Physical Properties of a Soluble Form of the Glycoprotein of Vesicular Stomatitis Virus at Neutral and Acidic pH†

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ABSTRACT: We have analyzed a soluble form of the glycoprotein (G) obtained from vesicular stomatitis virus (VSV) by treatment of intact virions with cathepsin D. This form lacks the carboxy-terminal and membrane-spanning domains and thus is analogous to the previously described secreted form of G, G_s. The molecular weight of the cathepsin D produced G, G(Cath D), measured by sedimentation equilibrium in the analytical ultracentrifuge is 57 600, indicating that it is a monomer. Intact G protein extracted from virions by octyl β-D-glucoside also is monomeric, based on sedimentation equilibrium analysis. These results suggest that G may be monomeric in virions. The Stokes radii (R_s) of the two forms of G were obtained from their migration in nondenaturing polyacrylamide gradient gels. The R_s of G(Cath D) in the absence of nonionic detergent was 37 Å; in the presence of nonionic detergent, it increased to 55 Å. The R_s of detergent-extracted intact G was 63 Å in nonionic detergent. From the molecular weight and R_s of G(Cath D), we calculated a sedimentation coefficient of 3.8 S; the value determined by centrifugation in a sucrose gradient was 3.7 S. Viruses such as VSV fuse with cell membranes at low pH [White, J., Matlin, K., & Helenius, A. (1981) *J. Cell Biol. 89*, 674–679]. We have used the fluorescent probe *cis,trans,trans,cis*-9,11,13,15-parinaric acid (*cis*-PnA) to detect a reversible conformational change in G(Cath D) when the protein was exposed to an acidic environment close to pH 5. *cis*-PnA binds to hydrophobic regions of protein, causing a quenching of the intrinsic tryptophan fluorescence and an increase in the fluorescence of the probe. Our results suggest that a conformational change in G occurs when VSV interacts with cellular membranes.

The membrane glycoproteins of enveloped viruses have a dual role in the infectious cycle. First, they are involved in the initial interaction of the virions with the host cell; second, during virus replication, the newly synthesized glycoproteins are usually required for the final stages of assembly and export of virus. Considerable evidence is now accruing about these proteins and how they function in these roles. In particular, the hemagglutinin (HA)[1] of influenza virus, the glycoprotein (G) of VSV, the glycoproteins (E1 and E2) of the α-viruses, Sindbis virus and Semliki Forest virus, and the fusion protein (F) of Sendai virus have been analyzed extensively. These proteins have three well-defined domains: a small cytoplasmic fragment containing the carboxy terminus, a membrane-spanning region close to the carboxy terminus, and the largest, amino-terminal domain which is that portion of the molecule exposed to the environment and containing the oligosaccharide moieties.

There is now convincing evidence that interaction of these virions with cells may involve fusion of viral and cell membranes. This fusion can occur either at the cell surface by lowering the pH of the medium or in endocytic vesicles, if the pH of the vesicles is acidic (White et al., 1981; Matlin et al., 1982). The common denominator is the acidic environment which seems to be required for these viruses to cause fusion, except for paramyxoviruses which cause cell fusion at neutral pH (Choppin & Scheid, 1980). The viral membrane glycoproteins are essential for fusion to occur although the precise mechanism by which they orchestrate this event is not understood. The acidic environment may induce a conformational change in the membrane protein permitting it to induce fusion. Conformational changes have been documented for BHA (Skehel et al., 1982) and the E2 protein of Sindbis virus (Edwards et al., 1983) after acidification and for the F protein at neutral pH (Hsu et al., 1981). In addition, the HA and F proteins must be proteolytically cleaved for efficient fusion (Homma & Ohuchi, 1973; Scheid & Choppin, 1974; Huang et al., 1981; White et al., 1981).

We have been investigating the physical properties of G protein of VSV (Crimmins & Schlesinger, 1982). Studies with G protein as well as with other membrane proteins are usually carried out in nonionic detergents to maintain a "native" soluble form of the protein (Helenius & Simons, 1975; Tanford & Reynolds, 1976). In the absence of detergent and lipid, these proteins form protein micelles, termed rosettes, which are large tail to tail aggregates (Simons et al., 1978; Petri & Wagner, 1979). It has been possible to obtain the HA protein in a water-soluble form by treating intact influenza virus with bromelain to remove the carboxy-terminal and membrane-spanning domains (Brand & Skehel, 1972). The G protein of VSV also exists in a soluble form, termed G_s, which is released into the medium during virus replication (Kang & Previc, 1970; Little & Huang, 1978; Chatis & Morrison, 1983). Recently, it became possible to obtain a water-soluble form of G directly from virions by treating intact VSV with cathepsin D (D. Miller, personal communication). We report here our studies on some of the properties of this form of G as well as those of the detergent-solubilized intact G. Using sedimentation equilibrium in the analytical ultracentrifuge to measure molecular weights, we found that both forms of G exist as monomers in solution. We have also been able to detect an acid-induced conformational change in G by using

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[1] Abbreviations: VSV, vesicular stomatitis virus; G, glycoprotein of VSV; HA, hemagglutinin of influenza virus; BHA, bromelain-treated HA; *cis*-PnA, *cis,trans,trans,cis*-9,11,13,15-parinaric acid; OG, octyl β-D-glucoside; emulphogen, poly(oxyethylene) 10-tridecyl ether; standard buffer, 140 mM NaCl, 10 mM sodium phosphate, and 1 mM disodium ethylenediaminetetraacetate, pH 7.4; $NaDodSO_4$, sodium dodecyl sulfate; Trp, tryptophan; Tris, tris(hydroxymethyl)aminomethane.

