Physical Properties of the Glycoprotein of Vesicular Stomatitis Virus Measured by Intrinsic Fluorescence and Aggregation†

Dan L. Crimmins and Sondra Schlesinger*

ABSTRACT: We have studied the denaturation and renaturation of the purified glycoprotein (G) of vesicular stomatitis virus by using intrinsic fluorescence spectroscopy and an aggregation assay. Our studies were carried out with G containing two complex oligosaccharide chains, with the asialo form of the protein, and for some experiments with G containing altered oligosaccharide structures. Fluorescence quenching using acrylamide showed no differences between the native and denatured states of G due to sialic acid content. Denaturation by guanidinium chloride (GdmCl) at 25 °C was reversible for the major transition region. The data analyzed by a two-state denaturation model gave a free energy of unfolding in the absence of denaturant of ~1.4 kcal/mol. For renaturation, two types of dialysis protocols were employed. The first (direct dialysis) involved dialysis against standard buffer [140 mM NaCl, 10 mM sodium phosphate, 1 mM disodium ethylenediaminetetraacetate, and 0.2% (w/v) poly(oxyethylene) 10-tridecyl ether, pH 7.4]. Recovery of the native emission maximum did not occur for any of the G proteins by using this procedure. The second (annealing dialysis) involved slow removal of GdmCl against decreasing concentrations of GdmCl in standard buffer over a period of 2–3 days. Only in this case was recovery of the native emission maximum and fluorescence intensity obtained. For those G proteins in which the oligosaccharide chains were decreased in size, this protocol led to extensive aggregation.

Glycoproteins of RNA-enveloped viruses can provide important models for studying the synthesis and structure of integral membrane proteins. These viruses contain limited genetic information and employ the same cellular organelles and enzymes for the synthesis and maturation of their glycoproteins as are required for the synthesis of cellular proteins. In addition to providing models for cellular membrane proteins, viral glycoproteins are important in their own right. They are required for the initial interaction between an enveloped virus and its host cell. Many of these proteins have a fusogenic activity which may be an essential part of the infectious process (White et al., 1981). Furthermore, it is these proteins, localized to the outer surface of virions and infected cells, to which the immune system responds in defense against a virus infection.

One of the most well-characterized viral glycoproteins is the G protein from the rhabdovirus, vesicular stomatitis virus (VSV).[1] The sequence of the mRNA encoding G is known (Rose & Gallione, 1981), permitting the localization of the signal peptide and the transmembrane segment. The virion polypeptide contains two asparagine-linked complex oligosaccharide chains (Tabas et al., 1978; Etchison & Holland, 1974; Etchison et al., 1977; Reading et al., 1978) and one to two covalently attached fatty acid molecules (Schmidt & Schlesinger, 1979; Schmidt et al., 1979). The molecular weight of the glycosylated polypeptide chain is about 60 000 (Rose & Gallione, 1981). Our previous studies had focused on the role of oligosaccharides in the maturation of G. They demonstrated that when G was synthesized in the absence of oligosaccharides the protein could be isolated from infected cells in an aggregated state (Gibson et al., 1978, 1979). The extent of aggregation was dependent both on the temperature of synthesis and on the primary structure of G. We also found that treatment of nonglycosylated or partially glycosylated G with GdmCl followed by removal of the denaturant by dialysis led to a temperature-dependent aggregation (Gibson et al., 1981). We suggested that oligosaccharides play an important role during the folding of G and that they may affect the ability of G to achieve a correct conformation (Gibson et al., 1981).

To define more accurately the factors required for the correct folding of G protein, we investigated conditions of denaturation and renaturation of the purified protein by using intrinsic fluorescence spectroscopy in addition to hydrodynamic properties. Most of the data reported here were obtained with G protein isolated from virions grown in BHK cells. Oligosaccharides on this protein are of the complex type containing *N*-acetylglucosamine, mannose, galactose, fucose, and variable amounts of sialic acid (Kornfeld et al., 1978). One potentially significant difference between G protein obtained from virions and newly synthesized proteins is the presence of sialic acid on the oligosaccharides of the former. Because these molecules add additional negative charges to the protein which could affect its stability during denaturation and renaturation, our studies were carried out with both the G protein [G(BHK)] and the asialo form. A limited amount of data is also presented for G protein with other alterations in oligosaccharide structure.

Materials and Methods

Cells, Viruses, and Purification of G. The cell line 15B (Gottlieb et al., 1975) is deficient in the enzyme GlcNAc transferase I, resulting in the accumulation of asparagine-linked oligosaccharides with the structure $Man_5GlcNAc_2$ (Kornfeld et al., 1978). Clone 6 (Gottlieb & Kornfeld, 1976) is deficient in α-mannosidase activity and accumulates asparagine-linked oligosaccharides containing $Man_8GlcNAc_2$. The growth and purification of VSV (Indiana serotype, San Juan strain) have been described (Gibson et al., 1979, 1981). G protein was extracted from purified VSV with octyl β-D-glucoside by using the method of Miller et al. (1980).

† From the Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110. *Received January 11, 1982.* This work was supported by Grants AI11377, AI12242, and P30 CA 16217 from the National Institutes of Health. D.L.C. was supported in part by Training Grant T32 CA 09129 from the National Institutes of Health.

