Subunit Interactions of Vesicular Stomatitis Virus Envelope Glycoprotein Influenced by Detergent Micelles and Lipid Bilayers†

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ABSTRACT: The envelope glycoprotein (G protein) of vesicular stomatitis virus is a transmembrane protein that exists as a trimer of identical subunits in the virus envelope. We have examined the effect of modifying the environment surrounding the membrane-spanning sequence on the association of G protein subunits using resonance energy transfer. G protein subunits were labeled with either fluorescein isothiocyanate or rhodamine isothiocyanate. When the labeled G proteins were mixed in the presence of the detergent octyl glucoside, mixed trimers containing both fluorescent labels were formed as a result of subunit exchange, as shown by resonance energy transfer between the two labels. In contrast when fluorescein- and rhodamine-labeled G proteins were mixed in the presence of Triton X-100, no resonance energy transfer was observed, indicating that subunit exchange did not occur in Triton X-100 micelles. However, if labeled G proteins were first mixed in the presence of octyl glucoside, energy transfer persisted after dilution with buffer containing Triton X-100. This result indicates that the G protein subunits remained associated in Triton X-100 micelles and that the failure to undergo subunit exchange was due to lack of dissociation of G protein subunits. Chemical cross-linking experiments confirmed that G protein was trimeric in the presence of Triton X-100. The efficiency of resonance energy transfer between labeled G protein was higher when G proteins were incorporated into dimyristoylphosphatidylcholine liposomes compared to detergent micelles. This result indicates that the labels exist in a more favorable environment for energy transfer in membranes than in detergent micelles. This appears to be due to a conformational difference in the G protein resulting in a closer packing of subunits in the region surrounding the label. These data also indicate that the fluorescein label is probably attached to the G protein near its membrane anchor. This idea was supported by showing that the fluorescein label remained associated with liposomes after proteolysis to remove the G protein external domain. The results presented here show that although the G protein is oligomeric in octyl glucoside or Triton X-100 micelles and in phospholipid bilayers, the subunit interactions are altered by the changes in the environment surrounding the membrane anchor region.

Enveloped viruses consist of a nucleoprotein core surrounded by a membranous envelope, which contains integral membrane glycoproteins that project from the surface of the envelope. Viral glycoproteins function in attachment to host cells and in fusion of the envelope with cellular membranes during virus penetration. During virus replication, viral glycoproteins are inserted into host cell membranes by way of the secretory pathway and participate in assembly and budding of mature virions [reviewed by Stephens and Compans (1988)]. Many viral and cellular integral membrane proteins have been shown to have an oligomeric structure that is essential for activity [reviewed by Hurtley and Helenius (1989)]. The subunit interactions of viral glycoproteins are often studied as models for the many integral membrane proteins that have an oligomeric structure. The glycoprotein (G protein)[1] of vesicular stomatitis virus (VSV) is a widely studied prototype viral envelope glycoprotein. The VSV G protein contains a large glycosylated external domain, a hydrophobic transmembrane sequence near the carboxy terminus, and a short carboxy-terminal sequence that extends from the inner surface of the envelope. G protein exists predominantly as a trimer of identical subunits in the virion and in infected cells (Dubovi & Wagner, 1977; Kreis & Lodish, 1986; Doms et al., 1987; Lyles et al., 1990). Mutational analysis of G protein has shown that formation of G protein trimers is essential for transport from the endoplasmic reticulum to the cell surface (Doms et al., 1988).

Analysis of G protein trimers in vitro has shown that stability of the trimers is dependent on the solubilizing detergent and pH. When G protein is solubilized from virions with Triton X-100 and analyzed by rate zonal centrifugation at pH 5.8, G protein sediments as a trimer, whereas if the centrifugation is performed at pH 7.4, the trimers irreversibly dissociate and sediment as monomers (Doms et al., 1987). In contrast, if G protein is solubilized using the detergent octyl glucoside, it primarily sediments as a trimer at both pH 5.8 and 7.5 (Lyles et al., 1990). Additionally, using the technique of resonance energy transfer between fluorescein- and rhodamine-labeled G proteins, it has been shown that trimers undergo a reversible dissociation and reassociation in the presence of octyl glucoside at pH 7.5 (Lyles et al., 1990, 1992). Similar subunit exchange has also been demonstrated in vivo using genetically distinct G proteins (Zagouras et al., 1991). More recently we have been able to analyze the reversible dissociation of G protein subunits directly, rather than through subunit exchange, by dilution of labeled G protein to concentrations in the range of the trimer dissociation constant (Lyles et al., 1992).

