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Articles

Dynamic Nature of the Quaternary Structure of the Vesicular Stomatitis Virus Envelope Glycoprotein†

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ABSTRACT: The envelope glycoprotein (G protein) of vesicular stomatitis virus probably exists in the viral envelope as a trimer of identical subunits. Depending on the conditions of solubilization, G protein may dissociate into monomers. G protein solubilized with the detergent octyl glucoside was shown to exist as oligomeric forms by sedimentation velocity analysis and chemical cross-linking. G protein was modified with either fluorescein isothiocyanate or rhodamine isothiocyanate. Resonance energy transfer between fluorescein and rhodamine labels was observed upon mixing the two labeled G proteins in octyl glucoside. This result provided further evidence that G protein in octyl glucoside is oligomeric and indicated that the subunits are capable of exchange to form mixed oligomers. Resonance energy transfer was independent of G protein concentration in the range examined (10–80 nM) and was not observed when labeled G proteins were mixed with fluorescein or rhodamine that was not conjugated to protein. Resonance energy transfer decreased upon incorporation of G protein into Triton X-100, consistent with sedimentation velocity data that G protein in Triton X-100 is primarily monomeric. Kinetic analysis showed that the subunit exchange reaction had a half-time of about 3 min at 27 °C that was independent of G protein concentration. These data indicate that the exchange occurs through dissociation of G protein trimers into monomers and dimers followed by reassociation into trimers. Thus, in octyl glucoside, G protein must exist as an equilibrium between monomers and oligomers. This implies that monomers are capable of self-assembly into trimers.

Many viral and cellular plasma membrane proteins consist of assemblies of polypeptide subunits; that is, they display some type of quaternary structure (Carlin & Merlie, 1986). Some surface proteins are composed of two or more different polypeptide chains, i.e., are heterooligomers. Examples include B cell surface immunoglobulins and T cell antigen receptors, class I and class II major histocompatibility antigens (MHC antigens),[1] and the acetylcholine receptor. Other membrane proteins consist of multiple copies of a single polypeptide. For example, the influenza virus hemagglutinin is synthesized as a single polypeptide precursor, which self-associates into a trimer of identical subunits (Wilson et al., 1981). Similarly, the influenza virus neuraminidase consists of a tetramer of identical subunits (Varghese et al., 1983). In some cases, such as surface immunoglobulins, the subunits are held together by disulfide bonds, while in other cases, such as MHC antigens, the subunit interactions are noncovalent.

Determination of the subunit structure of a membrane protein usually requires solubilization of the protein from the membrane either by detergent treatment or by proteolytic cleavage of the membrane-anchor domain. Such treatments may alter the subunit interactions, especially if they are noncovalent in nature. The vesicular stomatitis virus surface glycoprotein (G protein) was originally reported to be a monomer following solubilization with detergent or proteolytic cleavage of the membrane-anchor sequence (Crimmins et al., 1983). However, other evidence has suggested that G protein is oligomeric, most likely a trimer of identical subunits (Dubovi & Wagner, 1977; Kreis & Lodish, 1986; Doms et al., 1987).

Assembly of membrane protein subunits is thought to occur at the site of synthesis in the endoplasmic reticulum, and in several cases, it has been shown that acquisition of the correct quaternary structure is a regulatory step required for transport to Golgi membranes [reviewed by Rose and Doms (1988)]. Subunit assembly is usually a posttranslational event, and most polypeptides appear to exist as monomers in the endoplasmic reticulum membrane for a period of time following synthesis prior to interaction with other subunits. In the case of the

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[1] Abbreviations: DSS, disuccinimidyl suberate; G protein, envelope glycoprotein of VSV; MHC antigens, major histocompatibility antigens; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; VSV, vesicular stomatitis virus.

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influenza virus hemagglutinin, assembly of trimers occurs from a random pool of monomers, since mixed trimers can form in cells coinfected with two closely related influenza viruses (Boulay et al., 1988). However, once formed, the trimer structure appears to be very stable, since exchange of subunits did not occur following mixing of preformed trimers in detergent. This raises the question of whether the assembly of the subunit structure of membrane proteins is generally irreversible. Alternatively, the subunit interactions of some membrane proteins may be flexible so that subunits are in a dynamic equilibrium among oligomers and possibly in equilibrium with lower orders of association such as monomers. The data presented here provide evidence that such is the case for the VSV G protein. Subunit exchange among oligomers was detected by resonance energy transfer between fluorescent probes covalently attached to the G protein. Separate preparations of G protein were labeled with fluorescein and rhodamine and then were mixed in detergent solution. Resonance energy transfer between the fluorescein and rhodamine labels was detected upon mixing the two labeled G protein preparations. Since resonance energy transfer requires proximity of the labels (on the order of 50 nm), this experiment showed that mixed oligomers containing both fluorescein- and rhodamine-labeled G protein had formed as a result of subunit exchange between oligomers. Furthermore, analysis of the exchange kinetics indicated that the reaction was rapid (half-time of about 3 min at room temperature) and probably occurred through dissociation into monomers followed by reassociation into oligomers.