[1] Abbreviations: VSV, vesicular stomatitis virus; GdmCl, guanidinium chloride; emulphogen, poly(oxyethylene) 10-tridecyl ether; GlcNAc, *N*-acetylglucosamine; Man, mannose; endo H, endo-β-*N*-acetylglucosaminidase H; NATA, *N*-acetyl-L-tryptophanamide; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/82/0421-3518$01.25/0

Each preparation of G was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and based on both protein staining and radioactivity was more than 95% pure G (Gibson et al., 1981). G protein preparations were stored at 4 °C and generally used within 2 weeks.

Glycosidase Treatments. For removal of sialic acid from G, VSV (0.5 mg) was incubated for 1 h at 37 °C with 100 units of neuraminidase (Calbiochem-Behring) in 50 mM sodium acetate, pH 5.5, and 10 mM $CaCl_2$ in a final volume of 2 mL. The virus was pelleted by centrifugation for 90 min at 100000*g*, resuspended in 10 mM sodium phosphate, pH 8.0, recentrifuged as before, and finally resuspended in 10 mM sodium phosphate, pH 8.0, to a concentration of 2–3 mg/mL. When VSV was labeled with [^{3}H]glucosamine, 25–30% of the radioactivity was released after incubation with neuraminidase. (If all of the galactose residues were covered by sialic acid and if all of the sialic acid were released by neuraminidase treatment, 37% of the radioactivity would be released.) This result plus analysis of the glycopeptides by gel filtration and analysis of the protein by isoelectric focusing demonstrated that most of the sialic acid residues had been removed.

Lyophilized endo H, a gift from Dr. P. W. Robbins of Massachusetts Institute of Technology, was resuspended in water at 30 μg/mL and stored as frozen aliquots at −20 °C. Equal volumes of extracted G(15B) (5–15 μg) and 0.3 M citrate containing 0.2% (w/v) emulphogen, pH 5.5, were incubated for 18 h at room temperature with 0.3–0.9 μg of enzyme. We routinely analyzed the success of digestion by $NaDodSO_4$–polyacrylamide gel electrophoresis as the endo H treated material migrates faster than untreated protein (Tarentino et al., 1974; Gibson, 1981). The endo H treated protein, or the control treated in the same manner but without enzyme, was diluted into standard buffer or ≥7 M GdmCl in standard buffer to a final G protein concentration of ~5–20 μg/mL. These solutions were then used for denaturation, renaturation, or acrylamide quenching studies (see below).

Denaturation. G protein (final concentration of 5–20 μg/mL and volume of 1.2–1.5 mL) was diluted with 8 M GdmCl in standard buffer to the desired denaturant concentration and incubated at room temperature for 30 min–16 h. These samples were then used for fluorescence measurements or renaturation experiments.

Renaturation. The denatured protein (≥7 M GdmCl) was dialyzed against standard buffer at room temperature to remove the denaturant. Two types of dialysis protocols were used. The first (direct dialysis) involved dialysis of the denatured and control samples vs. a 200-fold volume excess of standard buffer for two 3-h periods (Gibson et al., 1981). In the second dialysis procedure (annealing dialysis), the denatured protein was successively dialyzed for the indicated times vs. a 200-volume excess of standard buffer containing 6 M GdmCl (4 h), 5 M GdmCl (4 h), 4 M GdmCl (16 h), 3 M GdmCl (4 h), 2 M GdmCl (4 h), and 1 M GdmCl (16 h) and finally against a 200-volume excess of standard buffer for two 3-h periods. A protein solution in standard buffer at the same protein concentration was *always* run as a control. In both protocols, the volume of the sample was measured after completion of dialysis, and samples were taken to determine radioactivity. Protein recovery was ≥90% in all cases. The denaturant-free sample was used for fluorescence measurements and then centrifuged at 100000*g* for 90 min at 4 °C (aggregation assay).

Fluorescence Measurements. An Aminco SPF spectrofluorometer operating in the ratio mode and an X-Y recorder were used to measure and record the protein intrinsic fluorescence. All measurements were performed at room temperature in acid-washed Teflon-stoppered, 1-cm pathlength quartz cuvettes. Instrumental conditions were as follows: time constant, 1 s; scan speed, 100 nm/min; exciting wavelength, 295 nm with a 1-nm band-pass; emission wavelength, 300–400 nm with a band-pass of 10 nm. The sensitivity scales (0.1 and 1.0) and the vernier adjustment were chosen to given an initial relative fluorescence of >80% full scale. Each sample (or solvent) was scanned a minimum of 2 times for averaging with the exciting lamp shutter closed between measurements to minimize heating or photodecomposition. Reported λ_{max} and relative intensity values have been manually subtracted from solvent at 1-nm intervals and have not been corrected for photomultiplier response vs. wavelength. The absorbance of all solutions was <0.1 at 295 nm. Instrument linearity and daily performance were checked by using NATA, 2–50 μM, in standard buffer. This concentration range more than spanned the response of G protein. During the course of these experiments, λ_{max} and the fluorescence intensity of the standard varied less than ±5%.

Acrylamide quenching studies were performed on G protein essentially as described (Eftink & Ghiron, 1981). Briefly, successive 10-μL aliquots of 8 M acrylamide in standard buffer were added to both G protein in the buffer of interest and an appropriate solvent blank at initial volumes of 1.5 mL. In initial experiments, 20–30 s after addition of acrylamide followed by gentle inversion of the cuvette the emission spectrum from 300 to 400 nm was monitored to check for anomalous behavior, especially denaturation. Later, only the fluorescence intensity at the appropriate λ_{max} was monitored.