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[1] Abbreviations: DSS, disuccinimido suberate; DMPC, dimyristoylphosphatidylcholine; DMSO, dimethyl sulfoxide; G protein, envelope glycoprotein; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline [50 mM tris(hydroxymethyl)aminomethane and 0.9% sodium chloride]; VSV, vesicular stomatitis virus.

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G protein can still form trimers following genetic deletion of the coding sequence for its membrane anchor (Crise et al., 1989). However, the differential stability of G protein trimers in the presence of octyl glucoside versus Triton X-100 suggests that the environment surrounding the membrane anchor sequence influences the interaction between G protein subunits. The goal of the experiments presented here was to investigate the mechanism of the differential stability of G protein trimers in buffers containing different detergents or different pH and in the presence of membrane phospholipids. Unexpectedly, Triton X-100 was found to slow G protein trimer dissociation, and little effect of incubation at pH 5.8 on G protein subunit exchange was observed. This contrasts with results from sedimentation velocity analysis and may be attributed to irreversible changes in G protein structure during sedimentation that are unrelated to the reversible dynamic equilibrium between G protein trimers, dimers, and monomers. The results with labeled G proteins incorporated into dimyristoylphosphatidylcholine liposomes indicate that G protein is oligomeric in membranes and suggest that it undergoes structural changes in the region near its membrane anchor sequence upon transfer from lipid bilayers to detergent micelles.

EXPERIMENTAL PROCEDURES

Isolation and Fluorescent Labeling of G Proteins. VSV (Indiana serotype) was grown in BHK cells. The purification and labeling of G protein with fluorescent probes has been described elsewhere (Lyles et al., 1990). Briefly, G protein was solubilized from purified virions with 50 mM octyl glucoside at pH 8.5. G protein was reacted with either fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate and purified by gel filtration and rate zonal centrifugation through a 5–17% (w/w) sucrose gradient containing 50 mM octyl glucoside in phosphate-buffered saline (PBS) at pH 7.5. The labeling conditions yielded approximately 1 fluorophore per G protein subunit. The concentration of the purified, labeled G protein was approximately 1 μM (total G protein subunits, i.e., about 60 μg/mL).

Chemical Cross-Linking of G Protein. Fluorescein-labeled G protein in 50 mM octyl glucoside and PBS, pH 7.5, was diluted with an equal volume of 50 mM borate buffer and 0.9% sodium chloride, pH 8.5, containing either 50 mM octyl glucoside or 1% Triton X-100. After a 30-min incubation, various concentrations of disuccinimido suberate (DSS) were added to the G protein solutions as a 1:100 dilution of the DSS stock solution in dimethyl sulfoxide and reacted at room temperature for 30 min. The reaction was terminated by addition of glycine (10 mM final concentration). The samples were then subjected to SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), and protein bands were visualized by silver staining (Wray et al., 1981).

Incorporation of G Protein into Liposomes. Fluorescein-labeled G protein was mixed with a 3-fold excess of either rhodamine-labeled or unlabeled G protein. The G protein solutions were added to dimyristoylphosphatidylcholine (dried into a thin film) to give lipid to protein weight ratios of 5, 10, and 25. The solutions were incubated at 37 °C for 1 h, followed by dialysis against three changes of PBS, pH 7.5. The resulting liposomes were diluted with 2 volumes of 65% (w/w) sucrose in PBS, pH 7.5, and a layer of 35% sucrose solution was placed on top of the liposome suspension followed by a layer of PBS, pH 7.5. The gradient was spun at 200000g_{max} in an SW41 rotor for 16 h. The liposomes were collected at the 35% sucrose/PBS interface, and the concentration of liposomes was estimated by light scattering at 550 nm. Liposomes containing rhodamine-labeled or unlabeled G protein were diluted to approximately equal concentrations for fluorescence measurements on the basis of the light scattering data. The ratio of rhodamine to fluorescein in the recovered liposomes was confirmed as 3:1 by measuring their absorbance at 554 and 495 nm, respectively, after treatment with 0.05 N sodium hydrodide and 0.1% SDS.

Proteolysis of G Protein in Liposomes with Trypsin. DMPC liposomes containing fluorescein-labeled G protein (0.55 mL, approximately 50 μg of protein/mL) were incubated with trypsin (5 μg/mL) for 1 h at room temperature. Soybean trypsin inhibitor was added to a final concentration of 25 μg/mL. The sample was mixed with 1.2 mL of 65% sucrose in PBS and overlaid with 3 mL of 35% sucrose in PBS followed by 0.5 mL of PBS. The gradient was spun at 150000g_{max} in an SW50.1 rotor for 16 h. Fractions of 0.5 mL were collected and diluted with 2 mL of 0.1 N NaOH and 0.1% SDS before determining fluorescein fluorescence intensity. The final concentration of sucrose was <10%, at which there is no significant quenching.