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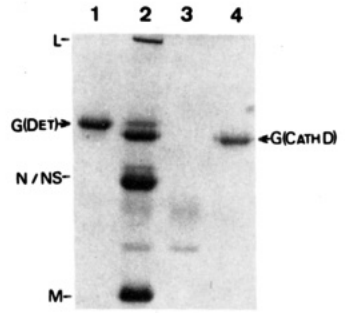

FIGURE 1: Purification of G and G(Cath D) from VSV analyzed by 10% $NaDodSO_4$–polyacrylamide gel electrophoresis. Lane 1, detergent-extracted G, G(Det); lanes 2–4, cathepsin D treated VSV after incubation for 4 h at 37 °C. Lane 2, total reaction mixture prior to the first centrifugation; lane 3, acidic supernatant; lane 4, pH 8 supernatant. Migration positions of G(Det), G(Cath D), and the viral proteins (L, N/NS, and M) are indicated. The gel was stained for protein; the material migrating between the N/NS and M proteins was only seen in acid-treated VSV samples containing cathepsin D. Electrophoresis was carried out for 4 h at 100 V.

cis-PnA, a fluorescent probe which binds to hydrophobic regions of proteins (Sklar et al., 1977; Kimelman et al., 1979).

Materials and Methods

Chemicals and Virus Purification. OG, emulphogen, and the protease cathepsin D (10 units/mg of protein) from bovine spleen were purchased from Sigma. Molecular Probes supplied *cis*-PnA and 1,6-diphenyl-1,3,5-hexatriene. The preparation, purification, and [^{35}S]methionine radiolabeling of the Indiana serotype, San Juan strain of VSV grown in baby hamster kidney cells have been described (Gibson et al., 1979, 1981).

Preparation and Purification of Various Forms of G. (i) Detergent-solubilized intact G [G(Det)]: G protein was extracted from purified VSV (2–3 mg/mL) with 50 mM OG–10 mM sodium phosphate, pH 8.0, essentially by the method of Miller et al. (1980). (ii) G in rosettes [G(Ros)]: G(Det) was first delipidated by centrifugation in a sucrose gradient containing 50 mM OG (Petri & Wagner, 1979, 1980). The peak fraction (~18–20% sucrose) was dialyzed at 4 °C against 500 volumes of standard buffer for 48–72 h with four to six changes of buffer at 12-h intervals. (iii) Cathepsin D treatment of intact VSV and purification of the soluble form of G [G(Cath D)]: Purified VSV (2 mg/mL) in 10 mM sodium phosphate, pH 8.0, was mixed with 200 mM Tris-maleate, pH 4, and cathepsin D (1.56 mg/mL) at a volume ratio of 1.6:2.5:0.9, respectively, and incubated for 4 h at 37 °C. After the reaction period, a small sample was removed for gel analysis (Figure 1, lane 2); the remainder was centrifuged at 100000*g* for 1.5 h at 4 °C. This centrifugation step removed cathepsin D but did not release the soluble form of G into the supernatant fraction (Figure 1, lane 3). The pellet was resuspended in the original reaction volume with 10 mM sodium phosphate, pH 8.0. After 15 min on ice, the sample was centrifuged as above. The resulting supernatant fraction containing the cleaved form of G was exhaustively dialyzed against standard buffer at 4 °C (Figure 1, lane 4). A second isolation procedure involved extraction in detergent. After centrifugation to remove the cathepsin D, the virus pellet was resuspended in the original reaction volume with 50 mM OG–10 mM NaOAc, pH 5.0, and shaken on an oscillating shaker at room temperature for 40 min. This sample was centrifuged, and the supernatant fraction was delipidated as described for G(Ros). The peak fraction (~14–16% sucrose) was exhaustively dialyzed against standard buffer at 4 °C. The detergent extraction of cathepsin D treated G was carried out at pH 5 rather than at pH 8 (Miller et al., 1980) because at the higher pH other viral proteins were detected in the supernatant fraction.

Gel Electrophoresis. Virus purity and the extent of the cathepsin D reaction were routinely checked by analysis on 10% $NaDodSO_4$–polyacrylamide gels (Gibson et al., 1981). To estimate the subunit molecular weight, the following protein standards were used (abbreviation and molecular weight in parentheses): β-galactosidase (β-Gal, 116 500); phosphorylase *b* (Phos *b*, 94 000); bovine serum albumin (BSA, 68 000); ovalbumin (Oval, 43 000); carbonic anhydrase (CA, 30 000); soybean bovine trypsin inhibitor (SBTI, 21 000); lysozyme (Lys, 14 000). Samples were reduced and run on a 10–20% linear acrylamide gradient in the presence of $NaDodSO_4$. Data were analyzed according to Lambin et al. (1976).