Data from acrylamide quenching experiments were analyzed in terms of a modified Stern–Volmer equation:

$$F_0/F = (1 + K_{eff}[Ac]) \exp(V_{eff}[Ac]) \quad (1)$$

where F_0 and F are the fluorescence intensity at λ_{max} in the absence and presence of acrylamide, respectively, K_{eff} is the effective collisional quenching constant, V_{eff} is the effective static quenching constant, and [Ac] is the molar concentration of acrylamide (Eftink & Ghiron, 1981). Correction was made for acrylamide absorbance at 295 nm and sample dilution (Cockle et al., 1978; Lehrer, 1978). A computer program was written to plot F_0/F vs. [Ac] and determine the slope and intercept by linear least-squares regression analysis. Where it was obvious that such plots were nonlinear, V_{eff} was varied from 0 to 3.0 M^{-1} in 0.05 M^{-1} increments until two conditions were satisfied, namely, the chosen V_{eff} gave an intercept of 1.00 ± 0.01 and the subsequent correlation coefficient was >0.99. In all cases, a unique value of V_{eff} was obtained.

Results

Intrinsic Fluorescence and Fluorescence Quenching by Acrylamide for G(BHK) and Asialo-G. The intrinsic fluorescence of native proteins is a fairly reliable measure of the environment of aromatic amino acids, with tyrosine and particularly tryptophan dominating. Fluorophores residing in a hydrophobic environment tend to show both a shift to shorter wavelengths and a greater quantum yield than those which are hydrophilic (Freifelder, 1976). The emission spectra for native and denatured G(BHK) are shown in Figure 1. In standard buffer, a structureless spectrum with a maximum at 340 nm was found.[2] In contrast, the denatured protein has

[2] The intrinsic fluorescence for native G(BHK) in two other nonionic detergents, octyl β-D-glucoside and Brij 58 [poly(oxyethylene) 20-cetyl ether], was identical with that shown, suggesting that the choice of detergent did not drastically influence the average tryptophan environment (data not shown).

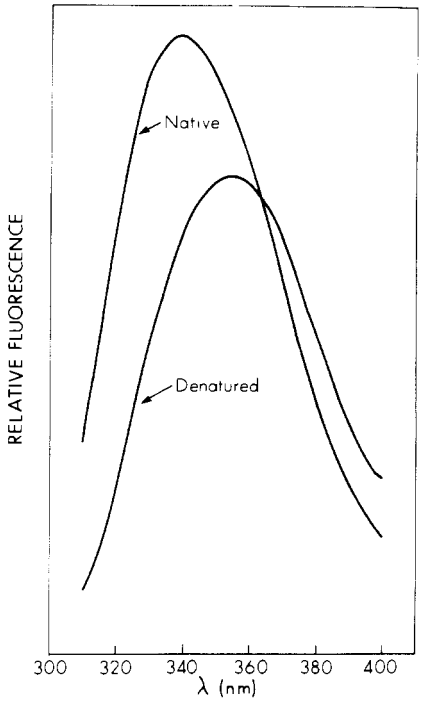

FIGURE 1: Uncorrected emission spectra for G(BHK) upon excitation at 295 nm. The concentration of G was 20 μg/mL in standard buffer (native) or standard buffer with 7.2 M GdmCl (denatured). The ordinate is in arbitrary units.

Table I: Intrinsic Fluorescence and Acrylamide Quenching Parameters for G(BHK), Asialo-G, and NATA[a]

	G(BHK)	asialo-G	NATA
standard buffer			
λ_{max}	340	340	358
K_{eff} (M^{-1})	2.75	2.56	13.3
	(9, 0.10)	(3, 0.15)	(4, 0.73)
V_{eff} (M^{-1})	<0.01	<0.01	1.53
	(9, <0.01)	(3, <0.01)	(4, 0.14)
standard buffer + ≥7.2 M GmdCl			
λ_{max}	356	356	360
K_{eff} (M^{-1})	3.03	3.15	7.14
	(5, 0.26)	(3, 0.20)	(6, 0.32)
V_{eff} (M^{-1})	0.55	0.47	1.46
	(5, 0.11)	(3, 0.18)	(6, 0.05)

[a] Reproducibility of λ_{max} was ± 1 nm. Acrylamide quenching parameters were determined from eq 1 as described under Materials and Methods. The numbers in parentheses are the number of determinations and the sample standard deviation, respectively, and are averages of at least two different virus preparations.

its maximum red shifted to 356 nm with a concomitant decrease in quantum yield. This GdmCl-induced shift to higher emission wavelength indicates an increased exposure of tryptophan residues while the decreased intensity reflects solvent quenching.

A summary of the emission maxima is presented in Table I for G(BHK), asialo-G, and the model compound NATA. Two points are noteworthy. First, λ_{max} values for both native and denatured asialo-G were identical with the λ_{max} value for G(BHK), showing that the negatively charged sialic acid residues did not influence the average tryptophan environment of this protein. Second, neither denatured protein had a λ_{max} identical with that of the model compound.

