Stability and Reconstitution of the Soluble Variant Surface Glycoprotein (sVSG) from *Trypanosoma brucei*†

Paul Rehaber,‡ Norbert Staudacher,‡ Robert Seckler,‡ Roland Bülow,§,‖ Peter Overath,§ and Rainer Jaenicke*,‡

Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, D-8400 Regensburg, West Germany, and Max-Planck-Institut für Biologie, 7400 Tübingen, West Germany

Received November 20, 1989; Revised Manuscript Received June 6, 1990

ABSTRACT: Soluble variant surface glycoprotein (sVSG) is the form of the coat protein of *Trypanosoma brucei* released by cleavage of its lipid anchor. As shown by ultracentrifugal analysis, the protein of the variant clone MITat 1.2 is a stable dimer of (117 ± 6)-kDa molecular mass. Its quaternary structure remains unaltered in the concentration range from 0.01 to ≈50 mg/mL. Further extrapolation to the in situ concentration on the cell surface points to no significant protein association beyond the dimer, because after correction for solution viscosity sedimentation velocity is independent of the protein concentration. The sedimentation constant, $s_{20,w} = 5.1 \times 10^{-13}$ s, together with the particle weight confirms the high anisotropy of the dimer. Circular dichroism and fluorescence spectra show the typical properties of an α-helical protein (51% α-helix) with fluorophores buried in the hydrophobic interior of the protein. Denaturation at extremes of pH leads to the monomer still maintaining a relatively compact structure. Increased concentrations of urea and guanidine hydrochloride cause randomization with cooperative transitions at 1.7 and 0.7 M, respectively. The yield of reconstitution of the denatured protein reaches 87% under optimum conditions. The final product is indistinguishable from the native protein in its spectral, hydrodynamic, and immunochemical properties. Immunological analysis included polyclonal antibodies as well as monoclonal antibodies raised against epitopes in the surface of the complete trypanosome, as well as cryptic epitopes exposed only on sVSG in solution. The kinetics of reconstitution involve sequential uni-bimolecular processes, corresponding to consecutive folding and subunit association. About 38% of the fluorescence of the native protein is recovered within the mixing time (secondary structure formation). Subsequent slow steps are concentration dependent and reflect the recovery of the native tertiary and quaternary structure. The second-order rate constant of reassociation observed at low protein concentrations is confirmed by direct measurements applying gel permeation chromatography (HPLC) or electrophoresis after chemical cross-linking: $k_2 = 9 \times 10^2$ L·mol^{-1}·s^{-1} at 20 °C.

The variable surface glycoprotein (VSG)[1] of the parasitic protozoan *Trypanosoma brucei* is arranged as a coat consisting of about 10^7 molecules that entirely covers the surface of the trypanosome (Vickerman, 1969; Cross, 1975). This coat shields the parasite from its environment but also triggers an immune response of the mammalian host. In adaptation to parasitism, trypanosomes possess the capability of antigenic variation. This enables the parasite to select and differentially express 1 of about 1000 genes encoding the different variants of the glycoprotein, this way evading the immune response. The VSGs are anchored in the membrane by a dimyristoylphosphatidylinositol residue that is linked to the C-terminus via a glycan moiety and an ethanolamine residue (Homans et al., 1989). Upon lysis of trypanosomes, the glycosylphosphatidylinositol (GPI) anchor is cleaved by a membrane-bound GPI-specific phospholipase C that converts the membrane form (mfVSG) of the protein to sVSG, the soluble form (Cardoso de Almeida & Turner, 1983; Bülow & Overath, 1986; Fox et al., 1986; Hereld et al., 1986). sVSG exists in solution as a dimer (Auffret & Turner, 1981) with a molecular mass of about 60 kDa per subunit. The length of the polypeptide chain varies between 450 and 500 amino acids and the carbohydrate content between 7 and 17% by weight among VSG variants (Johnson & Cross, 1977). The primary structures of several VSGs have been determined, and attempts have been made to resolve the three-dimensional structures. However, so far, only the N-terminal domains of some antigen types could be crystallized (Metcalf et al., 1988); structures were determined to a resolution of 6 Å (Freyman et al., 1984; Metcalf et al., 1987). The data suggest a rodlike structure made up of a bundle of four long, parallel α-helices. The variable "top" of the molecule, which is exposed to the extracellular surface of the trypanosome, has no pronounced α-helical structure. The connecting peptide between the two domains forms a flexible hinge region. In spite of the fact that the surface coat is densely packed in vivo, mfVSG shows rapid lateral diffusion (Bülow et al., 1988). For the soluble form, sVSG, no aggregation beyond the dimer could be detected so far (Strickler & Patton, 1982; Gurnett et al., 1986). However, in the corresponding experiments, the protein concentration was far from the physiological level, so that possible assemblies at higher concentrations could not be excluded. It remains unclear how a tight barrier around the parasite to components

† This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 43) and the Fonds der Chemischen Industrie.

‡ Universität Regensburg.

§ Max-Planck-Institut für Biologie.

‖ Present address: Immulogic, 855 California Ave., Palo Alto, CA 94304.

[1] Abbreviations: sVSG and mfVSG, soluble and membrane forms of the variant surface glycoprotein of *Trypanosoma brucei*, respectively; SDS, sodium dodecyl sulfate; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (registered trademark of Boehringer Mannheim GmbH); ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; N, native VSG; D, denatured VSG; N*, reconstituted VSG; MAb, monoclonal antibody.

0006-2960/90/0429-8217$02.50/0

of the host's immune system can be formed from mobile VSG dimers, and what role the membrane anchor plays for the arrangement of the glycoprotein in the surface coat. Reconstitution of soluble and membrane forms of the protein after unfolding in vitro might help to answer this question. In the present study, sVSG was characterized with regard to its structure, its state of association, and its stability under denaturing conditions. Applying physicochemical and immunological methods, reconstitution under optimum condition was found to recover the native conformation. Kinetic measurements were used to establish a model for the folding pathway of the protein.