Experimental Procedures

Isolation and Fluorescent Labeling of G Protein. VSV (Indiana serotype) was grown by infecting BHK cells at a multiplicity of 0.01 plaque forming unit per cell followed by incubation at 37 °C for 18–24 h. In some experiments, the virus was radioactively labeled by adding [^{3}H]leucine to the culture medium (10 μCi/mL). Virus was purified from the culture medium by differential centrifugation followed by equilibrium sedimentation in a sucrose gradient (Lyles, 1979). Purified virus (2 mg of protein), pelleted by centrifugation at approximately 100000g for 5 min in a Beckman airfuge (28 psi), was resuspended in 0.5 mL of 20 mM borate buffer, pH 8.5, containing 10 μg/mL aprotinin and then mixed with 0.5 mL of borate buffer containing 100 mM octyl glucoside. The extract was incubated 40 min at room temperature and then was spun for 10 min in the airfuge to pellet the nucleocapsids. The supernatant containing the crude G protein was labeled with fluorescein isothiocyanate (1 mM final concentration) for 1 h at room temperature or with tetramethylrhodamine isothiocyanate (0.15 mM) for 20 min at room temperature. Fluorophores were added by a 1:100 dilution of freshly prepared stocks in dimethylformamide. The reactions were terminated by addition of NaH_2PO_4 (21 mM final concentration) and glycine (5 mM) such that the final pH was 6.8. In some preparations, the glycine was omitted, since it was found not to influence the labeling ratio. The bulk of the unreacted fluorophore was removed by chromatography on Sephadex G-25 in 10 mM sodium phosphate/0.15 M NaCl (phosphate-buffered saline, PBS), pH 6.8, containing 2% sodium cholate and 2 mM phenylmethanesulfonyl fluoride. The change in detergent to cholate was found to be necessary to dissociate noncovalently bound label from the G protein. The cholate was replaced by octyl glucoside, and the G protein was purified from residual free fluorophore and contaminating proteins by rate zonal centrifugation in a 5–17% (w/w) sucrose gradient containing 50 mM octyl glucoside in PBS, pH 6.8. The gradient was spun at 300000g_{max} for 16 h at 5 °C in a Beckman SW50.1 rotor. The fluorescent bands were collected by puncturing the tube with a syringe. The fluorophore to protein ratio was determined by absorption spectroscopy and was usually 1.0 ± 0.15 per G protein monomer (67 000 molecular weight). The final G protein monomer concentration was usually 0.5–1.0 μM. The purity of the G protein was assessed by SDS–polyacrylamide gel electrophoresis in 10% polyacrylamide gels as described (Laemmli, 1970) followed by silver staining (Wray et al., 1981). To determine the extent of contamination with noncovalently bound label, fluorescently labeled G protein was mixed with G protein labeled with [^{3}H]leucine and then subjected to denaturation with SDS (Laemmli, 1970) followed by chromatography on a 1.1 cm × 50 cm column of Sepharose 6B equilibrated with PBS, pH 7.4, containing 0.1% SDS. Fractions were adjusted to 0.1 N NaOH; the fluorescence intensity was determined, and the radioactivity was determined by liquid scintillation counting.

Sedimentation Analysis of Labeled G Proteins. A 100-μL aliquot of labeled G protein was diluted with 200 μL of PBS containing either 50 mM octyl glucoside or 0.1% Triton X-100 at various pH values and then layered onto a 4.7-mL 10–20% (w/w) sucrose gradient containing the same buffer. The gradient was spun at 300000g_{max} for 16 h at 5 °C in an SW50.1 rotor and then collected into 15 fractions. The fractions were adjusted to 0.1 N NaOH, and the fluorescence intensity was determined. The sedimentation of standard proteins in parallel gradients was determined similarly except that the fractions were analyzed by the absorbance at 280 nm. The $s_{20,w}$ values of the standards used were 4.31 (bovine serum albumin), 7.35 (rabbit muscle aldolase), and 7.1 (mouse IgG) (Smith, 1970).

Chemical Cross-Linking of G Protein. A 100-μL aliquot of G protein was reacted with disuccinimidyl suberate (DSS) at various concentrations for 30 min at room temperature. DSS was added as a 1:100 dilution of stock solutions in dimethyl sulfoxide. The reactions were stopped by addition of glycine (10 mM final concentration), and the samples were subjected to SDS–polyacrylamide gel electrophoresis and silver staining as described above.

Spectroscopy. Absorption spectra were obtained with a Bausch and Lomb Spectronic 2000 spectrometer. The extinction coefficient for G protein (1.5×10^5 M^{-1} cm^{-1} at 280 nm) was calculated by using protein concentrations determined by the Lowry (1951) method corrected for the difference in tyrosine content between G protein and the standard. The extinction coefficients for fluorescein were determined to be 8.5×10^4 M^{-1} cm^{-1} at 495 nm (λ_{max}) and 3.0×10^4 M^{-1} cm^{-1} at 280 nm and for rhodamine were 5.5×10^4 M^{-1} cm^{-1} at 554 nm (λ_{max}) and 3.1×10^4 M^{-1} cm^{-1} at 280 nm. Fluorescence measurements were made with a Spex Fluorolog fluorometer as described (Lyles et al., 1985).

Results

Fluorescence Properties of Labeled G Proteins. G protein was labeled with fluorescein or rhodamine for use in resonance energy transfer experiments. G protein was solubilized from purified VSV with the detergent octyl glucoside. The detergent extract was labeled either with fluorescein isothiocyanate or with tetramethylrhodamine isothiocyanate to give approximately one fluorophore per G protein monomer. Labeling presumably results from random modification of free amino groups on the protein. The unreacted label was removed by gel filtration chromatography and rate zonal sedimentation in octyl glucoside. To assess the extent of contamination of labeled G protein with noncovalently bound label, the G