Spectroscopy. G protein concentrations and label ratios were calculated as described (Lyles et al., 1990). Fluorescence measurements were made using either a Spex Fluorolog or SLM/Aminco 8000C fluorometer as described (Lyles et al., 1985).

Table I: Effect of Detergents and pH on G Protein Trimer Subunit Exchange[a]

protocol	before mixing: detergent	before mixing: pH	after mixing: detergent	after mixing: pH	% energy transfer [mean ± SD (*N*)]
1	TX	7.5	TX	8.5	4.4 ± 2.1 (3)
2	OG	7.5	TX	8.5	26.0 ± 8.8 (5)
3	OG	7.5	OG	8.5	21.9 ± 3.5 (5)
4	OG	5.8	OG	8.5	16.0 ± 4.0 (4)
5	TX	5.8	TX	8.5	−2.5 (2)
6	OG	7.5	TX	5.8	
			+TX	8.5	14 (2)

[a] Fluorescein- and rhodamine-labeled G proteins were diluted separately with an equal volume of PBS containing either 50 mM octyl glucoside (OG) or 1% Triton X-100 (TX) and either 10 mM sodium phosphate, pH 7.5, or 100 mM sodium phosphate, pH 5.4, to give a final pH of 5.8. Fluorescein-labeled G protein and rhodamine-labeled G protein were mixed at a 1:3 molar ratio and incubated for 30 min in a total volume of 0.25 mL. The samples were then diluted to 2.5 mL with TBS containing the indicated detergent. The pH of the TBS was such that the final pH was 8.5. Fluorescence intensity measurements were made immediately after dilution and after a further 10-min incubation. There were no significant differences between these two measurements, and only the data obtained after 10 min are shown. The efficiency of resonance energy transfer was calculated by comparison with similarly treated controls in which unlabeled G protein was substituted for rhodamine-labeled G protein.

RESULTS

G Protein Subunit Association in Triton X-100. G protein was solubilized from purified VSV using the detergent octyl glucoside and labeled with either fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate to give approximately 1 fluorophore per G protein monomer. When fluorescein- and rhodamine-labeled G proteins are mixed in the presence of octyl glucoside, G protein subunits undergo a reversible dissociation and reassociation to form mixed trimers containing both fluorescein- and rhodamine-labeled subunits (Lyles et al., 1990). Experimentally this can be shown by quenching of fluorescein fluorescence due to resonance energy transfer between the fluorescein and rhodamine labels. The effect on G protein subunit association of changing detergents from octyl glucoside to Triton X-100 was tested (Table I). Labeled

G proteins, originally in buffer containing octyl glucoside, were diluted with buffer containing either Triton X-100 (line 1) or octyl glucoside (lines 2 and 3), and then the fluorescein- and rhodamine-labeled preparations were mixed and incubated for 30 min to allow for subunit exchange. The mixture was then diluted further in buffer containing either Triton X-100 (lines 1 and 2) or octyl glucoside (line 3) to measure the quenching of fluorescein fluorescence relative to identically-treated controls, which lacked rhodamine. The fluorescence measurements were made at pH 8.5 so that most of the fluorescein was in the highly fluorescent deprotonated form (see below). It has been shown previously that the efficiency of resonance energy transfer between labeled G protein subunits is independent of pH in the range from pH 6.5 to 8.5 (Lyles et al., 1990).

When fluorescein- and rhodamine-labeled G proteins were treated separately with Triton X-100 before mixing, little or no resonance energy transfer was observed (Table 1, line 1). This result could be due either to dissociation of G protein trimers to monomers in the presence of Triton X-100 or to absence of trimer dissociation, which would prevent the formation of mixed trimers. The latter was shown to be the case by incubation of fluorescein- and rhodamine-labeled G proteins in the presence of octyl glucoside to form mixed trimers before dilution into buffer containing Triton X-100 (line 2), in which the efficiency of resonance energy transfer was similar to that obtained in the presence of octyl glucoside alone (line 3). The results in Table I indicate that the effect of Triton X-100 was to slow the rate of trimer dissociation, so that mixed trimers did not form unless the labeled G proteins were premixed in the presence of octyl glucoside. In other experiments the efficiency of resonance energy transfer in mixed trimers was unchanged by a 5-h incubation at 25 °C, indicating that G protein trimers were stable for hours in the presence of Triton X-100. The results of these experiments contrast with the results of sedimentation velocity analysis, which indicated that G protein trimers were unstable in the presence of Triton X-100 unless the pH was below 6 (Doms et al., 1987; Lyles et al., 1990).