Materials and Methods

sVSG from the monomorphic trypanosome clone MITat 1.2 (also known as variant 221) was isolated and purified as described by Cross (1984) and Bülow (1987). The homogeneity of the protein was demonstrated by SDS–polyacrylamide gel electrophoresis. Concentrations of sVSG were determined by UV spectroscopy using an extinction coefficient of $A_{280nm}^{0.1\%,1cm} = 1.0$ calculated from the amino acid sequence of the VSG polypeptide (Turner, 1988; Wetlaufer, 1962). Polyclonal rabbit antibodies to VSG were produced by repeated immunization with purified sVSG. Production of monoclonal antibodies and mapping of epitopes have been described (Masterson et al., 1988). Peroxidase-conjugated polyclonal anti-mouse antibody and ABTS were from Boehringer (Mannheim). Activated CH-Sepharose-4B was purchased from Pharmacia (Uppsala); guanidine hydrochloride (grade "ultrapure") was from ICN/Schwarz-Mann (Orangeburg, NY). SDS and glutaraldehyde were products of Serva (Heidelberg); TWEEN 80 was supplied by Sigma (Munich). All other chemicals were of analytical grade and supplied by Merck (Darmstadt). For all solutions, quartz-bidistilled water was used.

Spectroscopy. Fluorescence spectroscopy made use of a Perkin-Elmer LS-5B luminescence spectrometer with a R100A recorder. Emission spectra were measured in semimicro cells in the wavelength range between 300 and 400 nm using an excitation wavelength of 280 nm.

CD spectra were monitored in a Jasco J500A spectropolarimeter with a DP-500 N data processor. Mean residue ellipticities were calculated by using a mean residue weight of 106.8 g/mol taken from the known amino acid sequence of MITat 1.2 VSG (Turner, 1988).

Analytical Ultracentrifugation. The sedimentation coefficient and the molecular mass of the native and denatured sVSG were determined in an analytical ultracentrifuge (Beckman Spinco Model E) equipped with a high-sensitivity scanning system and a high-intensity light source according to J. Flossdorf (GBF, Braunschweig). An AnG rotor with double-sector cells (12 mm, normal and capillary-type synthetic boundary) was used. Scanning wavelengths were 230 and 280 nm, respectively. Samples with a protein concentration higher than 5 mg/mL were detected by schlieren optics in an AnD rotor with ultrathin cells (1 mm). In order to determine the weight-average molecular weight, the meniscus depletion sedimentation equilibrium technique was applied (Yphantis, 1964). Weight-average molecular weights were calculated from ln c vs r^2 diagrams using a computer program kindly provided by G. Böhm (Regensburg). The diffusion coefficients were determined according to Elias (1961) making use of the height/area method and schlieren photographs. The partial specific volume of the VSG polypeptide as calculated from the amino acid composition is $\bar{v}_P = 0.740$ cm^3 g^{-1}; to account for glycosylation, a partial specific volume of $\bar{v}_{GP} = 0.72$ cm^3 g^{-1} was used (Durchschlag, 1986).

HPLC. Gel filtration HPLC was performed with the aid of an LKB 2156 solvent conditioner, an LKB 2152 controller, an LKB 2150 HPLC pump, and a Merck-Hitatchi F 1000 fluorescence detector ($\lambda_{exc} = 280$ nm; $\lambda_{em} = 330$ nm) making use of an LKB TSK G3000 SW (7.5 × 600) column and an LKB TSK GSW (7.5 × 75) precolumn. For the native samples, the elution buffer was 0.1 M sodium phosphate/0.1 M Na_2SO_4, pH 6.8; in order to denature the protein, 6 M urea was added. All samples were incubated for 24 h in the elution buffer. Protein concentrations were 60 μg/mL for native sVSG and 250 μg/mL for the protein in 6 M urea, respectively. Lysozyme, myoglobin, endoglycosidase F, lactate dehydrogenase, concanavalin A, catalase, bovine serum albumin, and human apotransferrin were used as standards.

ELISA. For sandwich ELISA measurements with mouse anti-MITat 1.2 VSG monoclonal antibodies, microtiter wells were coated with 100 μL of the polyclonal anti-sVSG 221 antibody diluted 1:1000 in 0.2 M TEA, pH 9.5, overnight at 4 °C. Remaining binding sites were blocked by incubation with 0.3 mL of 50 mM potassium phosphate, 0.1 M NaCl, 1% BSA, and 0.05% TWEEN 80, pH 7.2, for 20 min at 4 °C. All subsequent steps were done at room temperature.

Following one wash, 0.2 mL of sVSG antigens (5 μg/mL) was added and left for 1 h. After the antigens were washed twice, 0.2-mL serial dilutions of monoclonal antibodies (10–0.01 μg/mL) were added. Plates were again left for 1 h, washed twice, and incubated with 0.2 mL of a 1:1000 dilution of the peroxidase-conjugated anti-mouse serum for 1 h. After the plates were washed 3 times, 0.2 mL of ABTS in substrate buffer was added. The absorbance at 405 nm was measured after 45 min. All washes were done for 5 min with 0.15 M NaCl. Antibodies were diluted in 0.05 M potassium phosphate buffer, 0.1 M NaCl, 10 mg/mL BSA, and 0.5 mg/mL TWEEN 80, pH 7.2.

Carboxymethylation. sVSG was carboxymethylated after complete unfolding and reduction according to the method of Allen (1981).

Chemical Cross-Linking. Cross-linking was performed according to Hermann et al. (1981); sVSG was incubated with 0.3% (w/v) glutaraldehyde for 2 min at room temperature. The reaction was stopped with 30 mM $NaBH_4$ in 0.1 M NaOH.