Nondenaturing gels in a 3–30% linear acrylamide gradient (Crimmins & Schlesinger, 1982) were used to estimate the Stokes radius (R_s) of various forms of G protein. The following protein standards were used (abbreviation and Stokes radius in parentheses): thyroglobulin (Thy, 85 Å); ferritin (Fer, 61 Å); catalase (Cat, 52 Å); BSA (35.5 Å); and Oval (30 Å). Data were analyzed according to Nakashima & Makino (1980) and Nakashima et al. (1981).

Sedimentation Equilibrium. A Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner operating at 280 nm was used for all measurements. Double-sector cells were used in conjunction with an AnH rotor operating at 16 000–26 000 rpm at temperatures of 20 ± 2 °C. Protein samples, which had been exhaustively dialyzed against the buffer of interest, had an initial A_{280} (1-cm path-length cell) of 0.2–0.3 and were centrifuged for 18–66 h to check for equilibrium. (Most of the samples were at or very near equilibrium by 24 h.) Equation 1 was used to determine the

$$M_r = \left[\frac{2RT}{(1-\phi'\rho)\omega^2}\right]\left(\frac{\mathrm{d}\ln c}{\mathrm{d}r^2}\right) \qquad (1)$$

molecular weight, M_r, of the protein where R is the gas constant, T the absolute temperature, ϕ' the effective partial specific volume, ρ the solution density, ω the angular velocity, c the concentration of protein estimated from A_{280}, and r the distance from the axis of rotation. The slope resulting from a plot of ln A_{280} vs. r^2 was obtained by linear least-squares regression analysis. The correlation coefficient from this plot was always >0.99, indicating that the protein was homogeneous. If other substances bind to the protein, the term $1 - \phi'\rho$ can be expanded according to Tanford et al. (1974):

$$1 - \phi'\rho = (1 - \bar{v}_P\rho) + \sum_i \delta_i(1 - \bar{v}_i\rho) \qquad (2)$$

where $\bar{v}_P$ is the true partial specific volume of the protein, $\bar{v}_i$ is the partial specific volume of substance i when bound to protein, and δ_i is the amount (grams per gram) of substance i bound to protein. $\bar{v}_P$ for G was calculated from the derived amino acid sequence (Rose & Gallione, 1981) and carbohydrate composition (Etchison & Holland, 1974) by using the appropriate values given by Fish (1975) and was found to be 0.724 cm^3/g. This value was also used for G(Cath D).

Fluorescence Measurements. Instrumentation and general procedures have been described (Crimmins & Schlesinger, 1982). Quenching of the intrinsic Trp fluorescence with *cis*-PnA was essentially as described by Sklar et al. (1977) and Kimelman et al. (1979). Briefly, ethanolic stock solutions of *cis*-PnA (3.6×10^{-3} M) plus an equimolar amount of butylated hydroxytoluene added as an antioxidant were diluted with absolute ethanol to working concentrations of 10–0.1 μM. Successive 10–40-μL samples of this solution were added to

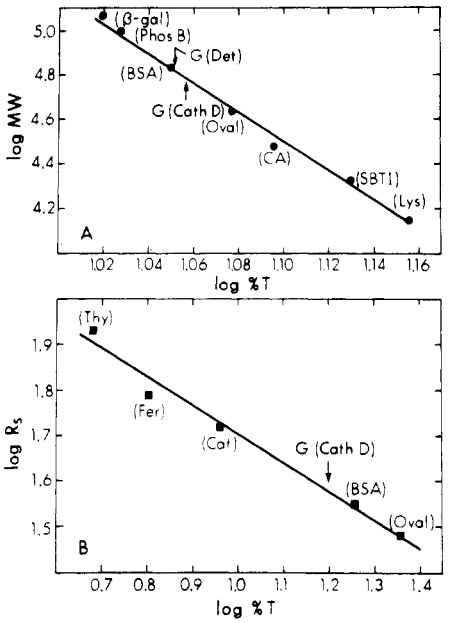

FIGURE 2: Calibration plots for molecular weights and Stokes radii: (A) log M_r vs. log %T for standard proteins and migration positions of G(Det) and G(Cath D). Samples were reduced and analyzed in 10–20% linear acrylamide gradient gels containing $NaDodSO_4$ for 6 h at 100 V. (B) log R_s vs. log %T for standard proteins and migration positions of G(Cath D) in a 3–30% nondenaturing linear acrylamide gradient gel run for 24 h at 100 V. Gel and electrophoresis buffer; 10 mM Tris–80 mM glycine, pH 8.3. %T, gel concentration reached by protein in polyacrylamide gradient gel electrophoresis (Nakashima & Makino, 1980).

the protein solution (0.5–5 μg/mL) and appropriate buffer blanks, both at initial volumes of 1.7 mL. Samples were incubated for 1 min in the dark prior to excitation at either 240 or 295 nm with emission subsequently monitored at 300–400 nm. Control experiments performed by adding the highest amount of absolute ethanol (<8% v/v) without *cis*-PnA showed less than a ±5% change in the peak emission intensity for all forms of G protein studied.