The quenching of fluorescence by acrylamide is a well-documented method for determining heterogeneity in the exposure of tryptophan residues to solvent (Eftink & Ghiron, 1975, 1976, 1977, 1981). The data are analyzed in terms of

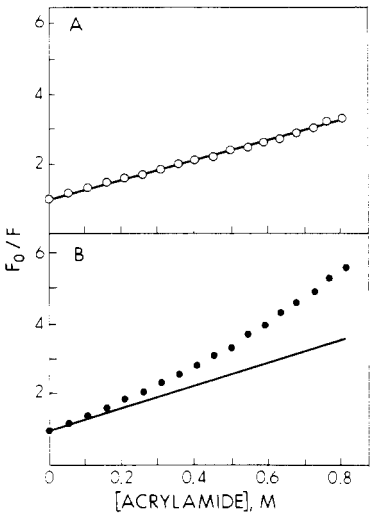

FIGURE 2: Stern–Volmer plot of F_0/F vs. [acrylamide] for G(BHK) in the native (○) and denatured (●) states. The solid line in both cases is the best fit to eq 1 as described under Materials and Methods. (A) Native protein: K_{eff} = 2.73 M^{-1}; V_{eff} = 0.0 M^{-1}; intercept = 0.996; correlation coefficient = 0.999. (B) Denatured protein: K_{eff} = 3.13 M^{-1}; V_{eff} = 0.55 M^{-1}; intercept = 0.992; correlation coefficient = 0.999.

a modified Stern–Volmer equation (see Materials and Methods). Two parameters emerge from such an analysis: K_{eff}, the effective collisional quenching constant (a product of the unquenched lifetime and the quenching rate constant), and V_{eff}, the effective static quenching constant. The last parameter is a measure of the probability of finding a quencher molecule close enough to a tryptophan residue to deactivate it instantaneously.

Results from a typical quenching experiment carried out with G are plotted in Figure 2 as F_0/F vs. [acrylamide]. Figure 2A shows that quenching in the native state is solely the result of a collisional mechanism as V_{eff} is essentially zero. This means (Eftink & Ghiron, 1976) that the native protein shows heterogeneous fluorescence with tryptophan residues differing slightly in accessibility [not an altogether surprising result for a protein which has 15 tryptophans per 495 residues (Rose & Gallione, 1981)]. Like other investigators (Eftink & Ghirion, 1976; Cockle et al., 1978), we also observed a slight blue shift during quenching. We attribute this to selective quenching of solvated tryptophans which emit at longer wavelengths. This blue shift also suggests that acrylamide does not denature the protein.

Quenching in the denatured state, however, shows that both static and collisional mechanisms were operative. The plot given in Figure 2B now shows positive deviation from linearity ($V_{eff} > 0$). Two possible explanations for this static quenching are that there is near equal accessibility of all tryptophan residues or that the fluorescence is being dominated by a single class of tryptophans (Eftink & Ghiron, 1976). K_{eff} also increases slightly for the denatured protein, but in the absence of lifetime measurements, it is difficult to interpret this result.

Table I lists the values for the two quenching parameters in both the native and denatured states. Again, like λ_{max}, there appears to be little difference between G(BHK) and asialo-G. Taken together, the similarity of λ_{max} and the quenching parameters allow us to conclude that the native (initial) and denatured (final) conformation of G(BHK) is not influenced by sialic acid content.

Denaturation of G by GdmCl. We have used two parameters of fluorescence to monitor the unfolding of G caused by exposure to GdmCl: the red shift in λ_{max} and F_{340}/F_{356}. The latter is an attempt to resolve each spectrum into native and

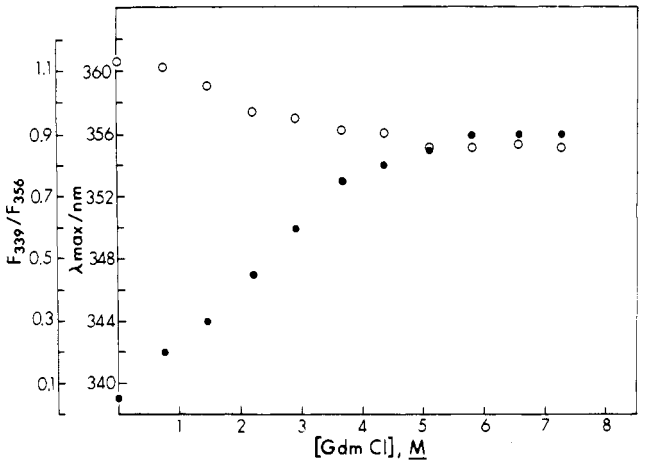

FIGURE 3: GdmCl-induced fluorescence changes for G(BHK). λ_{max} (●) and F_{339}/F_{356} (○) vs. [GdmCl].

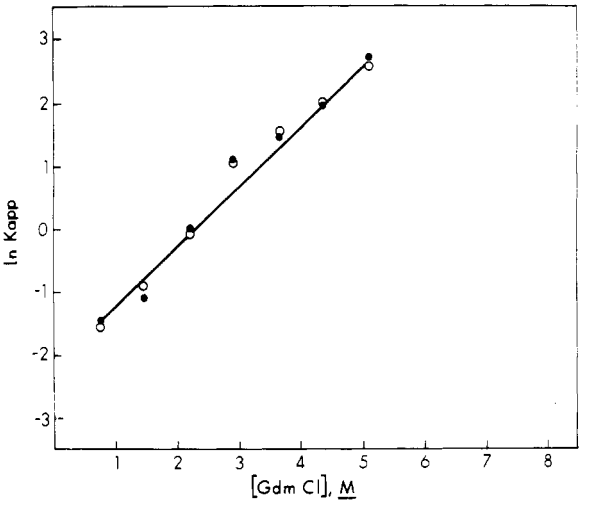

FIGURE 4: Two-state denaturation for G(BHK) plotted as ln K_{app} vs. [GdmCl]. $K_{app} = (1 - f_N)/f_N$, where f_N is the fraction of native protein. BHK (I) (○); BHK (II) (●). Two G(BHK) and two asialo-G preparations have been omitted to avoid clutter. Linear least-squares regression analysis of all six samples gives ln K_{app} = (0.95 M^{-1})-[GdmCl] − 2.18, with a correlation coefficient of 0.99.