Denaturation/Renaturation. The stability of sVSG against various denaturants was measured in 50 mM sodium phosphate buffer, pH 6.8. In order to accomplish equilibrium, samples were taken after 5 min, 8 h, 24 h, and 48 h. Renaturation was started by diluting the denatured protein to the desired denaturant concentration. Kinetic measurements under optimum conditions made use of the following set of parameters: denaturation for 30 min at 20 °C in 50 mM sodium phosphate buffer, pH 6.8, 2 M guanidine hydrochloride, and 2 mM EDTA; under these conditions, no disulfide isomerization should occur in the unfolded state. Renaturation was performed in 50 mM sodium phosphate buffer, pH 6.8, 2 mM EDTA, and ≤50 mM Gdn·HCl. The fast initial phase in the regain of native fluorescence emission could not be resolved by manual techniques. It is therefore ignored in the following kinetic analysis.

Results

Association. In a first series of experiments, sedimentation analysis was applied to determine the native solution structure of sVSG. As shown by sedimentation velocity measurements at concentrations between 10 μg/mL and 49 mg/mL, the state of association was found to be unaltered. Measurements of

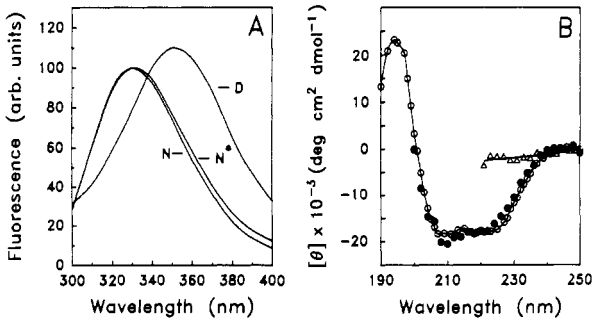

FIGURE 1: (A) Fluorescence emission spectra of native (N), denatured (D), and reconstituted (N*) sVSG (19.4 μg/mL). Spectra were recorded at 20 °C in 50 mM sodium phosphate and 2 mM EDTA, pH 6.8 containing 6.6 M Gdn·HCl (D) or no denaturant (N, N*). (B) Far-UV circular dichroism of sVSG: native (○), denatured in 6.6 M Gdn·HCl (△), and reconstituted from guanidine (●). CD spectra were taken at ambient temperature in 0.1-mm (○) or 1-mm (●, △) cells at a protein concentration of 1.9 mg/mL (○) or 44 μg/mL in 50 mM sodium phosphate, pH 6.8.

the sedimentation and diffusion coefficients at c = 49 mg/mL made use of ultrathin cells. The sedimentation coefficient decreased with increasing protein concentration, exhibiting a linear concentration dependence up to a concentration of ≈5%; this excludes the formation of aggregates beyond the dimer even at exceedingly high protein concentrations. The concentration dependence of the sedimentation coefficient in the given concentration range (with c milligrams per milliliter) is given by

$$s_{20,w} = 5.09(1 - 0.0096c) \times 10^{-13}$$

deviations are below 2%. Thus, the sedimentation constant is found to be $s^0_{20,w} = (5.09 \pm 0.09) \times 10^{-13}$ s. This value is anomalously low for a protein of the given size, indicating that sVSG must be highly anisotropic.

The apparent diffusion coefficient, D_{app}, was determined from sedimentation velocity experiments, and in synthetic boundary cells, applying the area/maximal gradient method. Extrapolation to zero time yielded $(2.03 \pm 0.10) \times 10^{-7}$ cm^2 s^{-1}. Combining $s^c_{20,w}$ and $D^c_{t=0}$, the molecular mass of native sVSG at ≈50 mg/mL was found to be $M_{s,D}$ = 117 kDa.

Ultracentrifugal analysis was clearly confirmed by gel filtration using HPLC. For native sVSG, the molecular mass was found to be 122 ± 3 kDa. Denatured and reduced sVSG was monomeric with M_r = 60K ± 1K. Again no assemblies beyond the dimer were detectable. Chemical cross-linking of the native protein with glutaraldehyde at a protein concentration of 25–100 μg/mL gave the same result [cf. Hermann et al. (1981) and Jaenicke and Rudolph (1986)]. SDS–polyacrylamide gel electrophoresis of cross-linked samples and subsequent densitometry yielded the dimer as the dominating species (>95%). Assemblies with higher molecular mass were not detectable.

Conformation and Equilibrium Transitions. In order to characterize the native protein and its equilibrium transitions in the presence of denaturants, fluorescence emission spectroscopy and dichroic absorption in the far ultraviolet were applied. As shown in Figure 1A, maximum fluorescence emission in the native state occurred at 330 nm; upon denaturation in 6 M guanidine hydrochloride, the maximum was shifted to 350 nm. The ratio F_{350}/F_{330} was a sensitive indicator for the native state of the protein and independent of protein concentration. In the native state, it amounted to 0.76, in the fully denatured state 1.41. The equilibrium transition could be monitored at 320 nm (cf. Figures 1A and 2B). The far-ultraviolet circular dichroism spectrum showed the typical profile commonly observed for helical proteins (Figure 1B).