Results

Molecular Weight Determinations. Treatment of intact VSV with cathepsin D cleaves the G protein of VSV, giving rise to a large molecular weight fragment (D. Miller, personal communication). Two lines of evidence established that it is the carboxy-terminal end which is removed: (i) After treatment with this protease, the G protein is released from virions (Figure 1, lane 4), and (ii) the small fragment of G remaining with the virus is the fragment to which palmitate is attached (A. Magee and M. Schlesinger, unpublished results). Palmitate is known to be covalently linked to G protein near the carboxy-terminal domain (Petri & Wagner, 1980). The identification of one small (palmitate-labeled) fragment in addition to the large molecular weight fragment suggests that cathepsin D cleaves the G protein at a single site.

The minimum chain molecular weight determined by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2A) was 61 300 for G(Cath D) and 67 000 for intact G. If the mean residue molecular weight is assumed to be 115, the results of the gel analysis indicate a loss of ~50 amino acids in the formation of G(Cath D). Similar results have been obtained for the soluble form of G released from cells (Chatis & Morrison, 1983). The removal of ~50 amino acids from the carboxy terminus of G would produce a polypeptide containing essentially all of the amino acids of the original protein except for the cytoplasmic tail and the membrane-spanning region.

Table I: Physical Properties of G(Det) and G(Cath D)[a]

	G(Det)	G(Cath D)
M_r(calcd)	62.4	
M_r(determined)		
$NaDodSO_4$-polyacrylamide gel	67 (2, 3)	61.3 (2, 2.5)
sedimentation equilibrium	monomer[b] (2)	57.6 (4, 4.2)
R_s (Å)	63[c] (7, 4)	55[c] (4, 4)
		37 (4, 3)
R_s/R_{min}[d]	1.89	1.45
	1.80[e]	1.31[e]
s(calcd)[f] (S)		3.8
s(determined)[g] (S)		3.7 (2, 0.3)

[a] Values presented have been determined from at least two different virus preparations. The numbers in parentheses refer to the number of determinations and the sample standard deviation, respectively. [b] See text. [c] The gel and sample contained 0.2% (w/v) emulphogen or 50 mM OG. [d] See eq 4. [e] Values have been corrected by assuming a hydration of 0.25 g of H_2O/g of protein. [f] See eq 5. [g] To estimate s for G(Cath D) from a sucrose gradient, the following proteins were included as standards: γ-globulin (7 S); BSA (4.6 S); Oval (3.6 S). Centrifugation in a 5–15% sucrose gradient in standard buffer was carried out for 24 h at 35 000 rpm in a Beckman SW41T1 rotor at 20 °C.

The molecular weight of G under nondenaturing conditions has not been determined, in part because of the difficulty of measuring molecular weights of proteins in detergents. Because G(Cath D) is soluble in aqueous buffers, it was feasible to determine its molecular weight by sedimentation equilibrium in the analytical ultracentrifuge. The value obtained, 57.6 kdalton (Table I), in standard buffer demonstrated that this form of G protein is a monomer.

The analogous determination of the molecular weight for proteins in detergent solutions is more complicated as the amounts of detergent and lipid bound to the protein should be taken into account in the molecular weight calculation. If we assume that the lipid is largely replaced by detergent in detergent extracts (Clarke, 1975), it becomes necessary to know the values of two additional parameters before determining the molecular weight by using eq 1: δ_D, the amount of bound detergent, usually obtained from equilibrium dialysis or gel filtration experiments, and $\bar{v}_D$, the partial specific volume of the detergent when bound to protein, usually determined in the absence of added protein (Tanford et al., 1974). For OG solutions above the critical micelle concentration, $\bar{v}_D$ = 0.95 ± 0.03 cm^3/g (J. Reynolds, personal communication). We did not make a direct determination of δ_D but instead proceeded as follows (McCaslin & Tanford, 1981). Rearrangement of eq 1 and 2 gives

$$\delta_D = \frac{A}{M_r} - B \qquad (3)$$

where $A = \{2RT/[\omega^2(1 - \bar{v}_D\rho)]\}(d \ln c/dr^2)$ and $B = (1 - \bar{v}_P\rho)/(1 - \bar{v}_D\rho)$. For a given experiment, the observed values of A and B were inserted into eq 3. M_r was then incremented in integer multiples of 62 400, the calculated molecular weight of intact G protein. For two different virus preparations, δ_D = 0.83 and 0.75 g/g for M_r = 62 400 (i.e., a monomer) and δ_D = −2.35 and −2.38 g/g, respectively, for M_r = 124 800 (i.e., a dimer). Higher multimers of G yielded even more negative δ_D values. Hence, physically unsatisfactory results were obtained for anything but a monomer. For both experiments, the observed slopes would have to be in error by >+75% for δ_D to be positive. This was so much greater than the actual error [<10%, see Table I for G(Cath D)] that we conclude that G(Det) is a monomer. The calculated average value of δ_D, 0.79 g/g, for monomeric G(Det) would correspond to ~95 mol of OG/mol of G. The aggregation number of OG is

~100 [quoted in Mimms et al. (1981)] so that the above molar ratio indicates a protein–detergent complex of approximately one G protein per one OG micelle. This ratio is a reasonable estimate in view of the micellar hypothesis proposed by Tanford & Reynolds (1976).