Further evidence supporting the existence of G protein trimers in the presence of Triton X-100 was obtained by using the chemical cross-linker DSS, as shown in Figure 1. G protein was purified in the presence of octyl glucoside and labeled with fluorescein isothiocyanate as above and then diluted to pH 8.5 with buffer containing either octyl glucoside or Triton X-100. The G protein samples were incubated with varying concentrations of DSS for 30 min before analysis by SDS–polyacrylamide gel electrophoresis (Figure 1). Cross-linking of G protein in the presence of octyl glucoside yielded prominent dimer bands that were progressively converted to trimer bands over the range of DSS concentrations used (6–50 μg/mL, lanes 1–4), which agrees with earlier work (Doms et al., 1987; Lyles et al., 1990). The G protein dimer band appeared as a doublet, while the trimer band was diffuse and migrated at a lower apparent molecular weight than expected. The aberrant mobility of cross-linked G protein dimers and trimers presumably resulted from heterogeneity in the formation of intramolecular cross-links. Similar results were obtained when G protein was incubated in Triton X-100 (lanes 5–8). The distribution of G protein subunits among monomer, dimer, and trimer species cannot be quantitated using chemical cross-linking; however, the cross-linking pattern in the presence of Triton X-100 is similar to that in octyl glucoside and in Triton X-100 at low pH (Doms et al., 1987; Lyles et al., 1990). These results indicate that G protein is oligomeric in the presence of Triton X-100, which supports the resonance energy transfer data presented in Table I.

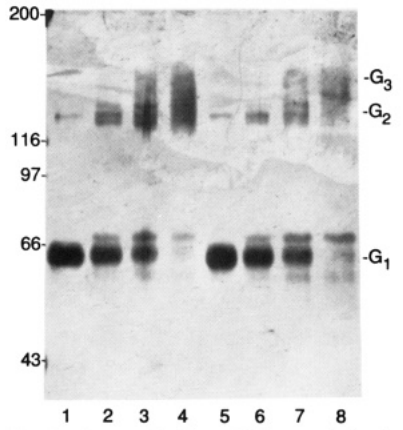

FIGURE 1: Chemical cross-linking of G protein in the presence of octyl glucoside or Triton X-100. Fluorescein-labeled G protein was diluted with an equal volume of buffer to give a pH of 8.5 and a final detergent concentration of either 50 mM octyl glucoside (lanes 1–4) or 0.5% Triton X-100 (lanes 5–8), and the samples were incubated for 30 min. Samples were treated with disuccinimido suberate (DSS) for 30 min at room temperature and then examined by SDS–polyacrylamide gel electrophoresis followed by silver staining. Positions of molecular weight markers ($\times 10^{-3}$) and G protein trimer, dimer, and monomer species are indicated. DSS concentrations were 0 (lanes 1 and 5), 5.6 μg/mL (lanes 2 and 6), 16.7 μg/mL (lanes 3 and 7), and 50 μg/mL (lanes 4 and 8).

Effect of Low-pH Treatment on G Protein Trimer Subunit Exchange. On the basis of sedimentation velocity analysis (Doms et al., 1987; Lyles et al., 1990), incubation of G protein at pH 5.8 should stabilize the trimer structure and might be expected to prevent subunit exchange by slowing the rate of trimer dissociation. This hypothesis was tested by mixing fluorescein- and rhodamine-labeled G proteins at pH 5.8 and assaying the resonance energy transfer as above. Unfortunately it was not practical to measure fluorescein fluorescence at low pH, since the quantum yeild of fluorescein fluorescence is low at pH 5.8. Therefore, labeled G proteins were incubated together at pH 5.8 for 30 min, and resonance energy transfer measurements were obtained immediately after the solution pH was raised to 8.5. After a further 10-min incubation, the fluorescence measurements were repeated to determine whether further subunit exchange occurred after the pH was raised to 8.5 (Table I, lines 4–6).

Unexpectedly, the results obtained by incubation of labeled G proteins at pH 5.8 were similar to those obtained by incubation at pH 7.5. Mixing fluorescein- and rhodamine-labeled G proteins at pH 5.8 in the presence of octyl glucoside resulted in the formation of mixed trimers as shown by resonance energy transfer betweeen the two labels (line 4). Although the efficiency of energy transfer following incubation at pH 5.8 was slightly less than that at pH 7.5 (about 16% versus 22%), the subunit exchange reaction had in fact gone to completion at pH 5.8, since no further increase in the efficiency of energy transfer was observed upon raising the pH. The results obtained at pH 5.8 in the presence of Triton X-100 were also similar to those at pH 7.5 in that no resonance energy transfer was observed following incubation in the presence of Triton X-100 (line 5) unless mixed trimers were first formed in the presence of octyl glucoside (line 6).