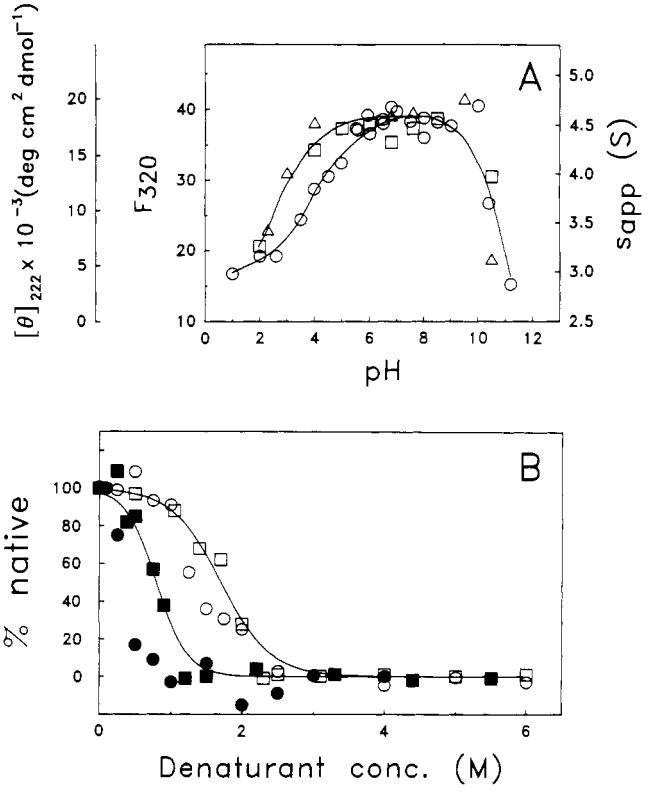

FIGURE 2: Stability of sVSG monitored by fluorescence (●, ○), circular dichroism (■, □), and sedimentation velocity (△). (A) pH dependence; (B) denaturation by Gdn·HCl (closed symbols) and urea (open symbols). All samples were incubated at the given solvent condition for 24 h at 20 °C. Solid lines in (B) represent linear fits of log (f_N/f_D) vs denaturant concentration to the CD data and indicate secondary structure transitions. Protein concentrations were 5 (●, ○), 30 (■, □), and 270 μg/mL (△). The size of the symbols corresponds to the ranges of error.

According to the method of Chen and Yang (1971), the native protein contains about 51% α-helix. In 6 M guanidine hydrochloride, the spectrum indicated complete randomization. As in the case of many other oligomeric proteins (Jaenicke, 1987), the N → D transition was not reversible at intermediate denaturant concentrations. Thus, to approach equilibrium, the protein was incubated under the respective conditions until no further change in spectroscopic or hydrodynamic parameters was observed (Jaenicke & Rudolph, 1986).

As indicated by the degree of flattening of the ellipticity at 222 nm (θ_{222nm}), neither low nor high pH led to complete denaturation (Table I, Figure 2A). In the case of the denaturation in the presence of urea or guanidine hydrochloride, unfolding transitions observed by CD spectroscopy occurred around 1.7 ± 0.2 and 0.7 ± 0.1 M, respectively (solid lines in Figure 2B). Beyond 1.5 M guanidine or 4 M urea, CD, fluorescence, and $s_{20,w}$ of VSG were characterizing a randomized monomeric polypeptide chain.

In all denaturants, transitions observed by fluorescence, CD, and sedimentation did not completely coincide, suggesting that the N → D transition did not obey the two-state model. As no systematic variation of transition midpoints with protein concentration was observed, and the concentration dependence of $s_{20,w}$ was linear down to ≤10^{-7} M VSG dimer, concentration-dependent dissociation does not appear important in the guanidine- or urea-induced denaturation.

Reconstitution. Optimum conditions of denaturation/renaturation were investigated, making use of extremes of pH, urea, and guanidine hydrochloride, respectively. Since reconstitution experiments were intended to simulate the folding

Table I: Characterization of sVSG in the Native (N) and Reconstituted State (N*) after Previous Denaturation in Various Denaturants (D)[a]

state	solvent	$s_{20,w}$ (S)	M (kDa)	λ_{max}[f] (nm)	$[\theta]_{222} \times 10^{-3}$ (deg·cm²·dmol⁻¹)[g]
N	sodium phosphate, pH 6.8–7.6	5.0 ± 0.2	122 ± 3[b] 117 ± 3[c]	330	17.7
$D_{pH\,2}$[e]	50 mM glycine/H_3PO_4, pH 2.3	3.5	61 ± 2[d]	346	9
$D_{pH\,10}$	0.1 M glycine/NaOH, pH 10.5	3.3	62 ± 2[d]	342	13
D_{Gdn}	50 mM sodium phosphate, pH 6.8, + 1.5 M Gdn·HCl	1.5	61 ± 2[d]	350	≤2
D_{urea}	50 mM sodium phosphate, pH 6.8, + 7.6 M urea	2.3	59 ± 2[d]	350	≤2
N*	sodium phosphate, pH 6.8–7.6	4.9 ± 0.2	124 ± 3[b]	330.5	17.8

[a] $s_{20,w}$, sedimentation coefficients, determined at c_P = 0.2–0.5 mg/mL. [b] M, molecular mass, determined by gel permeation HPLC. [c] M determined by sedimentation velocity/diffusion. [d] M determined by high-speed sedimentation equilibrium centrifugation. [e] For denatured states (D), corrections were determined according to Kawahara and Tanford (1966) and Durchschlag (1986). [f] λ_{max}, fluorescence emission maximum. [g] $[\theta]_{222}$, mean residue ellipticity at 222 nm.