Stokes Radius. The molecular shape of a protein can be estimated by determining the Stokes radius of the particle from experiments employing nondenaturing linear acrylamide gradient gels (Nakashima & Makino, 1980; Nakashima et al., 1981). Typical calibration plots are given in Figure 2B. In the absence of detergent, a R_s value of 37 Å was found for G(Cath D), and in the presence of nonionic detergent, this value shifted to 55 Å. The corresponding value for G(Det) was 63 Å. The increase in R_s which occurred when G(Cath D) interacted with nonionic detergent suggests that even with the hydrophobic membrane-spanning domain removed the G protein remained sufficiently hydrophobic to bind detergent. This result was not surprising in view of the report that the naturally occurring soluble form of G, G_s, retains hydrophobic properties (Little & Huang, 1978).

Measured R_s values in combination with the calculated R_{min} values (R_s/R_{min}) can be used to measure the asymmetry of a particle (Tanford et al., 1974; McCaslin & Tanford, 1981). R_{min} is the minimum radius of an equivalent spherical particle containing the known amount of bound detergent but no bound water and is expressed by (Tanford et al., 1974)

$$R_{min} = \left[\left(\frac{3}{4\pi N}\right) M_r(\bar{v}_P + \delta_D \bar{v}_D)\right]^{1/3} \quad (4)$$

Solving this equation for G(Cath D) in the absence of detergent gave R_{min} = 25.5 Å, and for G(Det), R_{min} = 33.3 Å. The value for G(Det) was obtained by using the average value obtained from two experiments for the calculated δ_D, assuming G(Det) is monomeric. The corresponding R_s/R_{min} ratios were then 1.45 and 1.89, respectively. Taking into account a hydration of 0.25 g of H_2O/g of protein (Tanford, 1961; Cantor & Schimmel, 1980) further reduced these ratios to 1.31 and 1.80, respectively. These latter values suggest that the water-soluble form of G is slightly asymmetric (Teller et al., 1979) while G(Det) appears to be a rather asymmetric particle, a result also found for other membrane proteins in nonionic detergent solutions (Tanford et al., 1974; McCaslin & Tanford, 1981). Modeling both proteins as ellipsoids of revolution yielded axial ratios of 6 (prolate) or 6.8 (oblate) for G(Cath D) and 15.3 (prolate) or 20.5 (oblate) for G(Det). The dimensions of hydration-free G(Cath D) with the same axial ratios and a molecular weight of 57 600 would be 168 × 28 Å (prolate) and 96 × 14 Å (oblate), with the values for hydration-free G(Det) having a molecular weight of 62 400 being 322 × 21 Å (prolate) and 143 × 7 Å (oblate).

Sedimentation Coefficient. The sedimentation coefficient of G(Cath D) was calculated in the usual manner from

$$s = \frac{M_r(1 - \phi'\rho)}{6\pi\eta N R_s} \quad (5)$$

yielding a value of 3.8 S (Table I). We also determined the s value from sucrose gradient centrifugation by using proteins of known s values as standards (data not shown). Although there are objections and limitations to this procedure (Tanford et al., 1974), the value we obtained, based on the averaging of two experiments, was 3.75 S and was in good agreement with the calculated value.

Effects of Acidification on G and on VSV. One objective of our analysis of G was to determine if exposure to acidic pH—a condition under which VSV causes cells to fuse (White et al., 1981; Matlin et al., 1982)—induced a conformational change in the protein. One difficulty in trying to identify conformational changes which are thought to involve increased accessibility of hydrophobic regions in the protein is that exposure of small hydrophobic regions may be largely obscured by the extremely hydrophobic membrane-spanning region. This difficulty was eliminated in the studies with the HA of influenza virus by working with a soluble form of the protein, BHA, lacking the membrane-spanning region (Skehel et al., 1982).