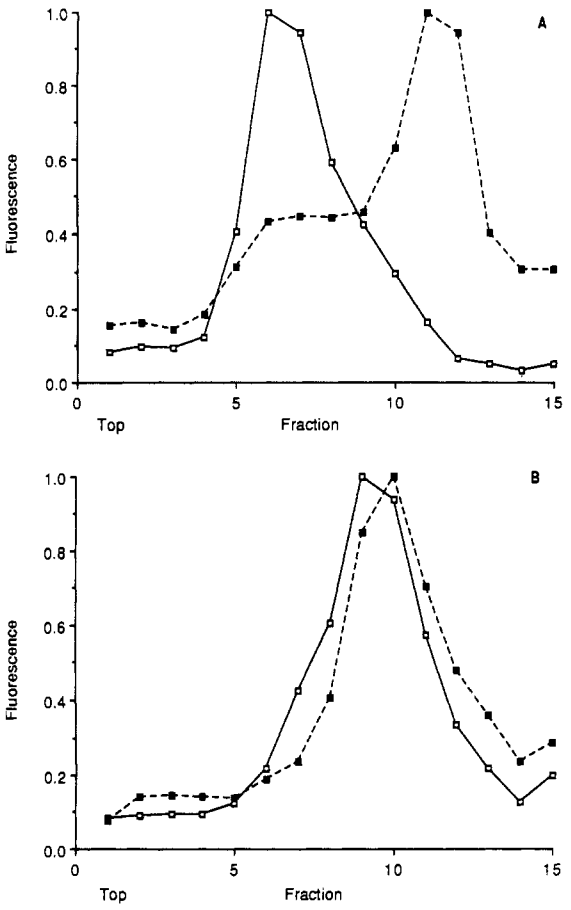

FIGURE 1: Sedimentation analysis of labeled G protein in detergent. Fluorescein-labeled G protein was sedimented through a 10–20% sucrose gradient containing 0.1% Triton X-100 (A) or 50 mM octyl glucoside (B) and PBS at pH 7.5 (□) or pH 5.8 (■). Fractions were collected, and the fluorescein fluorescence intensity was determined. Data were normalized to the intensity of the peak fraction.

protein was denatured by incubation at 100 °C in SDS. Gel filtration chromatography on Sepharose 6B in the presence of SDS showed that 96% ± 3% of the fluorescence cochromatographed with G protein labeled with [^{3}H]leucine (mean ± SD for three determinations). Fluorescence excitation and emission spectra were typical for similarly labeled proteins: fluorescein excitation λ_{max} = 498 nm and emission λ_{max} = 522 nm; rhodamine excitation λ_{max} = 554 nm and emission λ_{max} = 578 nm. The quantum yield for fluorescein-labeled G protein was found to be identical with that for unconjugated fluorescein, which has a quantum yield of about 0.3 (Cantor & Schimmel, 1980). Fluorescence polarization, analyzed as described (Lyles et al., 1985), for both labels was $p < 0.05$, indicating that they were highly mobile on the time scale of fluorescence lifetime. Fluorescein and rhodamine with these spectral properties have been shown to form a good donor–acceptor pair for resonance energy transfer. The critical distance (R_0) at which transfer efficiency is 50% is approximately 50 nm (Damjanovich et al., 1983), making these labels suitable for analysis of protein–protein interaction in which the separation between fluorophores is expected to be in this order of magnitude.

Sedimentation Analysis of Labeled G Protein. G protein isolated from virions with octyl glucoside was capable of undergoing structural transitions between monomeric and trimeric states in detergent solution. This ability was retained after modification of the G protein with fluorescent probes as shown in Figure 1. G protein was solubilized from purified virions in octyl glucoside and labeled with fluorescein or rhodamine as described above. The association state of the labeled G protein was analyzed by sedimentation in 10–20% sucrose gradients containing the detergent Triton X-100 at pH 7.5 and 5.8 (Figure 1A). Most of the labeled G protein sedimented approximately twice as fast at pH 5.8 as at pH 7.5 (peak fractions 5–6 versus 11–12). This difference in sedimentation in Triton X-100 has been shown to be due to the existence of G protein trimers at pH 5.8 and monomers at pH 7.5 (Doms et al., 1987). The sedimentation of G protein at both pH values was heterogeneous, with some slower sedimenting material present at pH 5.8 and some faster sedimenting species present at pH 7.5. This was due in part to the fact that the G protein was initially in octyl glucoside, which must undergo exchange for Triton X-100 in the gradients, and therefore the association/dissociation reactions may be incomplete.

Table I: pH Dependence of G Protein Sedimentation[a]

detergent	pH	app $s_{20,w}$ (S), mean ± SD (N)
octyl glucoside (50 mM)	8.5	4.3 (2)
	7.5	4.5 ± 0.3 (7)
	6.5	4.7 ± 0.5 (4)
	5.8	5.5 ± 0.4 (3)
Triton X-100 (0.1%)	7.5	3.2 ± 0.1 (4)
	6.5	3.2 (2)
	5.8	6.2 ± 0.2 (4)

[a] Fluorescein-labeled G protein was sedimented through 10–20% sucrose gradients in PBS under the indicated conditions at 300000g_{max} for 16 h at 5 °C. The apparent $s_{20,w}$ was calculated from the sedimentation of standard proteins of known $s_{20,w}$ in parallel gradients.

In contrast to the results of sedimentation in Triton X-100, the sedimentation of labeled G protein in octyl glucoside was not so markedly dependent on pH, as shown in Figure 1B. G protein reproducibly sedimented slightly faster at pH 5.8 than at pH 7.5. The sedimentation of G protein in both octyl glucoside and Triton X-100 was not altered by the labeling procedure, since results identical with those in Figure 1 were obtained by using unlabeled G protein (not shown). Since the sedimentation rate of G protein in octyl glucoside was intermediate between that of monomer and that of trimer in Triton X-100, it is difficult to deduce the association state of G protein in octyl glucoside from these data alone. For example, the change in detergent could influence the sedimentation velocity in either direction. However, the fact that G protein in octyl glucoside at pH 7.5 sediments nearly as rapidly as at pH 5.8 suggests that it is oligomeric at both pH values, since the low pH favors the oligomeric form in Triton X-100. The weight of the evidence presented below supports this conclusion.

The pH dependence of the sedimentation of G protein in Triton X-100 has been shown to correlate with a conformational change in G protein responsible for promoting fusion of the virus envelope with cellular membranes during virus penetration by endocytosis (Doms et al., 1987). The sedimentation of labeled G protein was analyzed as a function of pH in both octyl glucoside and Triton X-100, and the apparent $s_{20,w}$ was determined by comparison with the sedimentation of standard proteins (Table I). At pH 6.5 and above, the sedimentation rate of G protein is largely independent of pH in both detergents, while at pH below 6, the sedimentation rate increased in both detergents. This pH dependence is the same as that for fusion of the VSV envelope with cellular membranes as measured in a hemolysis assay (Bailey et al., 1984; and our unpublished data) in which VSV was hemolytically active only at pH below 6.