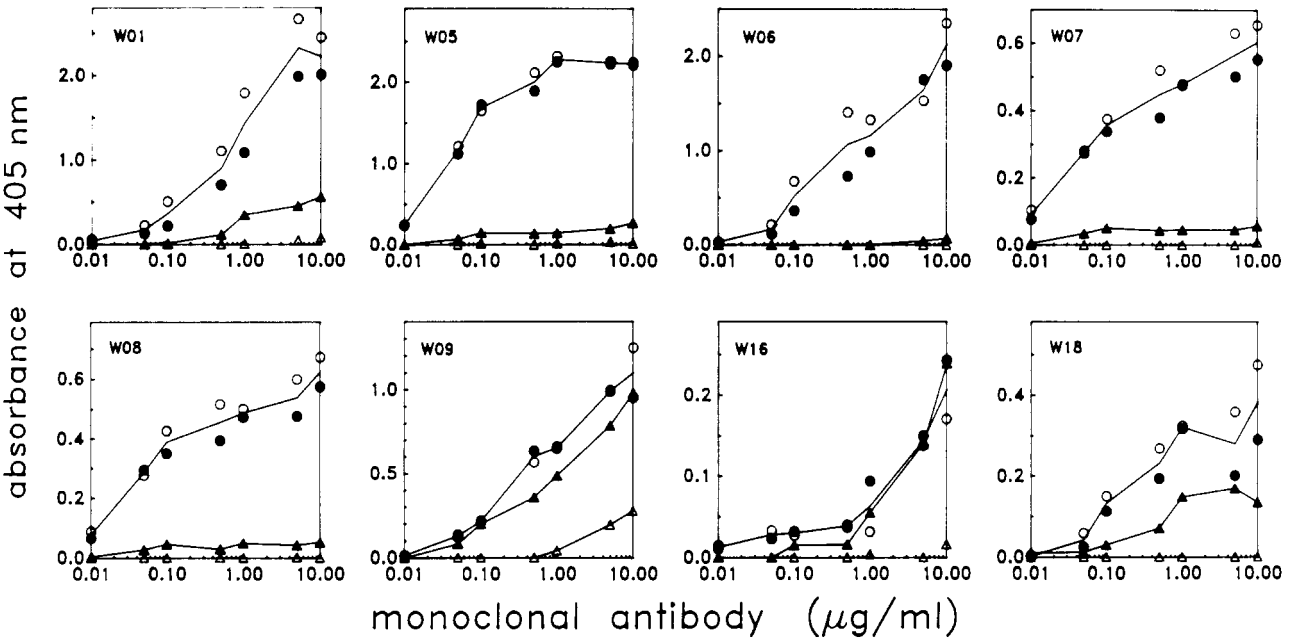

FIGURE 3: Immunochemical characterization of reconstituted sVSG. Native (○), reconstituted (●), denatured carboxymethylated (△), and partially denatured [(▲) cf. text] sVSG antigen were probed with a series of monoclonal antibodies by indirect sandwich ELISA as described under "Materials and Methods". Monclonal antibodies W01, W09, W16, and W18 recognize epitopes accessible on the surface of the intact trypanosome; W05, W06, W07, and W08 are directed toward "cryptic" epitopes exposed only on sVSG in solution (Masterson et al., 1988).

and association in vivo (Jaenicke, 1987, 1988), complete unfolding was considered to be the adequate starting condition. Systematic experiments showed that guanidine hydrochloride yielded maximum reconstitution. The protein was incubated at room temperature for 10–60 min. Renaturation was started by dilution in 50 mM sodium phosphate buffer, pH 6.8 at 0 °C, in the presence of 2 mM EDTA. Maximum renaturation was accomplished at 0 °C; increasing temperature strongly enhanced the renaturation rate, at the same time decreasing the yield.

Comparison of the Native and Reconstituted States. Reconstituted sVSG was compared to the native form using spectroscopic, hydrodynamic, and immunological criteria. As shown in Figure 1A, the fluorescence emission spectra of the native and reconstituted sVSG were indistinguishable within the range of error. The maximum of fluorescence emission and the ratio of the fluorescence amplitudes, F_{350}/F_{330}, of the native (N) and the reconstituted state (N*) were 330.0 nm (N) vs 330.5 nm (N*) and 0.76 (N) vs 0.77 (N*), respectively. Both native and reconstituted sVSG exhibited the same characteristic minima in the CD spectrum at 222 and 208 nm. The amplitude of the renatured sVSG at 208 nm exceeded the amplitude of the native protein insignificantly (Figure 1B). Glutaraldehyde cross-linking and subsequent quantification by HPLC or SDS–polyacrylamide gel electrophoresis confirmed the state of association of both N and N* to be the dimer. At 20 °C, aggregation as a side reaction of reconstitution did not exceed 12%. The corresponding sedimentation coefficient for N* was $s_{20,w} = (4.9 \pm 0.2) \times 10^{-13}$ s, compared to $(5.0 \pm 0.2) \times 10^{-13}$ s for the native protein (Table I).

Apart from the spectral and hydrodynamic data which only allow one to monitor the gross structure of the protein, attempts were made to characterize the native and reconstituted states applying immunochemical methods. Experiments using polyclonal antibodies showed that the reaction of the reconstituted sVSG was comparable with the reaction of the native protein. However, the carboxymethylated protein, which according to its CD and fluorescence properties is in the denatured state, also showed a strong reaction with the polyclonal serum. Thus, this result could not be taken as proof for the structural identity of native and renatured sVSG.

In order to obtain an unambiguous result, monoclonal antibodies had to be applied. As shown previously (Masterson et al., 1988), monoclonal antibodies may be raised (i) against epitopes on the surface of the intact trypanosome ("anti-surface MAbs") and (ii) against epitopes that become accessible when sVSG is present in solution, ("anti-cryptic MAbs"). As shown in Figure 3, within the range of error, both the native and the renatured sVSG showed identical reactions with the two types of monoclonal antibodies. In order to find out to what extent the reaction was specific for the native protein, carboxymethylated sVSG was applied as a control. The denatured protein showed only very weak or no interaction at all. However, carboxymethylated protein differs from the native

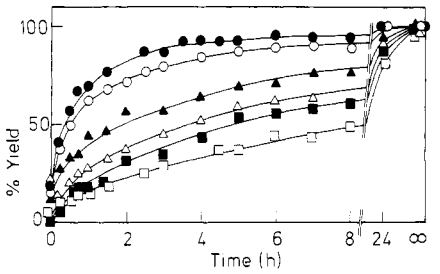

FIGURE 4: Kinetics of reconstitution of sVSG as determined by changes in F_{350}/F_{330}. Refolding and reassociation at 0 °C were initiated by dilution from 2 M Gdn·HCl. Protein concentrations were 9.7 (□), 19.4 (■), 38.7 (△), 87.1 (▲), 175 (○), and 250 μg/mL (●).

form with respect to its surface charge so that the binding constants of the antigen–antibody complex may be altered, thus perturbing the ELISA analysis.