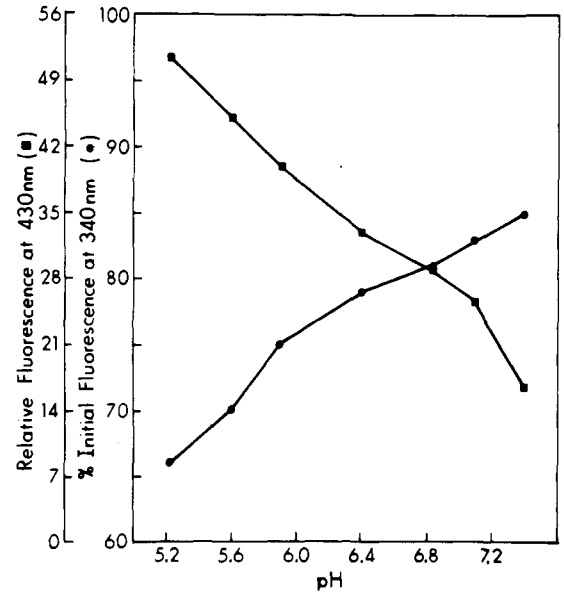

FIGURE 3: pH curve for the interaction of *cis*-PnA with G(Cath D). 1.7 mL of protein at 3 μg/mL and 0.14 mL of 4.5 μM *cis*-PnA in absolute ethanol were incubated at the indicated pH. Excitation was at 295 nm (band-pass = 1 nm) with emission at 340 nm (band-pass = 10 nm) (●), or excitation was at 325 nm (band-pass = 1 nm) with emission at 430 nm (band-pass = 40 nm) (■). Different pH values were obtained by mixing the protein sample in standard buffer with 140 mM NaCl, 10 mM NaOAc, and 1 mM disodium ethylenediaminetetraacetate, pH 5.0, and were measured directly in the cuvette with a Sensorex S900C combination electrode. The pH varied by <±0.05 pH unit before and after addition of *cis*-PnA.

We have also used a soluble form of G to search for conformational changes in this molecule brought about by acidification. The "lipid-like" fluorescent probe *cis*-PnA (Sklar et al., 1977) provides a valuable tool for detecting increases in accessibility of hydrophobic regions of proteins. The binding of this compound to such regions of a protein has two effects: an increase in the fluorescence emission intensity of the probe and a quenching of the intrinsic Trp fluorescence (Sklar et al., 1977; Kimelman et al., 1979). The effect of pH on these two parameters for G(Cath D) is seen in Figure 3. On the basis of these data, subsequent acidification experiments were carried out near pH 5, and Trp quenching was determined. The latter measurement was more sensitive than the increase in fluorescence of *cis*-PnA and permitted us to work at low concentrations of protein. It was necessary to use minimal protein concentrations because the isoelectric point of G(Ros) and G(Cath D) is close to pH 5 (Chatis & Morrison, 1983) and acidification to that pH should cause precipitation. At the concentrations of protein we used, we did not detect any aggregation based on the sensitive technique of intrinsic Trp fluorescence (Paladini & Weber, 1981). Our results obtained from a total of four experiments on two different G(Cath D) preparations showed that the averge Trp residue either was identical or was slightly more mobile for the acidified sample (polarization = 0.146 ± 0.007) than for the neutral pH control sample (polarization = 0.162 ± 0.008). If aggregation had

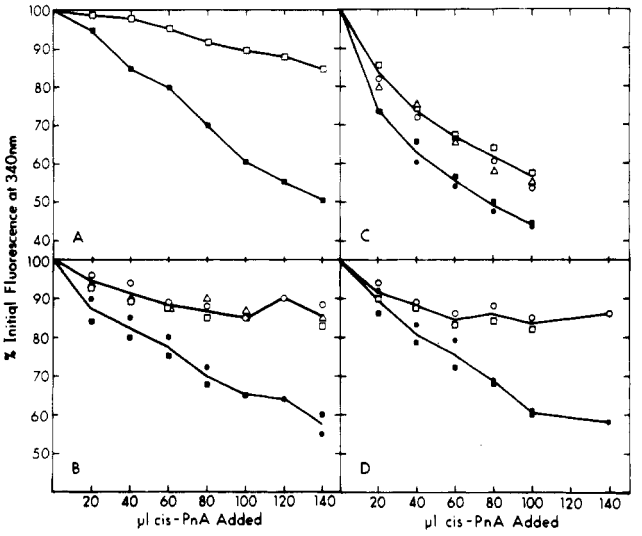

FIGURE 4: Quenching of intrinsic Trp fluorescence by *cis*-PnA for (A) detergent-extracted G(Cath D), (B) G(Cath D), (C) G(Ros), and (D) G(Cath D). Open symbols, pH 7.2–7.5; filled symbols, pH 4.8–5.4. Circles, sample preparation I; squares, sample preparation II. (A and B) Excitation at 295 nm (band-pass = 1 nm) with emission at 340 nm (band-pass = 10 or 20 nm). (C and D) Excitation at 240 nm (band-pass = 1 nm) with emission at 340 nm (band-pass = 40 nm). Protein concentrations ranged from 0.5 to 5.0 μg/mL. The final concentrations of *cis*-PnA were varied from 0.05 to 800 μM. To show reversibility, acidified samples were dialyzed against standard buffer (pH 7.4) before the addition of *cis*-PnA: G(Cath D) (B, triangles); G(Ros) (C, triangles).

occurred, an increase in Trp polarization would be expected (Anderson & Weber, 1966).