Chemical Cross-Linking of G Protein in Octyl Glucoside. Chemical cross-linking data support the hypothesis that G protein in octyl glucoside exists primarily as oligomeric forms

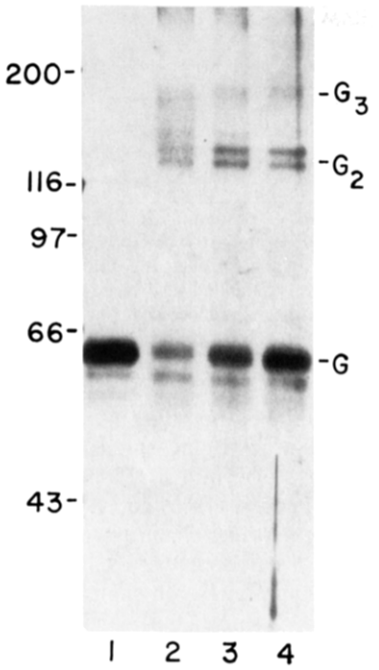

FIGURE 2: Chemical cross-linking of G protein in octyl glucoside. G protein was solubilized from virions and purified by sedimentation on a 5–17% sucrose gradient in PBS, pH 7.5, with 50 mM octyl glucoside. Pooled gradient fractions containing G protein were reacted with disuccinimidyl suberate (DSS) for 30 min at room temperature and then subjected to SDS gel electrophoresis, a silver stain of which is shown. Positions of molecular weight markers ($\times 10^{-3}$) and of G protein monomer, dimer, and trimer species are indicated. DSS concentrations were 0 (lane 1), 50 μg/mL (lane 2), 16.7 μg/mL (lane 3), and 5.6 μg/mL (lane 4).

as shown in Figure 2. G protein was solubilized in octyl glucoside and then purified by sedimentation on a 5–17% sucrose gradient containing octyl glucoside. Pooled gradient fractions containing G protein were reacted for 30 min at room temperature with the cross-linking agent disuccinimidyl suberate (DSS). The reactions were analyzed by SDS–polyacrylamide gel electrophoresis. As shown in Figure 2, both dimer and trimer forms of cross-linked G protein were observed even at the lowest concentration of DSS used (5 μg/mL). The dimer band appears as a doublet, presumably reflecting cross-linked products that differ in their conformation in SDS. Since chemical cross-linking does not give quantitative data, this experiment does not indicate the distribution of native G protein among monomer, dimer, and trimer species. However, the cross-linking pattern is very similar to published data for G protein in Triton X-100 at low pH (Doms et al., 1987). Some preparations of G protein contain a minor component (visible in Figure 2) of slightly lower molecular weight than intact G protein. This protein does not participate in the cross-linking reaction. Although the nature of this protein was not investigated, it is probably a proteolytic fragment of G protein generated during purification.

Resonance Energy Transfer between Labeled G Proteins. Further evidence that G protein in octyl glucoside consists of oligomeric species was obtained by resonance energy transfer between G protein subunits labeled with fluorescent probes. These experiments also demonstrated that G protein subunits are capable of exchange between oligomers. Fluorescein-labeled G protein (15 nM) in octyl glucoside was mixed with rhodamine-labeled G protein at various mole ratios, and the decrease in fluorescein fluorescence as a result of quenching by rhodamine was quantitated. The results are shown in Figure 3. Quenching was observed at all ratios examined up to about 30% quenching at a mole ratio of eight rhodamine

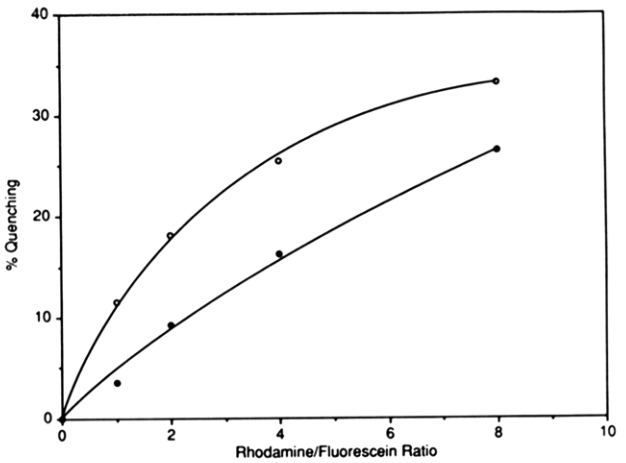

FIGURE 3: Resonance energy transfer between labeled G proteins. G protein was labeled with either fluorescein or rhodamine at a label to G protein (monomer) ratio of 1.0 (○) or 0.4 (●). Fluorescein- and rhodamine-labeled G proteins were mixed at the indicated label ratio in PBS, pH 7.5, with 50 mM octyl glucoside. Resonance energy transfer was quantitated as the percent quenching of fluorescein fluorescence. Data shown are from a representative of five separate experiments.

Table II: pH Dependence of Resonance Energy Transfer between Labeled G Proteins[a]

detergent	pH	% quenching, mean ± SD (*N*)
octyl glucoside (50 mM)	8.5	29 ± 6 (3)
	7.5	31 ± 6 (3)
	6.5	30 ± 6 (3)
Triton X-100 (0.1%)	7.5	10 (2)

[a] Fluorescence quenching of fluorescein-labeled G protein by rhodamine-labeled G protein was determined in PBS under the indicated conditions at a fluorescein:rhodamine ratio of 3:1.

labels per fluorescein. The quenching of fluorescein fluorescence by rhodamine-labeled G protein was competitively inhibited by unlabeled G protein. When the rhodamine labeling conditions were adjusted to give 0.4 label per subunit, the ability to quench the fluorescence of fluorescein-labeled G protein was correspondingly reduced (Figure 3). A similar decrease in quenching was observed when the label ratio was altered by mixing G protein containing one rhodamine label per subunit with unlabeled G protein (data not shown).