The time course of G protein subunit exchange was examined to determine whether incubation at pH 5.8 affected the ability of trimers to undergo subunit exchange after returning to high pH (Figure 2). Fluorescein- and rhodamine-labeled G proteins were incubated separately at pH 5.8 in the presence

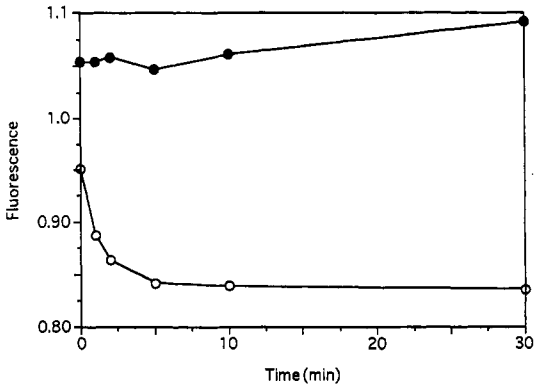

FIGURE 2: Kinetics of G protein subunit exchange in the presence of octyl glucoside or Triton X-100 after pretreatment at pH 5.8. Fluorescein- and rhodamine-labeled G proteins were diluted separately with an equal volume of buffer to give a pH of 5.8 and a final detergent concentration of either 50 mM octyl glucoside (○) or 0.5% Triton X-100 (●). After 30 min the fluorescein-labeled G protein solution was diluted 40-fold with TBS, pH 8.5, containing either 50 mM octyl glucoside or 0.1% Triton X-100, and the solution was equilibrated for 10 min. A 3-fold excess of low-pH-treated, rhodamine-labeled G protein was then added and the fluorescein fluorescence intensity was measured. The final G protein concentration for both detergent samples was 40 nM (total G protein subunits). Fluorescence intensities were normalized to those of similarly treated fluorescein-labeled G protein mixed with a 3-fold excess of unlabeled G protein. Data shown are representative of four separate experiments for G protein in the presence of octyl glucoside and two experiments for G protein in the presence of Triton X-100.

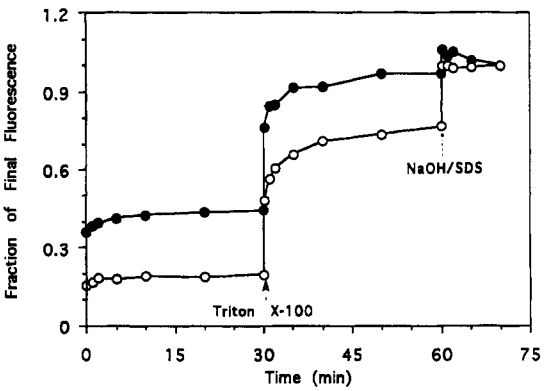

FIGURE 3: Subunit association of labeled G proteins in DMPC liposomes. DMPC liposomes containing fluorescein-labeled G protein and a 3-fold excess of rhodamine-labeled (○) or unlabeled (●) G protein were prepared with a weight ratio of DMPC to G protein of 5:1. Liposomes were diluted 25-fold with TBS, pH 8.5, and fluorescein fluorescence was measured as a function of time. At the indicated time, Triton X-100 (final concentration 0.1%) was added. Total fluorescein fluorescence intensity was then determined by treatment of the samples as indicated with 0.05 N sodium hydroxide and 0.1% SDS for 10 min. Data were normalized to the final fluorescence.

of either octyl glucoside or Triton X-100. After 30 min the pH was raised to 8.5, and the labeled G proteins were immediately mixed. Subunit exchange, detectable as a decrease in fluorescein fluorescence intensity with time, occurred in the presence of octyl glucoside but not Triton X-100 (Figure 2). The half-time for the subunit exchange reaction in the presence of octyl glucoside, determined from the data in Figure 2, was 2 min, which is similar to that observed without the incubation at low pH (Lyles et al., 1990). The data in Table I and Figure 2 indicate that incubation of G protein at pH 5.8 had relatively little effect on the reversible dissociation and reassociation of G protein subunits.