Two excitation wavelengths were used to monitor Trp quenching by *cis*-PnA; 240 nm, where no inner-filter correction due to *cis*-PnA is needed (Sklar et al., 1977), and 295 nm, where a correction is necessary (Kimelman et al., 1979). Excitation at either 295 nm (Figure 4B) or 240 nm (Figure 4D) gave similar results. In the absence of *cis*-PnA, Trp quenching of acidified G(Cath D) was ~5%.

One concern we had about analyzing G(Cath D) was that it had been generated by incubation at low pH with cathepsin D and this treatment might induce an irreversible change in the protein. Two observations suggest that our analysis with G(Cath D) was valid. First, when the pH of the G(Cath D) protein solution was returned to neutral, the addition of *cis*-PnA no longer caused an increase in the quenching of Trp (Figure 4B, triangles). Second, an increase in Trp quenching was also found when preparations of G(Ros) were exposed to pH 5 (Figure 4C). G(Ros) was prepared by extraction of VSV with OG (Materials and Methods), and the first exposure of the protein to low pH was in these quenching experiments. There was, however, significant background quenching at neutral pH which we attribute to the presence of the hydrophobic tail regions of G. The increased Trp quenching by *cis*-PnA for G(Ros) was also not observed when the sample was neutralized before the addition of *cis*-PnA (Figure 4C, triangles).

The preparation of G(Ros) involved many more steps than that of G(Cath D). To establish that the observed differences were not due to the method of preparation, we performed experiments on detergent-extracted G(Cath D) (Materials and Methods) which had been extracted from virus and purified in a manner identical with that for G(Ros). The data obtained with this preparation of G(Cath D) were virtually indistinguishable from those found with the protein released directly from the virus (Figure 4A).

We have also carried out experiments with 1,6-diphenyl-1,3,5-hexatriene (Shinitzky & Barenholz, 1978) similar to those described above with *cis*-PnA. The latter compound is an acid and might be affected by changes in pH, whereas the binding properties of 1,6-diphenyl-1,3,5-hexatriene should not be influenced by pH changes. The quenching data (not shown) obtained with this probe were qualitatively the same as those found with *cis*-PnA.

In addition to analyzing purified G protein, we also examined the effect of exposing intact VSV to an acidic environment ranging from pH 4.8 to 5.6. Incubation over this pH range caused extensive aggregation of the virus, as measured both by rate zonal centrifugation in a sucrose gradient and by electron microscopy. Aggregation was observed over a concentration range from 0.01 to 2 mg of viral protein per mL, and even at the lowest concentrations, more than 50% of the virus had aggregated. Furthermore, exposure to neutral pH did not cause disassociation of the aggregates although the G protein extracted by OG from aggregated virions was not itself aggregated.

Discussion

We have described several properties of a soluble form of the G protein of VSV as a goal toward understanding what characteristics of this molecule are essential for the functions carried out by the intact membrane-bound form of G found in virions and in infected cells. Our sedimentation equilibrium studies demonstrate that both a water-soluble form of G and the detergent-solubilized intact molecule exist in solution as monomers. These findings distinguish the soluble form of G from an equivalent form of HA from influenza virus. The latter protein is a trimer on the basis of X-ray crystallographic studies (Wilson et al., 1981), and appears to be a trimer also in influenza virions on the basis of chemical cross-linking studies (Wiley et al., 1977). For the two closely related α-viruses, Sindbis virus and Semliki Forest virus, cross-linking studies show that the two spike glycoproteins, E1 and E2, exist as a heterodimer in intact virions (Ziemiecki & Garoff, 1978; Rice & Strauss, 1982). Furthermore, extraction of these glycoproteins with the mild nonionic detergent Triton X-100 maintains the fidelity of their association (Ziemiecki & Garoff, 1978; Rice & Strauss, 1982). Electron microscopy (von Bonsdorff & Harrison, 1978) of Sindbis virus glycoproteins after treatment with low concentrations of Triton X-100 and the chemical cross-linking studies of Rice & Strauss (1982) suggest that a trimer of heterodimers is the "functional" complex for these proteins.

The proposal that G is monomeric in virions was first made by Cartwright et al. (1972) on the basis of electron microscopy. Treatment of VSV with cross-linking agents, however, caused the formation of dimers and trimers of G and led to the belief that G is oligomeric in virions (Dubovi & Wagner, 1977; Mudd & Swanson, 1978). Both reports were cautious in their conclusions and pointed out some of the pitfalls of cross-linking studies. Our results showing that G is a monomer in solution bring back into consideration the possibility that G is a monomer in virions. The alternative explanation that an oligomeric form of G was disrupted by our isolation procedures requires that subunit interactions were not stable either to nonionic detergent or to low pH. Protein–protein interactions are usually stable to nonionic detergents (Helenius & Simons, 1975) although OG can cause some dissociation of cytochrome oxidase (Rosevear et al., 1980). One of the more prevalent intermolecular interactions between polypeptide chains is a coiled-coil arrangement of α-helices (Crick, 1953; Talbot & Hodges, 1982). The crystal structure of BHA shows that a