As an alternative, ELISA reactivity of the MAbs was tested using an sVSG antigen that had been partially denatured during lyophilization, as indicated by fluorescence spectroscopy. As shown in Figure 3, five out of eight of the monoclonal antibodies reacted poorly with the partially denatured antigen, while exhibiting normal reactivity toward sVSG reconstituted after unfolding in guanidine hydrochloride.

Kinetics of Reconstitution. The kinetics of refolding and reassociation were monitored by fluorescence spectroscopy or cross-linking with glutaraldehyde. As has been previously reported for a variety of proteins with sizes close to VSG (Jaenicke, 1987), the regain of native fluorescence displays two phases: a fast one which cannot be resolved on the time scale of the present measurements and a second slow phase. In the case of oligomeric proteins, the slow phase has frequently been found to depend on protein concentration, thus proving that the fluorescence signal reflects both folding *and* association. Fluorescence emission spectra taken after completion of the rapid phase exhibited an isofluorescent point at 330 nm. Thus, the amplitude ratio F_{350}/F_{330} may be used to monitor reconstitution independent of small amplitude variations induced by temperature fluctuations.

At very low VSG concentrations, the initial rapid phase should correspond to folding in the absence of association. This assumption was confirmed by chemical cross-linking (see below). Thus, the shift in the fluorescence ratio immediately after starting renaturation at minimum protein concentration is defined as 0% reconstitution; the final value of the slow phase corresponds to the native protein and may, therefore, be identified with 100% reconstitution. Figure 4 summarizes the data under optimum temperature conditions (0 °C) at concentrations up to 250 μg/mL. Attempts to linearize the set of profiles according to second-order kinetics revealed that the association of monomers to form the dimer governed the reaction only at exceedingly low concentrations. Beyond $c \approx 10$ μg/mL, the apparent second-order rate constants decreased significantly, which clearly proves that folding *and* association are involved in the overall reconstitution reaction.

The assembly at low protein concentrations could be quantified by $k_{2,app} = 9 \times 10^2$ L·mol^{-1}·s^{-1} (20 °C). The temperature dependence of the reaction yielded an activation energy of 52 kJ/mol.

Since the kinetic analysis of the assembly based on the slow phase of the regain of fluorescence emission may be affected by folding steps, reassociation was measured directly using chemical cross-linking with glutaraldehyde (Figure 5). When the cross-linked samples are separated by HPLC or SDS–polyacrylamide gel electrophoresis, the decrease of monomers and the synchronous increase of dimers can be measured with

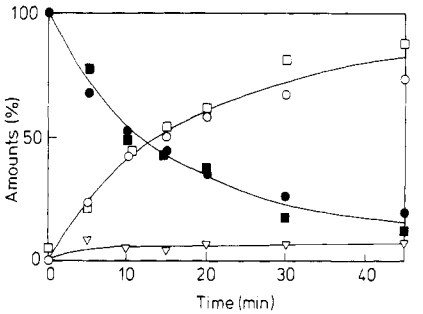

FIGURE 5: Reassociation of sVSG (69 μg/mL) after denaturation in 2 M Gdn-HCl as determined by cross-linking with glutaraldehyde and subsequent separation by SDS–polyacrylamide gel electrophoresis [(■) monomers; (□) dimers] and HPLC [(●) monomers; (○) dimers; (▽) aggregates].

high precision (Hermann et al., 1981). Both procedures yielded the same results: At $c = 69$ μg/mL and 20 °C, the second-order rate constants of the association reaction were $k_2 = 9.4 \times 10^2$ L·mol^{-1}·s^{-1} (SDS–PAGE) and $k_2 = 1.0 \times 10^3$ L·mol^{-1}·s^{-1} (HPLC), in accordance with the rate constant obtained for the slow phase of the fluorescence kinetics. Early during reconstitution, after completion of the rapid initial phase of the change in fluorescence emission, the protein is essentially monomeric, in accordance with the fact that the rapid phase can be attributed to the unimolecular folding of the monomer.

Discussion

Surface layers of microorganisms differ widely in the composition and molecular structure of their building blocks (König & Stetter, 1986; Sleytr & Messner, 1988). In most eubacteria, a covalent proteoglycan network allows the cells to cope with osmotic stress while in archaebacteria noncovalent protein assemblies prevail. The cell surface of the parasitic protozoon *Trypanosoma brucei* shows still another pattern: a closely packed glycoprotein coat made up of 60-kDa protomers fixed to the plasma membrane by a dimyristoylphosphatidylinositol residue which is covalently linked to the C-terminus of the protein via a polysaccharide moiety. The structural features of this topology serve two purposes: (i) they allow the extreme morphological flexibility which is a prerequisite of the type of cell motion characteristic for *Trypanosoma brucei*; (ii) they provide a simple mechanism of antigenic variation by alternative expression of VSG structural genes and dilution of the preexisting surface coat. The physicochemical properties of the released form of the variable surface glycoprotein of *Trypanosoma brucei* have not been studied in detail, and how the assembly of the protein coat occurs remains an open question. The present experiments deal with the solution properties of sVSG, and its in vitro reconstitution after solubilization and subsequent reconstitution. MITat 1.2 sVSG was chosen for the present study, because this variant has been the subject of X-ray crystallography of its N-terminal domain (Freyman et al., 1984) and secondary structure analysis by Raman spectroscopy (Jähnig et al., 1987).

The result may be interpreted in terms of an attempt to model the in vivo assembly of the cell surface. That this interpretation has its limitations is obvious because of the fact that assembly in vivo occurs virtually as a polymerization to a preexisting matrix, while reconstitution in vitro merely consists of folding and association of proteins in solution. In this connection, two questions deserve investigation: First, is the preexisting matrix a necessary requirement for the self-organization of VSG; i.e., do cell surface components serve as morphopoetic factors? Second, do the protomers show a tendency to form two-dimensional assemblies resembling the

surface layer of *Trypanosoma brucei*? The answer to both questions is negative. Reconstitution of sVSG after denaturation in 6 M guanidine hydrochloride yields the protein in its native state; in solution, this represents the 120-kDa dimer as a homogeneous entity which does not tend to form higher assemblies even at extreme concentrations.