G Protein Subunit Interactions in Dimyristoylphosphatidylcholine Liposomes. Fluorescein- and rhodamine-labeled G proteins were mixed in the presence of octyl glucoside, and the mixed trimers were incorporated into liposomes by removal of the detergent by dialysis in the presence of DMPC. Liposomes containing a mixture of fluorescein-labeled and unlabeled G protein served as the control for fluorescence in the absence of resonance energy transfer. Since the recovery of liposomes varied between the two preparations, the total amount of fluorescein fluorescence was determined at the end of the experiment by treatment of the liposomes with 0.5 N NaOH and 0.1% SDS, which disrupts quenching mechanisms dependent on interaction between subunits or interaction with lipids. Fluorescence measurements were normalized to the total fluorescein fluorescence so that intensities in the presence versus the absence of the rhodamine label could be compared directly. The fluorescence of fluorescein-labeled G protein incorporated into liposomes with rhodamine-labeled G protein was substantially quenched relative to that in liposomes containing unlabeled G protein (FIgure 3), indicating that the G protein in liposomes is oligomeric. The efficiency of resonance energy transfer between labeled G proteins in liposomes (58% in the experiment shown in Figure 3) was approximately 3-fold higher than that observed in the presence of detergents (Table I). Upon addition of Triton X-100 to a final concentration of 0.1%, the fluorescence intensities of

Table II: Effect of Surface Density of G Protein in Dimyristoylphosphatidylcholine Liposomes on Subunit Association[a]

lipid/protein	pH treatment	% energy transfer [mean ± SD (*N*)]
5	8.5	58 ± 7 (4)
10	8.5	65 (2)
25	8.5	60 (2)
5	5.8	54 ± 10 (3)

[a] Dimyristoylphosphatidylcholine (DMPC) liposomes were prepared as described under Experimental Procedures with the indicated lipid to G protein weight ratios. Fluorescence was measured after 10-fold dilution of the liposome preparations with TBS, pH 8.5. The fluorescence intensities of fluorescein-labeled G protein were normalized to the fluorescence intensity after treatment with 0.05 N sodium hydroxide and 0.1% SDS for 10 min. The percentage of resonance energy transfer was then determined by comparing the normalized fluorescence intensity of the fluorescein-labeled G protein in liposomes containing rhodamine-labeled G protein with the normalized intensity of fluorescein-labeled G protein in liposomes containing unlabeled G protein. Liposomes treated at low pH were incubated for 30 min at pH 5.8 by dilution with an equal volume of 100 mM phosphate buffer, pH 5.4. The pH was then raised to 8.5 by 10-fold dilution with TBS, pH 8.7.

both liposome preparations increased by a factor of approximately 2.5; however, the efficiency of resonance energy transfer decreased to the level observed prior to incorporation into liposomes (about 20%). Subsequent addition of NaOH and SDS to the labeled G proteins abolished the resonance energy transfer but had little further effect on the fluorescence of fluorescein-labeled G protein. The dramatic difference in quantum yield of fluorescein fluorescence between labeled G protein in liposomes versus Triton X-100 was presumably due to an interaction of the fluorescein label with the lipid bilayer (see below). The fluorescence of rhodamine-labeled G protein was even more extensively quenched (>90%) upon incorporation into liposomes (data not shown). However, this would not affect its ability to act as an energy transfer acceptor from fluorescein, since there was no detectable change in its absorption spectrum.

The higher efficiency of resonance energy transfer between labeled G proteins in liposomes compared to detergent micelles could be due to energy transfer between (rather than within) trimers. The effect of surface density of the G protein in liposomes was therefore examined by increasing the ratio of DMPC to protein (Table II). The efficiency of resonance energy transfer between fluorescein- and rhodamine-labeled

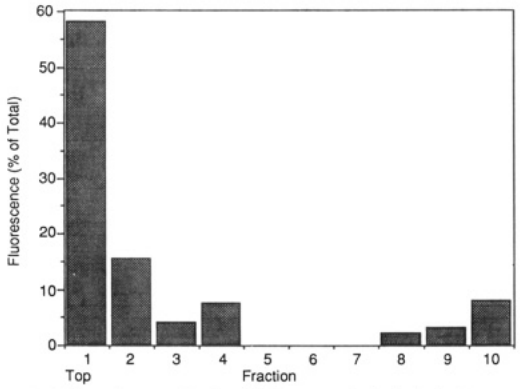

FIGURE 4: Flotation analysis of fluorescein-labeled G protein in DMPC liposomes after treatment with trypsin. DMPC liposomes containing fluorescein-labeled G protein were incubated in the presence of trypsin for 1 h at room temperature. The sample was mixed with 65% sucrose and overlaid with 35% sucrose followed by PBS. The gradient was spun at 150000g_{max} for 16 h to separate liposomes (which float to the top of the gradient) from nonassociated proteolytic fragments (which remain at the bottom). Fractions were collected and the fluorescein fluorescence intensity was determined. Data are presented as the percentage of the sum of all the intensities.

