exposure of the various structural elements. Such speculation must await analysis when the tertiary structures of these VSG's become available.

Gomes et al. (1986) have shown that the CD spectrum of *T. brucei* 8 AnTat 1.1A VSG indicates an α-helix content of 43% and a β-sheet content of 8%. In addition, the CD spectrum of the amino-terminal tryptic fragment (43 kDa) showed that it possessed virtually all of the ordered structure of the intact molecule. These observations suggest that the VSG molecule possesses two distinct domains. A similar α-helical structure has been proposed for other membrane glycoproteins (Cohen & Phillips, 1981). Because of packing constraints, it is likely that among antigenically diverse VSG's the α-helical content will be conserved. Therefore, differences in α-helix content may be entirely due to minor structural variations within the amino-terminal domain of VSG. Our results suggest that perturbations to the structure of the amino-terminal domain of VSG which are even below the detection limit of CD can affect the integrity of individual antigenic determinants.

The process of unfolding induced by denaturants such as Gdn·HCl may intially involve alterations to the surface of the molecule as nonpolar groups access solvent more easily. These slight structural changes, not detectable by CD, clearly denature individual surface-oriented antigenic determinants with the resultant loss of antibody binding. Attempts at mapping these epitopes onto chemically or enzymatically derived fragments of VSG have failed owing to the complex topographically assembled structures of the native epitopes (Clarke et al., 1987). Other workers have noted a direct correlation between the conformational stability of protein fragments and their reactivity with anti-protein antibodies (Vita et al., 1985).

Previous efforts at mapping surface-oriented antigenic determinants of VSG's (Miller et al., 1984a) have localized one epitope to the N-terminal domain but not to any CNBr-derived fragments. We have attempted to distinguish the fine specificity of VSG-specific monoclonal antibodies by analyzing their reactivity with VSG that had been partially denatured with a variety of reagents. By identifying identical patterns of reactivity within the monoclonal antibody panel, it is possible to assign the same fine specificity (i.e., the binding of the same epitope on VSG) to two or more antibodies.

The differing patterns of reactivity shown by the panel of monoclonal antibodies suggest that there are four distinct and separate epitopes defined. TRYP 1E1 and WAT 24A1 share identical patterns of reactivity as do WAT 8A1 and WAT 20A1. The remaining three antibodies exhibit unique patterns of reactivity, suggesting that each binds a unique and separate epitope all of which are topographically assembled and highly

sensitive to structural perturbations.

As the assay is described in this report, the antigen is applied to NC paper following treatment with the various denaturants. Following this, the NC paper is washed thoroughly with PBS-T. Thus, there is the possibility that a degree of renaturation may occur as the denaturant is removed. This is no doubt possible upon treatment with 10 mM 2-ME where reoxidation of reduced sulfhydryls can occur. In addition, treatment with urea, Gdn·HCl, and GITC was for 1 h only at room temperature. Clearly, there are effects that will not be seen under these conditions. However, the fact that the method does indeed discriminate between individual monoclonal antibodies indicates that structural modifications are occurring to specific regions of the polypeptide involved in epitope formation.

Differential denaturation provides a means by which monoclonal antibodies can be grouped according to shared specificities in a noncompetitive assay. In addition, information regarding structural features of determinants can be established. The two monoclonal antibodies WAT 8A1 and WAT20A1 appear to bind to the same antigenic determinant on both VSG's, a conclusion supported by competitive binding studies (Liu and Clarke, unpublished observations). The determinant is stable in the presence of 6 M Gdn·HCl, 6 M GITC, 8 M urea, and 0.1% SDS and even to heating to 100 °C for 10 min. The epitope is not exposed on the surface of the molecule and may consist of a carbohydrate determinant or is a linear continuous site on the polypeptide backbone. Competitive assays (Liu and Clarke, unpublished observations) showed competitive interaction among TRYP 1E1, TRYP 2B1, and WAT 24A1. It appears from the present results that TRYP 2B1 binds a distinct but neighboring epitope to that bound by TRYP 1E1 and WAT 24A1. Therefore, our panel of surface-specific monoclonal antibodies map to four distinct determinants on WaTat 1.1 VSG, three of which are surface oriented.

We have noted subtle structural differences between WaTat 1.1 and WaTat 1.12 VSG's. In spite of the near identity of their amino-terminal sequences, there is now substantial evidence for divergence of structure at the primary and secondary levels between the two molecules. These differences are apparent in their tryptic (Barbet et al., 1982) and CNBr (Clarke et al., 1987) peptide maps. Our present results show that the 50% transition point in the unfolding of WaTat 1.1 and WaTat 1.12 in the presence of Gdn·HCl is also different. On this basis, WaTat 1.12 is slightly more stable to the effect of this denaturant. Cohen et al. (1984) have described a high degree of α-helical coiled-coil structure in the amino terminal of two VSG's on the basis of the predictive method of Crick (1953). This correlates with the work of Gomes et al. (1986), who showed that the amino-terminal tryptic fragment of a VSG contained all the ordered structure of the entire molecule. Thus, the amino terminal (approximately two-thirds of the VSG molecule), which contains the variant specific epitopes, appears to consist largely of α-helical structure. Subtle differences in the α-helix content of the amino terminals of WaTat 1.1 and WaTat 1.12 may be correlated with the presence of an epitope on WaTat 1.1 (that bound by TRYP 1E1) which is not found on WaTat 1.12 (see Figure 8) although this is highly speculative.