Several control experiments (not shown) demonstrated the specificity of quenching by rhodamine-labeled G protein. First, unlabeled G protein had no effect on the fluorescence of fluorescein-labeled G protein. Second, the quenching observed by using a 3:1 ratio of rhodamine- to fluorescein-labeled G protein was independent of the G protein concentration in the range examined (10–80 nM total G protein). This control demonstrates that the quenching is not due to random collisions between G protein monomers. Third, no quenching was observed upon mixing fluorescein-labeled G protein with a 3-fold molar excess of rhodamine-labeled glycine or conversely by mixing fluorescein-labeled glycine with a 3-fold excess of rhodamine-labeled G protein. These controls rule out the possibility that the quenching was due to exchange of noncovalently bound labels (which would be present as the glycine conjugate) rather than exchange of labeled subunits. As described above, such noncovalently bound label comprises at most a few percent of the total label associated with the G protein.

Resonance energy transfer between fluorescein- and rhodamine-labeled G protein was examined as a function of pH in octyl glucoside and in Triton X-100. The data are shown

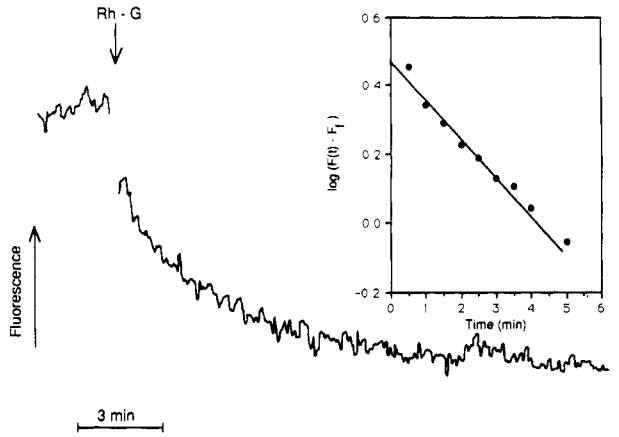

FIGURE 4: Kinetics of G protein subunit exchange. Fluorescein-labeled G protein (5 nM) was equilibrated in PBS, pH 7.5, with 50 mM octyl glucoside. At the indicated time, a 3-fold molar excess of rhodamine-labeled G protein (Rh-G) was added. Fluorescein fluorescence is shown. Inset: Semi-log plot of the data. $F(t)$ = fluorescence at indicated time; F_f = final fluorescence intensity.

in Table II. Quenching of fluorescein fluorescence by rhodamine-labeled G protein in octyl glucoside was independent of pH in the range of pH 6.5–8.5. When rhodamine- and fluorescein-labeled G proteins were mixed in Triton X-100, resonance energy transfer was reduced considerably to about 10% quenching of fluorescein fluorescence. Thus, the extent of energy transfer was correlated with the presence of oligomeric forms of G protein as detected by sedimentation. Resonance energy transfer was difficult to quantitate reliably at pH 5.8 for two reasons. First, the pK_a of fluorescein is near neutral pH, and the protonated form has a very low quantum yield (Leonhardt et al., 1971). Thus, the intensity of fluorescein fluorescence is low at low pH. Second, and more importantly, the fluorescein fluorescence intensity undergoes a slow decrease upon shift to pH 5.8 in the absence of rhodamine-labeled G protein, making it difficult to compare precisely the intensity in the presence versus the absence of rhodamine. The basis for this fluorescence change is currently being investigated, since it may reflect conformational changes in the G protein associated with the acquisition of membrane fusion activity.

Kinetics of G Protein Subunit Exchange. The time course of the exchange of G protein subunits was determined. Fluorescein- and rhodamine-labeled G proteins were mixed in octyl glucoside, and the decrease in fluorescein fluorescence was monitored as a function of time. The data obtained using 20 nM total G protein are shown in Figure 4. The reaction had a half-time of about 3 min at room temperature (27 °C). A semi-log plot of the data gave a straight line (Figure 4 inset), as expected for a label exchange reaction. As shown in Table III, the reaction half-time is independent of the total G protein concentration in the concentration range examined (10–80 nM). These data indicate that the exchange of subunits is not a simple second-order reaction in which two G protein oligomers collide in order for the subunit exchange to take place. Instead, they are consistent with a mechanism in which the G protein oligomers must first dissociate before the mixed oligomers can form (see Discussion). Thus, the kinetic data also argue for flexibility in the association state of the G protein and suggest that monomeric and oligomeric forms of G protein coexist in dynamic equilibrium in detergent solution.

Table III: Concentration Dependence of G Protein Subunit Exchange Kinetics[a]

[G protein] (nM)	$t_{1/2}$ (min)
10	3.1
20	3.1 ± 0.4
40	3.2 ± 1.2
80	2.7

[a] Fluorescein-labeled G protein at one-fourth the indicated final concentration was equilibrated at room temperature (27 °C) in PBS, pH 7.5, with 50 mM octyl glucoside. A 3-fold molar excess of rhodamine-labeled G protein was added to bring the total G protein to the indicated final concentration. Fluorescein fluorescence was monitored as a function of time for at least 15 min. Data shown are mean ± SD for three determinations or mean of duplicate determinations.