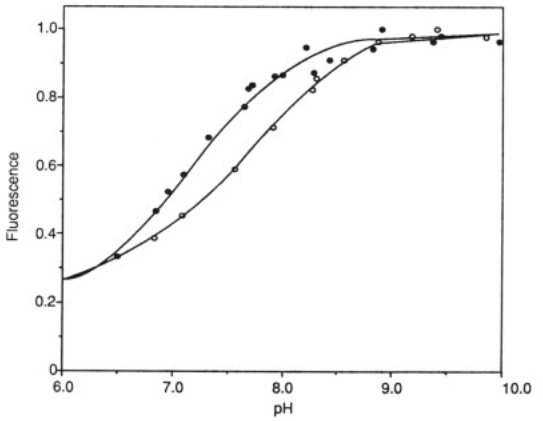

FIGURE 5: Effect of lipid and detergent environments on the pH titration of fluorescein label bound to G protein. DMPC liposomes containing fluorescein-labeled G protein were diluted in 10 mM Tris and 0.9% NaCl, pH 8.5. Fluorescein fluorescence was determined as a function of pH titrated to pH 10 with 0.1 N NaOH, then to pH 6.5 with 0.1 N HCl, and then to pH 8.5 with 0.1 N NaOH. Triton X-100 was added to a final concentration of 1% and the titration was repeated. The fluorescence intensities in the presence of Triton X-100 (●) or incorporated into DMPC liposomes (○) were normalized to the maximum fluorescence in each titration.

G protein subunits was approximately the same for all lipid to protein ratios. As in Figure 3, solubilization of these liposomes with Triton X-100 increased the overall fluorescence, and the efficiency of energy transfer was reduced to approximately 20% (not shown). These data imply that high protein surface density in liposomes was not responsible for resonance energy transfer between labeled G protein subunits. These data do not rule out the possibility that G protein trimers form small clusters that are independent of protein to lipid ratio in liposomes. However, lateral diffusion measurements have indicated that if clusters of G protein exist in liposomes, they are not large (Cartwright et al., 1982). Incubation of liposomes at pH 5.8 for 30 min had no effect on the resonance energy transfer in liposomes or Triton X-100 micelles (Table II).

Additional experiments showed that the same results were obtained both below and above the DMPC phase transition temperature at 22 °C (not shown).

The enhancement of resonance energy transfer in liposomes and its subsequent decrease after solubilization with Triton X-100 suggest that the fluorescein and rhodamine labels were attached to a region of the protein whose conformation differs when G protein is in a lipid bilayer versus a detergent micelle. Thus the labels were likely to be attached close to the membrane anchor sequence of G protein. Such a location might also be responsible for the dramatic difference in the quantum yield of fluorescein fluorescence when liposomes containing labeled G protein were solubilized with Triton X-100 (Figure 3). Further evidence for the location of the fluorescein label in a membrane-associated region of the G protein is presented in Figure 4. Fluorescein-labeled G protein was incorporated into DMPC liposomes and the liposomes were treated with trypsin, which cleaves the G protein external domain and leaves the membrane anchor sequence associated with the liposomes. The cleavage products were analyzed by SDS–polyacrylamide gel electrophoresis, which showed that nearly all of the G protein was converted to small peptides (<10 000 molecular weight; data not shown). Thus, the DMPC liposomes containing G protein consisted primarily of liposomes with the large external domain of the G protein on the outside. Trypsin-treated liposomes were isolated from released peptides by flotation in a sucrose gradient. Gradient fractions were collected, and the fluorescence of each fraction was measured (Figure 4). Approximately 80% of the fluorescence floated to the top of the gradient with the liposomes (fractions 1–4). These data are consistent with attachment of the fluorescein label near the G protein membrane anchor sequence.

When the influenza virus hemagglutinin is modified with fluorescein isothiocyanate at lysine residues that border the membrane anchor sequence, the properties of the label's fluorescence are consistent with placement in the head group regions of lipid bilayers or detergent micelles (Lyles et al., 1985). Furthermore, the environment surrounding the label in the presence of detergent is more polar than in lipid bilayers. The pK_a of the fluorescein group is a particularly sensitive indicator of this difference in polarity, with the less polar environment in liposomes favoring the less charged protonated form. The pK_a of the fluorescein in labeled G protein displayed similar behavior (Figure 5). Fluorescein-labeled G protein was incorporated into DMPC liposomes and the fluorescein fluorescence intensity was determined as a function of pH. The pK_a of the fluorescein was determined to be approximately 7.7; however, after solubilization with Triton X-100 the pK_a was shifted to approximately 7.3. The experiment shown in Figure 5 was performed at 30 °C, which is above the transition temperature for the gel to fluid phase transition to DMPC (22 °C). Similar results were obtained below the phase transition temperature at 15 °C (not shown). The difference in fluorescein pK_a between liposomes and detergent micelles provides further evidence that the fluorophore is attached close to the membrane anchor sequence of the G protein.