denatured components. Both of these parameters are plotted as a function of [GdmCl] in Figure 3 for G(BHK). As [GdmCl] increases, we see a monotonic parallel increase in λ_{max} and a decrease in F_{339}/F_{356}. Note that in both cases, neither parameter changes significantly after ~5 M GdmCl. We interpret this to mean that denaturation, as followed by these fluorescence parameters, is essentially complete after 5 M GdmCl. The curve for asialo-G using both parameters is very similar to that shown in Figure 3, providing further evidence that sialic acid content does not appear to influence the unfolding properties of this protein (data not shown).

In denaturation studies, it is desirable to make measurements under equilibrium conditions and be able to reverse the effect of the denaturant. Only when these two conditions are met can a meaningful thermodynamic analysis of the data be attempted. The protein was incubated at room temperature at given denaturant concentrations for various lengths of time between 30 min and 16 h before the fluorescence was measured. All changes in spectra had occurred by 30 min, indicating that we were making equilibrium measurements at each GdmCl concentration used. (The incubation time was then chosen as a matter of convenience.)

The strategy for checking reversibility involved preparing protein solutions equal in concentration but at different [GdmCl]. These different GdmCl solutions were then mixed in various proportions to produce a new [GdmCl] which was then compared to a protein solution originally prepared at this new [GdmCl]. Over the major range of [GdmCl] used, denaturation was reversible as evidenced by spectral properties (data not shown). These mixing experiments rule out any anomalous effect that GdmCl may have on the detergent such as changing the critical micelle concentration (Gratzer & Beaven, 1969) and indicate that any observed spectral change due to GdmCl is the result of the denaturant acting on the protein and *not* on the detergent properties. Dialysis experiments where native and denatured (≥7.2 M GdmCl) proteins were dialyzed vs. intermediate [GdmCl] and then compared to a sample made up at that intermediate [GdmCl] gave the same results as the mixing experiments. Thus, for the major part of the denaturation curve, we were making equilibrium measurements and achieved reversibility.

Since the denaturation curves are apparently monophasic (Figure 3), we tested the applicability of a two-state denaturation mechanism (Lapange, 1978). In this case, we used λ_{max} as the parameter of interest as it showed the greatest variation with [GdmCl] and so should be subject to less experimental error than F_{340}/F_{356}. The apparent equilibrium constant, K_{app}, between native (N) and denatured (D) species given in terms of λ_{max} is then

$$K_{app} = \frac{\lambda_{max,N} - \lambda_{max}}{\lambda_{max} - \lambda_{max,D}} \quad (2)$$

where $\lambda_{max,N} \sim 340$ nm, $\lambda_{max,D} \sim 356$ nm, and λ_{max} is at intermediate [GdmCl]. A plot of ln K_{app} vs. [GdmCl] is given in Figure 4 for two different preparations of G(BHK). (The data from two other samples of G(BHK) and from two asialo-G samples were omitted to avoid clutter.) A linear least-squares fit of the data for all six samples gives a slope of 0.95 M^{-1} with the midpoint of the transition at 2.28 M GdmCl. In order to obtain the free energy of unfolding in the absence of denaturant, $\Delta G°$, we followed Schellman (1978) by using the relationships:

$$\Delta G° = -RT\Delta b_{23} C°_m \quad (3)$$

and

$$\Delta b_{23} = [\partial(\ln K_{app})/\partial C_3]_{T,P} \quad (4)$$

where $C°_m$ is the denaturant concentration at the midpoint of the transition and Δb_{23} in our case is simply the slope of the plot in Figure 4. Substitution of the experimental data into these last two equations gives a free energy of unfolding in the absence of denaturant at 25 °C of ~+1.4 kcal/mol. If F_{340}/F_{356} is used to obtain $\Delta G°$ instead of λ_{max}, a value of ~+0.4 kcal/mol is obtained. The difference in these two $\Delta G°$ values is probably due to the small change in the ratio parameter as a function of [GdmCl] manifesting itself in a larger experimental error. In either case, this is an extremely small stabilization energy.

Renaturation of G. The denatured protein was dialyzed directly against standard buffer in an attempt to regain native spectral features. The dialyzed samples were analyzed both by fluorescence measurements and by determining the extent of aggregation after centrifugation at 100000*g* for 90 min at 4 °C (Table II). The aggregation assay was included since improper renaturation of some proteins can lead to aggregation (Orsini & Goldberg, 1978) (see also Table III). The most striking feature of the data obtained for direct dialysis was that the putative renatured protein *did not* possess a native λ_{max} (column N″) when monitored at an exciting wavelength of 295 nm. Once again, there was little, if any, effect of sialic

Table II: Fluorescence and Aggregation Properties of Renatured G(BHK) and Asialo-G