DISCUSSION

Evidence presented here indicates that the quaternary structure of the VSV G protein is sufficiently flexible that (1) exchange of subunits between oligomers readily occurs, (2) G protein isolated in octyl glucoside can give rise to either trimers or monomers in Triton X-100 depending on the pH, and (3) G protein in octyl glucoside may exist as an equilibrium between monomers and oligomers. The observation that exchange of subunits can occur between G protein oligomers is not unique among proteins with quaternary structure. For example, in the case of hemoglobin, which is a tetramer of two α chains and two β chains, the ability to form hybrid tetramers upon mixing of different hemoglobin electrophoretic variants has been known for many years (Bunn & McDonough, 1974). Like soluble proteins, membrane proteins are capable of subunit exchange. Class I MHC antigens consist of a membrane-spanning α chain noncovalently associated with β_2-microglobulin, which is also present in serum in a soluble form. The β_2-microglobulin associated with the α chain is capable of exchange with soluble β_2-microglobulin (Hyafil & Strominger, 1979; Parker & Strominger, 1985). However, the exchange of subunits containing membrane-anchor sequences could conceivably be more difficult, since sequences in and around the membrane-spanning domain have been implicated in stabilization of the quaternary structure of both the influenza virus hemagglutinin (Doms & Helenius, 1986) and the VSV G protein (Crimmins et al., 1983; Doms et al., 1987). These two viral glycoproteins appear to differ in the stability of the subunit interaction, since subunit exchange among hemagglutinin trimers does not occur (Boulay et al., 1988), while G protein oligomers readily undergo subunit exchange, as shown here.

Further evidence for the flexibility of the quaternary structure of the G protein is its sensitivity to the type of detergent and pH. At neutral pH, Triton X-100 favors the formation of monomers while octyl glucoside favors the formation of oligomers. At pH below 6, the G protein undergoes a conformational change necessary to mediate fusion of the virus envelope with membranes of endocytic vesicles during virus penetration into host cells. The lower pH enhances the stability of the G protein quaternary structure, even in Triton X-100, as originally shown by Doms et al. (1987) and confirmed here. Such a change in the stability of subunit interactions in response to physiological stimuli is not unprecedented. The stability of the hemoglobin tetramer is greatly enhanced upon deoxygenation such that the tetramer to dimer dissociation constant is decreased by 6 orders of magnitude (Ip et al., 1976).

The data presented here also indicate that G protein in octyl glucoside probably exists as an equilibrium between monomers and oligomers. The strongest argument for such an equilibrium is derived from the kinetics of the subunit exchange reaction, which showed that the reaction half-time was independent of the G protein concentration. Two general classes of mechanism for subunit exchange could be considered,

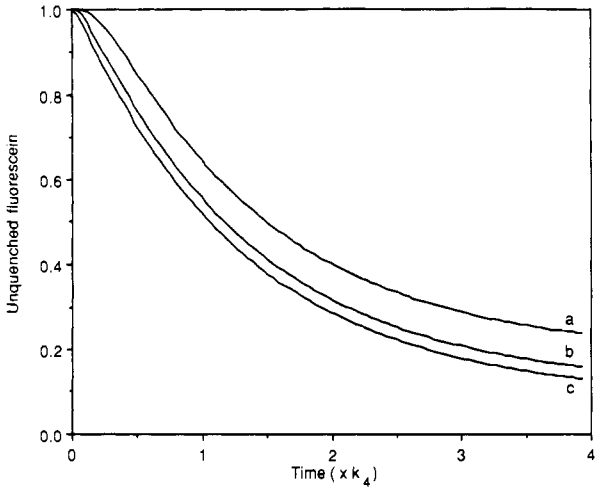

FIGURE 5: Kinetic model of G protein subunit exchange: Effect of G protein concentration. Calculation of the proportions of different molecular species upon mixing fluorescein-labeled G protein trimers with a 3-fold excess of rhodamine-labeled G protein trimer was performed as described in the Appendix as a function of time relative to the trimer dissociation rate constant (k_4). Initial concentrations of fluorescein-labeled G protein were 10 (a), 100 (b), and 1000 (c) times the equilibrium constant for trimer dissociation. In this example, it was assumed that trimers and dimers dissociated and reassociated at equal rates. The proportion of unquenched fluorescein was calculated as the sum of pure fluorescein-labeled trimers, dimers, and monomers divided by the total fluorescein: $(f3 + f2 + f)/f3(0)$. Reaction half-times ($\div k_4$) were determined to be 1.08 (a), 0.92 (b), and 0.86 (c).

namely, collisional mechanisms and dissociative mechanisms. Collisional models would include a classical second-order reaction in which two trimers must collide in a particular orientation and then separate such that the subunits are redistributed. In such a case, the collision rate and thus the reaction rate would be highly dependent on the total G protein concentration. Thus, our results (Table III) rule out collisional models. Dissociative mechanisms include a reaction path in which trimers must first dissociate into monomers and dimers, which then reassociate to form trimers. The equations that describe several simpler dissociative models have been solved explicitly and resemble those of first-order reactions in that the reaction half-time is independent of concentration (Nichols & Pagano, 1982; Jones & Thompson, 1989). We have not attempted to solve explicitly the equations for the complicated set of reactions involved in the formation of mixed trimers. However, computer modeling of these reactions as described in the Appendix indicates that, like other dissociative models, the reaction half-time is independent of G protein concentration. Figure 5 shows the results of such a calculation assuming the initial concentration of fluorescein-labeled G protein to be 10, 100, or 1000 times the trimer dissociation constant. Although the reaction end points differ slightly as a result of differences in the equilibrium distribution of labeled species, the reaction half-time is largely independent of G protein concentration. Thus, our data indicate that labeled G protein trimers must first dissociate into monomers and dimers before forming mixed trimers. A similar dissociative mechanism is responsible for subunit exchange among hemoglobin tetramers (Bunn & McDonough, 1974) and probably for class I MHC antigens (Hyafil & Strominger, 1979). As in most reaction mechanisms that involve an initial dissociation step, the half-time of the reaction is determined by the rate constant for the dissociation step (Bunn & McDonough, 1974; Roseman & Thompson, 1980; Nichols & Pagano, 1982; Jones & Thompson, 1989). This rate constant is about 0.2 min^{-1} (half-time approximately 3 min) for G protein in octyl glucoside at room temperature and neutral pH. Although this reaction is quite rapid, it is similar to magnitude to other subunit dissociation rates, which range in half-time from minutes to hours depending on temperature and pH (Bunn & McDonough, 1974; Parker & Strominger, 1985).