We have previously described the purification of the membrane form of VSG (Cardoso de Almeida & Turner, 1983) by reverse-phase HPLC (Clarke et al., 1985). We have shown that VSG prepared in this way can be bound by monoclonal antibodies which react with the surface of the living trypanosome (Clarke et al., 1987). Present results clearly show that there is a lack of ordered structure of the HPLC-purified material as determined by CD. Loss of enzymatic activity frequently accompanies purification by reverse-phase HPLC due most likely to binding to the hydrophobic alkyl chains of the matrix. This is true in the present case since exposure of VSG to the HPLC solvents did not destroy the surface-oriented antigenic determinants (Table II). In the present work, attempts were made to induce refolding of the denatured VSG by dialysis into saline. This was not successful. There is, therefore, a degree of irreversible structural alteration to VSG when purified by HPLC. It should be noted that the CD spectrum of the denatured HPLC-purified membrane form of WaTat 1.1 (Figure 3) is quite different from that of WaTat 1.1 denatured by Gdn·HCl (Figure 1). The fact that HPLC VSG binds surface-specific monoclonal antibodies and Gdn·HCl-denatured VSG does not indicates that the structural alterations induced by the two denaturing treatments are quite different. Clearly, however, the membrane form of VSG prepared by HLPC is not suitable for X-ray crystallographic studies.

Although others have reported that VSG's may exhibit diverse tertiary structures (Lalor et al., 1984; Strickler et al., 1987) based on predicted secondary structure variation, our evidence suggests that there are constraints on the diversity of possible tertiary structures and that a general pattern of VSG structure will emerge. The present data on the antigenically related WaTat 1.1 and WaTat 1.12 VSG's show that the molecules show only subtle differences in secondary structure.

We are in the process of extending these studies, including complete primary structure determinations, to other VSG molecules derived from the WaTat serodeme some of which are antigenically related to WaTat 1.1 and WaTat 1.12 and others of which bear no common epitopes. It is hoped that the expected conserved structural features may be exploited in the development of a molecularly defined vaccine.

Added in Proof

Recently, Jahnig et al. (1987) have reported measurements of VSG secondary structure using Raman spectroscopy. Their conclusions regarding the high α-helix content and the conservation of higher order folded structure among diverse VSG's are confirmed by results of this work.

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Time-Resolved Europium(III) Luminescence Excitation Spectroscopy: Characterization of Calcium-Binding Sites of Calmodulin†

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ABSTRACT: Pulsed-dye laser excitation and lifetime spectroscopy of the $^7F_0 \rightarrow {}^5D_0$ transition of Eu^{3+} reveals details of the binding of this ion to the calcium-binding sites of calmodulin (labeled I–IV, starting at the N-terminus). For 10 μM calmodulin Eu^{3+} binds quantitatively at sites I and II and more weakly at sites III and IV with K_d values of approximately 0.5 μM and 1.0 μM at the latter sites. In D_2O solution the time course of luminescence emission of Eu^{3+}-loaded calmodulin can be separated into three exponential components with lifetimes of 2.50 (sites I and II) and 1.70 and 0.63 ms (sites III and IV). This finding permits the time resolution of the excitation spectrum by determination of the amplitudes of the three components as the excitation wavelength is scanned across the spectral profile in 0.1-nm increments. The amplitudes (intensities at time $t = 0$) are plotted as a function of wavelength and the results fitted to three Lorentzian peaks centered at 579.20, 579.40, and 579.32 nm in order of decreasing lifetimes. In H_2O solution only two exponential luminescence decay components are resolvable with lifetimes of 0.41 and 0.27 ms, corresponding to sites I and II and sites III and IV, respectively. These results indicate that two water molecules are coordinated to the Eu^{3+} ions at sites I and II and at either site III or site IV, with three water molecules at the remaining site. By observation of Förster-type energy transfer in D_2O solutions of calmodulin containing Eu^{3+}/Nd^{3+} energy donor–energy acceptor mixtures, the distances between sites I and II and between sites III and IV were found to be 12.1 ± 0.5 and 11.6 ± 0.8 Å, respectively.

Excitation spectroscopy of the $^7F_0 \rightarrow {}^5D_0$ transition of europium(III) ions bound at calcium ion binding sites in proteins provides a wealth of information regarding the metal ion binding characteristics and structure of calcium-binding proteins (Horrocks & Sudnick, 1979a, 1981). This experiment involves excitation of the $^5D_0$ excited state of Eu^{3+} while a tunable dye laser is scanned in the 578–580-nm region.

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