	λ_{max}				$F_{N''}/F_{N'}$[b]	cpm in supernatant[c]	
	N[a]	D	N′	N″		N′	N″
direct renaturation							
G(BHK) (I)[d]	339	357	340	348	0.86	92	96
G(BHK) (I)	339	357	342	349	0.95	92	97
G(BHK) (II)	339	356	341	348	0.86	98	95
G(BHK) (III)	340	356	340	347	0.83	99	98
asialo-G (III)	340	356	340	348	0.84	99	99
annealing renaturation							
G(BHK) (I)	340	357	339	340	0.88	96	84
G(BHK) (II)	340	357	339	340	0.79	95	75
G(BHK) (III)	339	356	339	339	0.94	98	96
asialo-G (III)	339	356	338	339	0.91	98	86

[a] N, native protein in standard buffer; D, denatured protein in standard buffer + ⩾7.2 M GdmCl; N′, native protein dialyzed vs. standard buffer; N″, denatured protein dialyzed vs. standard buffer. [b] The ratio of the fluorescence intensities corrected for recovery after dialysis at the corresponding λ_{max} of species N″ to species N′. [c] The percent of the total radioactivity remaining in the supernatant after centrifuging at 100000*g* for 90 min at 4 °C. [d] The Roman numerals refer to different virus preparations.

acid. Aggregation was surely not the cause of improper renaturation as the last column shows >92% of the protein remains soluble after pelleting. Surprisingly, when the renatured protein from direct dialysis was monitored at λ_{ex} = 280 nm recovery of *both* λ_{max} and fluorescence intensity was found (Schlesinger & Gibson, 1981; D. L. Crimmins and S. Schlesinger, unpublished results).

Rapid removal of the denaturant might explain the inability of the protein to attain the correct native conformation. In contrast, slow removal of the denaturant (annealing) may allow true equilibrium to be reached at each point along the folding pathway, the net result being a return to the native conformation. Annealing experiments were designed to remove the denaturant at ~1 M decrements (see Materials and Methods for the exact protocol). In all cases, with excitation at 295 nm, the renatured protein (N″) has the same λ_{max} as does the control (N′) (Table II). We observed a small and variable amount of aggregation in these experiments which appeared to be related to the recovery of fluorescence intensity ($F_{N''}/F_{N'}$). Our subsequent analysis, however, demonstrated that aggregation did not affect the recovery of fluorescence intensity (Table III). Analysis of the renatured protein by NaDodSO$_4$–polyacrylamide gel electrophoresis demonstrated that no proteolysis had occurred during dialysis (data not shown).

An indication of the size distribution of aggregates is shown in Figure 5. G(BHK) and asialo-G samples from an annealing experiment were analyzed on a nondenaturing gel in a polyacrylamide gradient. There were only two populations for both of the renatured species (N″), those which did not enter the 3% stacking gel and those which migrated like the control samples (N′). Thus, the majority of the protein after annealing renaturation had the same molecular size and shape and most likely molecular weight as the control samples. Identical results were obtained for samples from direct dialysis experiments, except in this case all the material from the renatured samples (N″) entered the gel and migrated identically with the control samples (N′) (data not shown).

Analysis of G with Alterations in Oligosaccharides. We had demonstrated previously that the size of the oligosaccharide chain on G influenced the refolding of this protein at 40 °C as measured by aggregation (Gibson et al., 1981). To determine if oligosaccharides had any effect on denaturation or renaturation as measured by fluorescence, we carried out a series of experiments in which the oligosaccharide structures on G were $Man_8GlcNAc_2$, $Man_5GlcNAc_2$, and GlcNAc. The latter structure (GlcNAc) was obtained by treating G($Man_5GlcNAc_2$) with endo H as described under Materials and Methods.

Alterations in the oligosaccharides on G did not change any of the parameters of fluorescence we measured (Table III). As with G(BHK), the annealing protocol was required for

Table III: Fluorescence and Aggregation Properties of G Proteins Possessing Different Oligosaccharide Structures[a]

(A) Intrinsic Fluorescence and Acrylamide Quenching Parameters

	G($Man_5GlcNAc_2$)	G(GlcNAc)	G($Man_8GlcNAc_2$)
standard buffer			
λ_{max}	340	340	339
K_{eff} (M^{-1})	2.49 (4, 0.13)	2.60 (3, 0.15)	2.70 (4, 0.17)
V_{eff} (M^{-1})	<0.01 (4, <0.01)	<0.01 (3, <0.01)	<0.01 (4, <0.01)
standard buffer + ⩾7.2 M GdmCl			
λ_{max}	356	356	356
K_{eff} (M^{-1})	3.11 (4, 0.26)	nd[b]	3.03 (4, 0.12)
V_{eff} (M^{-1})	0.47 (4, 0.19)	nd	0.33 (4, 0.10)

(B) Renaturation

	λ_{max}					cpm in supernatant	
	N	D	N′	N″	$F_{N''}/F_{N'}$	N′	N″
direct renaturation							
G($Man_5GlcNAc_2$) (I)	340	357	341	350	0.99	98	96
G($Man_5GlcNAc_2$) (I)	341	357	342	352	0.90	98	89
G($Man_5GlcNAc_2$) (II)	339	357	342	354	1.16	88	95
G($Man_5GlcNAc_2$) (III)	339	356	342	348	0.77	95	89
G($Man_5GlcNAc_2$) (IV)	340	356	342	352	0.92	89	96
G($GlcNAc_1$) (III)	339	356	341	353	1.18	89	89
G($Man_8GlcNAc_2$) (III)	340	356	342	352	0.78	97	93
annealing renaturation							
G($Man_5GlcNAc_2$) (III)	338	356	339	339	1.19	95	58
G($Man_5GlcNAc_2$) (IV)	340	358	340	340	0.91	95	73
G(GlcNAc) (III)	338	356	339	339	0.95	93	44
G($Man_8GlcNAc_2$) (III)	340	357	339	340	0.88	98	82

[a] See Tables I and II for a description of the symbols used. [b] nd, not determined.