The conclusion that G protein oligomers are in equilibrium with monomers may possibly explain the variety of results that have been obtained in different laboratories that have examined the association state of the G protein. For example, Crimmins et al. (1983) concluded that G protein is a monomer in octyl glucoside based on sedimentation equilibrium experiments, although the argument was indirect, based on consideration of the effect of detergent binding. The distribution of subunits between monomers and oligomers could be shifted by a variety of factors, including the type of detergent, the pH, and the G protein concentration, and possibly by other factors such as VSV strain differences and differences in host cells used to propagate the virus. Presumably the environment provided by a membrane lipid bilayer favors the formation of oligomers, since G protein in cellular membranes and in the virus envelope appears to be a trimer (Doms et al., 1987).

Finally, our results provide a model for the association of trimers during G protein biosynthesis. The assembly of some oligomeric membrane proteins following synthesis in the endoplasmic reticulum may involve accessory proteins such as the heat shock related protein BiP (Bole et al., 1986). BiP is known to bind to incompletely or incorrectly folded proteins and thus may play a role in acquisition of the correct tertiary or quaternary structure of membrane and secretory proteins [discussed by Hurtley et al. (1989)]. The observation that G protein monomers are in equilibrium with oligomers implies that monomers are not denatured, at least for the lifetime required for reassociation. Thus, G protein subunits are capable of self-assembly, at least in detergent solution. Conceivably, the environment of the endoplasmic reticulum membrane makes the participation of other proteins necessary for formation of the final quaternary structure. However, our results would be consistent with an assembly model in which once the G protein monomer is correctly folded, self-association can be spontaneous.

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APPENDIX

Suppose fluorescein- and rhodamine-labeled G protein trimers must dissociate into dimers and monomers in order to reassociate into mixed trimers. Conceptually, the reaction path would be $F_3 + R_3 \rightleftarrows F_2 + F + R_2 + R \rightleftarrows F_2R + FR_2$, where F_3, F_2, and F are fluorescein-labeled trimers, dimers, and monomers, respectively. However, this reaction ignores the association of monomers into dimers and is cumbersome for the calculation of the individual species of labeled G. Instead, the complete reaction scheme can be written as a series of six trimer dissociation reactions (eq A1–A6) and three dimer dissociation reactions (eq A7–A9).

$$F_3 \rightleftarrows F_2 + F \quad \text{(A1)}$$

$$R_3 \rightleftarrows R_2 + R \quad \text{(A2)}$$

$$F_2R \rightleftarrows F_2 + R \quad \text{(A3)}$$

$$F_2R \rightleftarrows FR + F \quad \text{(A4)}$$

$$FR_2 \rightleftarrows R_2 + F \quad \text{(A5)}$$

$$FR_2 \rightleftarrows FR + R \quad \text{(A6)}$$

$$F_2 \rightleftarrows 2F \quad \text{(A7)}$$

$$R_2 \rightleftarrows 2R \quad \text{(A8)}$$

$$FR \rightleftarrows F + R \quad \text{(A9)}$$

For simplicity, assume that the reaction rates do not depend on the nature of the label, then we can define:

k_1 = association rate constant for dimer formation from monomers

k_2 = dissociation rate constant for dimers into monomers

k_3 = association rate constant for trimer formation

k_4 = dissociation rate constant for trimers

The hypothesis to be tested was that the reaction time course would be governed primarily by trimer dissociation, i.e., by k_4, and would be independent of protein concentration, by analogy with other dissociative mechanisms. Therefore, time was expressed in units of $1/k_4$ so that $k_4 = 1$, and the other rate constants, k_1, k_2, and k_3, were expressed as multiples of k_4, with k_1 and k_3 having arbitrary units of 1/concentration. Since k_4 is defined as 1, then k_3 defines the dissociation constant for trimers ($K_d = k_4/k_3$). All concentrations of reactants were defined in the same arbitrary units of concentration as $1/k_3$. Thus, for convenience, k_3 was defined as 1, so that all concentrations could be expressed as multiples of the trimer dissociation constant.

Now $f3$ was defined as the concentration of G protein subunits present as the species F_3, $r3$ as the concentration of subunits present as R_3, etc., so that $[F_3] = f3/3$, $[R_3] = r3/3$, etc. Also, $J1$, $J2$, $J3$, etc. were defined as the flux through reactions 1, 2, 3, etc. in units of concentration per time, so that

$$J1 = k_4 f3/3 - k_3 f1 f2/2$$

$$J2 = k_4 r3/3 - k_3 r1 r2/2$$

$$J3 = k_4 f2r/9 - k_3 r1 f2/2$$

$$J4 = 2k_4 f2r/9 - k_3 f1 fr/2$$

$$J5 = k_4 fr2/9 - k_3 f1 r2/2$$

$$J6 = 2k_4 fr2/9 - k_3 r1 fr/2$$

$$J7 = k_2 f2/2 - k_1 f1^2$$

$$J8 = k_2 r2/2 - k_1 r1^2$$

$$J9 = k_2 fr/2 - k_1 f1 r1$$

Note that for mixed trimers, one dissociation path occurs one-third as frequently as dissociation of pure trimers, e.g., $F_2R \rightarrow F_2 + R$, and the other occurs two-thirds as frequently, e.g., $F_2R \rightarrow FR + F$. This is reflected in the equations for $J3$, $J4$, $J5$, and $J6$. The rate equations for the different molecular species are