Sedimentation analysis of the protein in the analytical ultracentrifuge demonstrates that the concentration dependence of the sedimentation coefficient is linear. The negative slope confirms the general correlation between sedimentation rate and reciprocal viscosity. As pointed out by Lauffer (1944), particles in concentrated solutions sediment through the solution instead of through the solvent. Correcting for solution viscosity (Lauffer, 1989), the concentration dependence vanishes so that the sedimentation profile gives no limit for either dissociation of the dimer at low concentration or association at high concentration. The high stability of the MITat 1.2 sVSG dimer may be unique to this variant, as molecular weights between 79 000 and 113 000 have been determined in recent analytical ultracentrifugation studies for three other sVSGs (Gurnett et al., 1986; Clarke et al., 1988).

In contrast to the intact native glycoprotein with its phosphatidylinositol anchor (mfVSG), which requires detergents for its solubilization, sVSG is a soluble protein with spectral properties similar to those of common globular proteins (Figure 1). The circular dichroism spectrum yields an α-helix content of $\approx$51%, in close agreement with results from Raman spectroscopy (Jähnig et al., 1987). Both fluorescence and circular dichroism allow the stability of the protein to be determined and provide a means to compare the protein in its native and reconstituted state. The spectral changes accompanying denaturation in 6 M guanidine hydrochloride are reversed upon renaturation, suggesting that the native state of the protein is reconstituted. This conclusion is supported by the fact that the hydrodynamic properties of the native and reconstituted protein are found to be indistinguishable.

Since evidence from spectral and hydrodynamic data merely reflects the gross structure of the protein, immunochemical methods were applied in order to gain additional information regarding common structural features at higher resolution. Figure 3 clearly shows that eight monoclonal antibodies recognizing with widely differing affinities at least four nonoverlapping "surface" as well as "cryptic" epitopes of VSG (Masterson et al., 1988) show identical interactions with both native and reconstituted sVSG. In order to eliminate the possibility that the two classes of antibodies are sequence-specific rather than conformation-specific, or recognize the carbohydrate moiety instead of the protein, carboxymethylated sVSG was applied as a control. The modified protein was fully denatured and, therefore, will only react with sequence-specific antibodies. The lack of reaction of the antibodies with the carboxymethylated protein suggests that they recognize conformational epitopes. In addition, most of the monoclonal antibodies reacted very poorly with partially denatured unmodified VSG. Thus, we may draw the conclusion that native and reconstituted sVSGs are structurally indistinguishable with respect to their physicochemical and immunochemical properties.

The N $\rightarrow$ D equilibrium transitions of sVSG show different profiles depending on the denaturant (Figure 2). In the case of the pH-dependent denaturation and dissociation, the fluorescence profile at low pH deviates from the profile describing the change in the sedimentation coefficient and ellipticity. Comparing fluorescence emission and $\Delta s_{20,w}$, it becomes clear that the alterations in the accessibility of fluorophores occur at the level of the dimer while the dissociation of the dimer and the breakdown of the native secondary structure (ellipticity) run parallel. In this context, it is interesting to note that the relatively high value of $s_{20,w}$ at extremes of pH must belong to the monomeric protein in a highly compact conformation; on the other hand, the anomalously low s value observed for the native dimer points to a high anisotropy of the molecule. The denaturation profile in the presence of urea or guanidine hydrochloride appears to deviate from a simple two-state equilibrium. Again, changes in fluorescence emission occur before complete unfolding. Considering the "double-head" structure of the protein (Turner, 1988), it is not surprising that the denaturation transition does not occur in a monophasic highly cooperative fashion. Compared to bacterial surface layer proteins, sVSG shows relatively low stability; the urea and guanidine concentrations where unfolding of MITat 1.2 sVSG takes place are 1.7 and 0.7 M, respectively. Other sVSG variants have been observed to unfold at similar guanidine concentrations.

As mentioned, reconstitution leads back to the protein in its native state. Since the reaction starts from unfolded monomers, the overall mechanism of reconstitution must involve first-order folding and second-order association steps. When the regain of native fluorescence is used to monitor both processes, biphasic kinetics are observed. A fast first phase which has been generally observed in the refolding of globular proteins (Jaenicke, 1987) may be attributed to secondary structure formation. The subsequent slow processes reflect both tertiary and quaternary structure formation. Clear evidence for rate-determining association comes from both the concentration dependence of the regain of native fluorescence (Figure 4) and chemical cross-linking (Figure 5). At low protein concentration, where folding does not govern the overall reaction, dimerization is the rate-determining step. The corresponding apparent second-order rate constant of the fluorescence change is in excellent agreement with the constant determined by chemical cross-linking where the dimerization is monitored directly. On the other extreme, reconstitution at high concentration is limited by first-order folding reactions. Thus, the assembly pathway of sVSG may be summarized in terms of an essentially irreversible uni-bi-unimolecular sequential mechanism corresponding to consecutive folding, subunit association, and reshuffling.

The question how the phosphoinositol anchor and the cell membrane affect the overall kinetic mechanism, i.e., how VSG is assembled at the cell surface of the trypanosome, is still open. One way to approach this problem could be to determine the role of the dimyristoyl moiety for the assembly of VSG and to study the integration of the protein into lipid vesicles or membranes.

Acknowledgments

We thank Dr. Wayne J. Masterson (Dundee) for kindly providing monoclonal antibodies to MITat 1.2 sVSG.

References

Allen, G. (1981) *Lab. Tech. Biochem. Mol. Biol. 16*, 28–31.

Auffret, C. A., & Turner, M. J. (1981) *Biochem. J. 193*, 647–650.

Bülow, R. (1987) Ph.D. Thesis, University of Tübingen.

Bülow, R., & Overath, P. (1986) *J. Biol. Chem. 261*, 11918–11923.