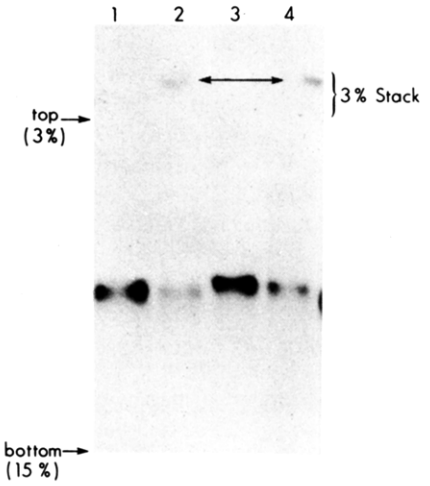

FIGURE 5: Nondenaturing polyacrylamide gel electrophoresis in a 3–15% linear acrylamide gradient. Fluorogram of [^{3}H]glucosamine-labeled G(BHK) and asialo-G after annealing renaturation and prior to the aggregation assay. Nondenaturing gels in a 3–15% linear gradient were prepared according to Nakashima & Makino (1980) and Nakashima et al. (1981) with the following modifications: the use of 0.2% (w/v) emulphogen instead of 0.1% (w/v) nonaethylene dodecyl ether and the incorporation of a 3% acrylamide stacking gel. The buffer was 10 mM Tris and 80 mM glycine, pH 8.3. Electrophoresis was for 18 h at 125 V at room temperature. Lane 1, G(BHK) species N′; lane 2, G(BHK) species N″; lane 3, asialo-G species N′; lane 4, asialo-G species N″. Top and bottom of gradient gel and 3% stack are indicated. Note that some material from both N″ species is not entering the stacking gel (arrow).

these G proteins to recover the native fluorescence spectra. However, the G proteins with decreased oligosaccharide size showed extensive aggregation after the annealing dialysis. The recovery of fluorescence intensity was more than 90% even when about 50% of the protein had aggregated.

Discussion

The goal of protein denaturation–renaturation experiments is to understand the complicated forces which serve to stabilize a protein's native conformation. Of the many possible conformational states available during protein folding, only a few are utilized. Experimental and theoretical results have shown the native state to be only marginally stable relative to the unfolded state (Tanford, 1970). Membrane proteins pose a particularly difficult challenge in this kind of study because they are extremely hydrophobic and are generally insoluble in an aqueous environment. Physicochemical studies of these proteins are usually carried out in detergents, and the interactions of these proteins with detergents introduce further complexities (Helenius & Simons, 1975; Tanford & Reynolds, 1976).

As a first step in determining the requirements for the correct folding of the G protein of VSV, we investigated the denaturation and renaturation of this protein by using intrinsic fluorescence spectroscopy. Most of our work was performed with G containing two complex oligosaccharide chains. This is the carbohydrate structure on the mature form of G found in virions (Etchison et al., 1977; Reading et al., 1978). We also carried out these experiments with the asialo form of G and determined that the removal of sialic acid from this protein had no effect on any of the properties we measured. As discussed below, other changes in oligosaccharide structure did affect some of these properties.

The denaturation curves for G and asialo-G were analyzed in terms of a two-state model, and a free energy of unfolding in the absence of denaturant ($\Delta G°$) at 25 °C of +1.4 kcal/mol was calculated. An estimate of $\Delta G°$ can also be made from the amino acid composition of a protein (Brandts, 1964; Kawaguchi & Noda, 1977). This method divides amino acid side chains into five groups, three of which are hydrophobic and are presumed to make a significant contribution to the overall stability. Recent sequence data (Rose & Gallione, 1981) on G protein allow us to place 146 residues in group I (ΔG_{tr} = 2.5 kcal/mol), 68 residues in group II (ΔG_{tr} = 1.5 kcal/mol), and 39 residues in group III (ΔG_{tr} = 0.6 kcal/mol) out of a total of 495 residues. ΔG_{tr} is the per mole free energy contribution to the stability of the native structure for an amino acid side chain from each group. Performing such a calculation gives $\Delta G° \sim +1$ kcal/mol, in good agreement with our experimental results. These values for stabilization energy are small, but the denaturation experiments were carried out under nonreducing conditions and the conformational states accessible to the molecule during refolding should be limited. Thus, the small $\Delta G°$ may reflect an increased stability of the denatured nonreduced molecule relative to the denatured reduced molecule. Since G protein contains 13 sulfhydryl residues (Rose & Gallione, 1981) in unknown disulfide linkage, we have purposely omitted reducing agent in these initial physical studies to avoid incorrect disulfide bond pairing during refolding. Our preliminary data indicate that reducing agents have no effect on the fluorescence properties of either the native or the denatured state of G (D. L. Crimmins and S. Schlesinger, unpublished results).