coiled-coil arrangement of three long stretches of α-helices allows interchain packing of hydrophobic side chain residues which are then "protected" from solvent (Ward & Dopheide, 1980; Wilson et al., 1981). Presumably, this interaction stabilizes the BHA trimer. If a similar type of interaction existed for G, it should not be disrupted by exposure of VSV to acid since coiled-coils increase in stability as the pH is lowered (Noelken & Holtzer, 1964; Lowey, 1965; Crimmins et al., 1981). In contrast to the coiled-coil interaction of BHA which is external to the membrane, it is the membrane-spanning region of glycophorin A which forms a dimeric coiled-coil as the hydrophobic residues of each chain interact primarily with the lipid environment of the membrane and not with the corresponding residues on the adjacent chain (Dunker & Jones, 1978; Furthmayr et al., 1978). Recent studies of Capone et al (1983) provide evidence against this type of interaction for G. They incorporated a photoreactive fatty acid analogue into G and found that after photoactivation of virions only a very low level of G had been converted to dimers or higher molecular weight oligomers. Much more extensive cross-linking would be expected if extensive interactions between G monomers existed in the membrane-spanning domain of the protein. Thus, if G is oligomeric in virions, the forces holding the subunits together must be weak ones and are unlikely to involve coiled coils.

Cohen & Phillips (1981) recently proposed two possible models to explain how proteins can form projections on the surface of microorganisms. One of these is a multiple-chain parallel molecule and is illustrated by the HA trimer. The other is an antiparallel single-chain molecule and provides an excellent model for the G monomer. The latter model requires that a polypeptide chain folds back on itself to form an intramolecular coiled coil. Although there is no direct evidence for such a structure in G protein, the algorithm of Chou & Fasman (1974) detects two α-helical stretches in G protein which would lie just outside of the membrane in the virion. These putative α-helical stretches occur from residues 392 to 407 and from residues 411 to 417. A proline residue (408) interrupts these helices and would provide a means for the polypeptide chain to fold back on itself, thus forming an antiparallel coiled coil possessing the proper pattern of hydrophobic contacts.

From our measurements of the R_s of G(Cath D), we calculated the dimensions of G(Cath D) to be 168 × 28 Å, assuming a prolate ellipsoid. These values are similar to those which have been reported for the dimensions of the spikes of VSV. The dimensions of the spikes should be most comparable to those of the amino-terminal domain [G(Cath D)], since the other domains would be internal to the virus membrane. Cartwright et al. (1972) determined the spikes to be 100 Å in length and 35 Å in diameter by electron microscopy. A completely different method for determining the length of the spikes, that of quasi-elastic light scattering and a resistive pulse technique, gave a value of 175 ± 25 Å (Feuer et al., 1978).

Studies with intact VSV demonstrated that acidification increases the binding of VSV to Madin-Darby canine kidney cells (Matlin et al., 1982), causes fusion of cells previously exposed to VSV (White et al., 1981), and enhances hemolysis (Mifune et al., 1982). The pH profile reported for fusion of BHK cells with VSV showed a sharp transition close to pH 6 (White et al., 1981). In contrast, the pH dependence of hemolysis by VSV (Mifune et al., 1982) followed a curve almost identical with that shown in Figure 3. We found that decreasing the pH caused a change in purified G protein, making one or more hydrophobic regions of the protein more accessible to the environment. Most of our results were obtained with G protein lacking the membrane-spanning and carboxy-terminal regions, demonstrating that the hydrophobic regions involved are distinct from the ones present in those domains. We used the fluorescent reagent *cis*-PnA to detect an increase in accessibility of hydrophobic regions of G. Our choice of this type of probe was governed by several factors. First, we wanted to measure conformational changes in G which were distinct from aggregation. Because Trp quenching by *cis*-PnA is a sensitive assay, we were able to use concentrations of protein at which aggregation was not detectable. Second, reagents such as *cis*-PnA can be used over a wide range of pH values. Our results indicated that the conformational changes which occurred upon acidification of G were reversed when the sample was neutralized. It is not feasible to use agents such as proteases to probe for conformational changes if the putative changes are reversed at the pH required for protease activity. In comparing samples of G protein which had been exposed to pH 5 and then neutralized to pH 7.4 with untreated controls, we were unable to detect any change in sensitivity to the proteases trypsin, chymotrypsin, or *Staphylococcus aureus* V8. This result supports our contention that the conformational change was reversible, but it could also be explained by a lack of accessibility of protease-sensitive sites. Conformational changes at low pH have been demonstrated for two other viral glycoproteins. The BHA from influenza virus irreversibly aggregates at pH $<\sim 5.6$ with changes in physical properties and protease sensitivity (Skehel et al., 1982). Exposure of Sindbis virus to low pH leads to an irreversible change in the E2 glycoprotein detected by increased sensitivity to protease (Edwards et al., 1983). These results taken together with our finding that low pH caused a change in the G protein support the conclusion that these types of conformational changes are important in the interaction of viral and cellular membranes.

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