Bülow, R., Overath, P., & Davoust, J. (1988) *Biochemistry 27*, 2384–2388.

Cardoso de Almeida, M. L., & Turner, M. J. (1983) *Nature 302*, 349–352.

Chen, Y. H., & Yang, J. T. (1971) *Biochem. Biophys. Res. Commun. 44*, 1285–1291.
Clarke, M. W., McCubbin, W. D., Kay, C. M., & Pearson, T. W. (1988) *Biochemistry 27*, 405–413.
Cross, G. A. M. (1975) *Parasitology 71*, 393–417.
Cross, G. A. M. (1984) *J. Cell. Biochem. 24*, 79–90.
Durchschlag, H. (1986) in *Thermodynamic Data for Biochemistry and Biotechnology* (Hinz, H.-J., Ed.) pp 45–128, Springer-Verlag, Berlin.
Elias, H. G. (1961) *Ultrazentrifugen Methoden*, Beckman Instruments, München.
Fox, J. A., Duszenko, M., Ferguson, M. A. J., Low, M. G., & Cross, G. A. M. (1986) *J. Biol. Chem. 261*, 15767–15771.
Freymann, D. M., Metcalf, P., Turner, M. J., & Wiley, D. C. (1984) *Nature 311*, 167–169.
Gurnett, A. M., Raper, J., & Turner, M. J. (1986) *Mol. Biochem. Parasitol. 18*, 141–153.
Hereld, D., Krakow, J. L., Bangs, J. D., Hart, G. W., & Englund, P. T. (1986) *J. Biol. Chem. 261*, 13813–13819.
Hermann, R., Jaenicke, R., & Rudolph, R. (1981) *Biochemistry 20*, 5195–5201.
Homans, S. W., Edge, C. J., Ferguson, M. A. J., Dwek, R. A., & Rademacher, T. W. (1989) *Biochemistry 28*, 2881–2887.
Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol. 49*, 117–237.
Jaenicke, R. (1988) in *Protein Structure and Protein Engineering* (Winnacker, E.-L., & Huber, R., Eds.) pp 16–36, Springer Verlag, Berlin.
Jaenicke, R., & Rudolph, R. (1986) *Methods Enzymol. 131*, 218–250.
Jähnig, F., Bülow, R., Baltz, T., & Overath, P. (1987) *FEBS Lett. 221*, 37–42.
Johnson, J. G., & Cross, G. A. M. (1977) *J. Protozool. 24*, 587–591.
Kawahara, K., & Tanford, C. (1966) *J. Biol. Chem. 241*, 3228–3232.
König, H., & Stetter, K. O. (1986) *Syst. Appl. Microbiol. 7*, 300–309.
Lauffer, M. A. (1944) *J. Am. Chem. Soc. 66*, 1195–1201.
Lauffer, M. A. (1989) *Motion in Biological Systems*, pp 48–50, A. R. Liss Inc., New York.
Masterson, W. J., Taylor, D., & Turner, M. J. (1988) *J. Immunol. 140*, 3194–3199.
Metcalf, P., Blum, M., Freymann, D., Turner, M. J., & Wiley, D. C. (1987) *Nature 325*, 84–86.
Metcalf, P., Down, J. A., Turner, M. J., & Wiley, D. C. (1988) *J. Biol. Chem. 263*, 17030–17033.
Sleytr, U. B., & Messner, P. (1988) *J. Bacteriol. 170*, 2891–2897.
Strickler, J. E., & Patton, C. L. (1982) *Exp. Parasitol. 53*, 117–132.
Turner, M. J. (1988) *The biology of parasitism*, pp 349–369, A. R. Liss Inc., New York.
Vickerman, K. (1969) *J. Cell Sci. 5*, 163–193.
Wetlaufer, D. B. (1962) *Adv. Protein Chem. 17*, 303–390.
Yphantis, D. A. (1964) *Biochemistry 3*, 297–317.

Extraction of the Energetics of Selected Types of Motion from Molecular Dynamics Trajectories by Filtering

Pnina Dauber-Osguthorpe and David J. Osguthorpe*
Molecular Graphics Unit, University of Bath, Claverton Down, Bath BA2 7AY, U.K.
Received March 15, 1990; Revised Manuscript Received May 11, 1990

ABSTRACT: A novel method for analyzing molecular dynamics trajectories has been developed which enables the study of selected motions and the corresponding energetics. In particular, it is possible to filter out the high-frequency motions and focus on the structural and energetic features of low-frequency collective motions. The trajectories of the properties of interest are Fourier transformed to the frequency domain, a filtering function is applied, and then an inverse transformation back to the time domain yields the filtered trajectory. The method is demonstrated for harmonic fluctuations and conformational transitions of acetamide and *N*-acetylalanine *N*-methylamide, as models for peptides and proteins.

In recent years the theoretical and experimental study of the dynamics of molecules has expanded dramatically. In particular, attention has been drawn to the flexibility and motion of biomolecules such as proteins and nucleic acid polymers and the role of the dynamic features in their biological function (Karplus & McCammon, 1981; McCammon & Harvey, 1987). Phenomena such as domain closure on ligand binding to enzymes (Remington et al., 1982), DNA unwinding when bound to drugs (Neidle & Waring, 1983), allosteric effects (Fletterick & Madsen, 1977), etc. all involve the collective motion of large regions of the molecules. Atomic and molecular mobility are of importance in other fields as well, for example, the diffusion of adsorbed molecules in zeolites, ion migration in superionic materials (Richards, 1989), the dynamic properties of bulk liquids and gases (Allen & Tildesley, 1987), and more. Thus, the investigation of such motion is of great interest.

In order to understand the dynamic behavior of molecular systems, it is important to be able to characterize the various motions in terms of changes in structure and energy. The theoretical investigation of conformational motion has involved three approaches to date. First, there is adiabatic (or flexible geometry) mapping in which steps along a conformation change path are taken (e.g., incrementing torsion angles or

0006-2960/90/0429-8223$02.50/0