Renaturation of G was measured by fluorescence and by the extent to which the protein aggregated. The rapid removal of GdmCl by dialysis did not lead to the recovery of the native fluorescence spectra for any of the G proteins examined (Table II). These renatured molecules were indistinguishable from the native protein in size. We were able to recover the native fluorescence spectra of G by stepwise removal of GdmCl. In some instances, however, this procedure led to aggregation of the protein. The extent of aggregation was very small for those G proteins with oligosaccharide chains of normal size but was quite significant for G proteins with oligosaccharide chains of reduced size. Our results using the assays of fluorescence spectroscopy and aggregation emphasize the need to monitor renaturation by several parameters. In the direct dialysis protocol for renaturation, fluorescence was a more sensitive indicator of incorrect renaturation than aggregation. In contrast, refolding of G which led to a return of the native fluorescence also led in some cases to improper folding detected by aggregation.

The aggregation observed by slow removal of GdmCl at room temperature parallels the results we obtained previously by direct dialysis of GdmCl at 40 °C (Gibson et al., 1981). G proteins with the oligosaccharide chain structure of either $Man_5GlcNAc_2$ or $Man_1GlcNAc_2$ aggregated after the removal of GdmCl at 40 °C, whereas G protein with the larger oligosaccharide structure of $Man_8GlcNAc_2$ did not. Thus, the present data provide further support for our proposal (Gibson et al., 1981) that for some proteins the size of the oligosaccharide chains can be an important factor in the ability of a protein to achieve the correct conformation.

Acknowledgments

We thank Dr. Paul Schlesinger (Department of Physiology and Biophysics) for allowing us to monopolize his spectrofluorometer and Steve Monroe, who wrote the computer program which permitted us to analyze the acrylamide fluorescence quenching data. Susan Peirce and Bill Mehard provided excellent technical assistance.

References

Brandts, J. F. (1964) *J. Am. Chem. Soc. 86*, 4302–4314.

Cockle, S. A., Epand, R. M., & Moscarello, M. A. (1978) *Biochemistry 17*, 630–637.
Eftink, M. R., & Ghiron, C. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3290–3294.
Eftink, M. R., & Ghiron, C. A. (1976) *Biochemistry 15*, 672–680.
Eftink, M. R., & Ghiron, C. A. (1977) *Biochemistry 16*, 5546–5551.
Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199–227.
Etchison, J. R., & Holland, J. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 75*, 715–719.
Etchison, J. R., Robertson, J. S., & Summers, D. F. (1977) *Virology 78*, 375–392.
Freifelder, D. (1976) in *Physical Biochemistry: Applications to Biochemistry and Molecular Biology*, pp 415–417, W. H. Freeman, San Francisco, CA.
Gibson, R. (1981) Ph.D. Thesis, Washington University, St. Louis, MO.
Gibson, R., Leavitt, R., Kornfeld, S., & Schlesinger, S. (1978) *Cell (Cambridge, Mass.) 13*, 671–679.
Gibson, R., Schlesinger, S., & Kornfeld, S. (1979) *J. Biol. Chem. 254*, 3600–3607.
Gibson, R., Kornfeld, S., & Schlesinger, S. (1981) *J. Biol. Chem. 256*, 456–462.
Gottlieb, C., & Kornfeld, S. (1976) *J. Biol. Chem. 251*, 7761–7768.
Gottlieb, C., Baenziger, J., & Kornfeld, S. (1975) *J. Biol. Chem. 250*, 3303–3309.
Gratzer, W. B., & Beaven, G. H. (1969) *J. Phys. Chem. 73*, 2270–2273.
Helenius, A., & Simons, K. (1975) *Biochim. Biophys. Acta 415*, 29–79.
Kawaguchi, H., & Noda, H. (1977) *J. Biochem. (Tokyo) 81*, 1307–1317.
Kornfeld, S., Li, E., & Tabas, I. (1978) *J. Biol. Chem. 253*, 7771–7786.
Lapange, S. (1978) in *Physicochemical Aspects of Protein Denaturation*, pp 187–188, Wiley, New York.
Lehrer, S. (1978) *J. Mol. Biol. 118*, 209–226.
Miller, K. D., Feuer, B. I., Van Deroef, R., & Lenard, J. (1980) *J. Cell Biol. 84*, 421–429.
Nakashima, H., & Makino, S. (1980) *J. Biochem. (Tokyo) 88*, 933–947.
Nakashima, H., Nakagawa, Y., & Makino, S. (1981) *Biochim. Biophys. Acta. 643*, 509–518.
Orsini, G., & Goldberg, M. E. (1978) *J. Biol. Chem. 253*, 3453–3458.
Reading, C. L., Penhoet, E. E., & Ballou, C. E. (1978) *J. Biol. Chem. 253*, 5600–5612.
Rose, J. K., & Gallione, C. J. (1981) *J. Virol. 39*, 519–528.
Schellman, J. A. (1978) *Biopolymers 17*, 1305–1322.
Schlesinger, S., & Gibson, R. (1981) in *The Replication of Negative Strand Viruses* (Bishop, D. H. L., & Compans, R. W., Ed.) pp 665–672, Elsevier/North-Holland, New York.
Schmidt, M. F. G., & Schlesinger, M. J. (1979) *Cell (Cambridge, Mass.) 17*, 813–819.
Schmidt, M. F. G., Bracha, M., & Schlesinger, M. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1687–1691.
Tabas, I., Schlesinger, S., & Kornfeld, S. (1978) *J. Biol. Chem. 253*, 716–722.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95.
Tanford, C., & Reynolds, J. A. (1976) *Biochim. Biophys. Acta 457*, 133–170.
Tarentino, A. L., Plummer, T. H., Jr., & Maley, F. (1974) *J. Biol. Chem. 249*, 818–824.
White, J., Matlin, K., & Helenius, A. (1981) *J. Cell Biol. 89*, 674–679.