$$d(f3)/dt = -3J1$$

$$d(r3)/dt = -3J2$$

$$d(f2r)/dt = -3(J3 + J4)$$

$$d(fr2)/dt = -3(J5 + J6)$$

$$d(f2)/dt = 2(J1 + J3 - J7)$$

$$d(r2)/dt = 2(J2 + J5 - J8)$$

$$d(fr)/dt = 2(J4 + J6 - J9)$$

$$d(f1)/dt = J1 + J4 + J5 + 2J7 + J9$$

$$d(r1)/dt = J2 + J3 + J6 + 2J8 + J9$$

For each molecular species y, the concentration at time t is

$$y(t) = y(0) + \int [d(y)/dt]\, dt$$

where $y(0)$ is the initial concentration of y. These equations were approximated by digital integration:

$$y(t) = y(0) + \sum [d(y)/dt]_i \Delta t$$

where Δt is a sufficiently small interval.

Since the reactions were initiated by a 10–100-fold dilution of fluorescein- and rhodamine-labeled G protein together, and since the ratio of rhodamine-labeled G protein to fluorescein-labeled G protein was always 3:1, the initial conditions were always defined as $r3 = 3f3$, and all other species as 0. The concentrations of each species were calculated from $t = 0$ to $t = 4/k_4$. Since the final percent quenching was the same regardless of the G protein concentration, it can be concluded that the G protein concentration is at least an order of magnitude greater than the trimer dissociation constant ($1/k_3$). Therefore, calculations were performed at various protein concentrations with the lowest being $f3 = 10$. Assume that F_2R, FR_2, and FR are equally quenched, so that the spectroscopic assay can be modeled by calculating the sum of the unquenched species ($f3 + f2 + f$) as a function of time. Finally, since we have no information about the dimer rate constants, the effect of G protein concentration was tested by assuming various values for k_1 and k_2, including $k_3:k_2:k_1$ = 1:1:1 (dimer and trimer equally stable), $k_3:k_2:k_1$ = 1:100:1 (dimer dissociates more rapidly than trimer), $k_3:k_2:k_1$ = 1:1:100 (dimer associates more rapidly than trimer), $k_3:k_2:k_1$ = 1:0.01:1, and $k_3:k_2:k_1$ = 1:1:0.01. In each series of calculations, the reaction end points differed slightly as a result of differences in the equilibrium distribution among the different labeled species, but in each case, the reaction half-time was largely independent of the initial G protein concentration and was slightly larger than $(\ln 2)/k_4$. Minor differences in half-time were due primarily to differences in the lag time required for kinetic intermediates to reach a steady-state level. Figure 5 shows an example of a calculation assuming $k_3:k_2:k_1$ = 1:1:1 for $f3 = 10K_d$, $100K_d$, and $1000K_d$.

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Quaternary Structure of the M_r 46 000 Mannose 6-Phosphate Specific Receptor: Effect of Ligand, pH, and Receptor Concentration on the Equilibrium between Dimeric and Tetrameric Receptor Forms†

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ABSTRACT: The M_r 46 000 mannose 6-phosphate specific receptor exists in solution as a mixture of noncovalently associated dimeric and tetrameric forms. The two quaternary forms were separated by sucrose density centrifugation, and their composition was assessed by cross-linking with bifunctional reagents followed by SDS–polyacrylamide gel electrophoresis. The dependence of equilibrium between the dimeric and tetrameric forms on pH, receptor concentration, and presence of mannose 6-phosphate was studied. The formation of tetrameric forms is favored by pH values around 7, high receptor concentration, and presence of mannose 6-phosphate ligand. Tetrameric forms bind stronger at pH 7 to phosphomannan–Sepharose 4B than dimeric forms. Both quaternary forms dissociate at the same pH from a mannose 6-phosphate affinity matrix. When starting with dimeric or tetrameric forms, the equilibrium between dimeric and tetrameric forms is reached at pH 7.5 and 4 °C after 6–8 days. The presence of 5 mM mannose 6-phosphate shifts the equilibrium toward tetrameric forms. At pH 4.5 and 4 °C, the association of dimeric to tetrameric forms is negligible, while tetrameric forms dissociate to dimeric forms within 12 h. The results demonstrate that oligomerization is an intrinsic property of MPR-46 that is affected by ligand binding, pH, and receptor concentration.

Mannose 6-phosphate specific receptors are involved in the sorting of newly synthesized lysosomal enzymes [for reviews, see von Figura and Hasilik (1986) and Dahms et al. (1989)]. They bind mannose 6-phosphate containing lysosomal enzymes in the trans Golgi and direct their transport to the prelysosome. Due to the acidic pH in the prelysosome, the lysosomal enzymes dissociate from the receptors and are than sorted to lysosomes by an yet unknown mechanism, whereas the receptors recycle back to the trans Golgi.

Two different mannose 6-phosphate recognizing receptors with apparent sizes of 46 kDa (MPR-46)[1] (Hoflack & Kornfeld, 1985a; Stein et al., 1987b; Dahms et al., 1987; Pohlman et al., 1988) and 215 kDa (MPR-215) (Sahagian et al., 1981; Oshima et al., 1988; Lobel et al., 1988) have been characterized. The larger of the two receptors binds insulin-like growth factor II in addition to mannose 6-phosphate and is therefore now referred to as M6P/IGF II receptor (Waheed et al., 1988; Kiess et al., 1988). Both receptors mediate transport of newly synthesized lysosomal enzymes from the Golgi to prelysosomes (Gartung et al., 1985; Stein et al., 1987c). The M6P/IGF II receptor mediates in addition the

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[1] Abbreviations: MPR-46, M_r 46 000 mannose 6-phosphate specific receptor; MPR-215, M_r 215 000 mannose 6-phosphate specific receptor; ECD, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; DSS, disuccinimidyl suberate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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