Aside from the change in quantum yield and pK_a, other fluorescence properties of the fluorescein label did not change markedly when G protein in liposomes was solubilized with Triton X-100. There was no significant difference in the wavelength dependence of fluorescence emission or excitation (λ_{max} = 522 and 498 nm, respectively). Steady-state fluorescence polarization values were slightly higher when labeled G protein was incorporated into liposomes (P = 0.31) than after solubilization with Triton X-100 (P = 0.23).

DISCUSSION

In the present study the subunit interactions of G protein were investigated by resonance energy transfer between

fluorescent labels convalently attached to the G protein. Four experiments indicate that most of the label was near the membrane anchor sequence: (1) Most of the label remained associated with lipid bilayers following proteolysis of labeled G protein in liposomes to remove the external domain (Figure 4). (2) The pK_a of the fluorescein was shifted by incorporation of labeled G protein into liposomes compared to that in detergent micelles, suggesting that the fluorescein interacted directly with the lipid bilayer (Figure 5). (3) The quantum yield of fluorescein or rhodamine fluorescence was enhanced when labeled G protein incorporated into liposomes was solubilized with detergents (Figure 3), indicating that the labels were subjected to quenching mechanisms in lipid bilayers that were decreased upon solubilization with detergents. (4) The efficiency of resonance energy transfer between fluorescein- and rhodamine-labeled subunits was enhanced by incorporation into lipid bilayers compared to detergent micelles (Table II). The most likely explanation for the difference in energy transfer is that the G protein conformation differs in liposomes compared to detergent micelles, so as to decrease the distance between labels. It is possible that other factors besides the distance between labels could account for the difference in energy transfer by changing by Förster transfer distance (R_0) characteristic of the fluorescein–rhodamine donor–acceptor pair, which is about 45 Å for labeled G proteins in the presence of detergents (Lyles et al., 1990). However, most of the observed changes in the properties of the probes, such as the decrease in quantum yield of the donor, would have the effect of decreasing rather than increasing the efficiency of energy transfer when G protein is incorporated into liposomes.

The results of our previous study with the influenza virus hemagglutinin (HA) (Lyles et al., 1985) suggest a mechanism for the preferential labeling of lysine residues near the membrane anchor sequence that interact with the head group region of lipid bilayers or detergent micelles. Upon solubilization in octyl glucoside, the relatively less polar environment of the micelle compared to the aqueous phase would be expected to decrease the pK_a of the boundary lysine amino groups, favoring deprotonation of the ϵ-amino group and increasing their reactivity with the fluorescent isothiocyanates. By limiting the reaction to 1 label per G protein subunit, the fluorescent labels should preferentially react with the G protein near its membrane anchor sequence. There are several possible sites within the G protein sequence at which a fluorescein label might have the properties described above. On the basis of the similarity of the fluorescence properties to those of fluorescein-labeled HA (Lyles et al., 1985), the most likely site of labeling in the G protein is Lys 463, which is the last charged amino acid prior to the hydrophobic region of the membrane anchor sequence (Ross & Gallione, 1981). However, it is also possible that other sites in the protein that interact with lipid bilayers as a result of the tertiary structure of the G protein might be labeled.

Crise et al. (1989) have shown that mutant G proteins that contain only the ectodomain of the protein can form trimers, indicating that the membrane anchor sequence is not essential for trimer formation. However, in the present study the effect of changing detergents on the rate of subunit dissociation suggests that the membrane anchor sequence can also modulate subunit interactions. An alternative possibility is that detergents alter the subunit dissociation by binding to sites in the ectodomain in addition to binding to the membrane anchor sequence. Additional evidence is provided by studies on the interactions of the VSV matrix protein with the G protein (Lyles et al., 1992). In these experiments the viral matrix protein, which presumably binds to the cytoplasmic domain of the G protein, was found to enhance trimer stability, providing further support for the idea that sequences outside the ectodomain can influence the G protein subunit interaction. This hypothesis is supported by work on the influenza virus HA protein in which the transmembrane and cytoplasmic sequences were removed by bromelain treatment. The resulting water-soluble ectodomain fragment is still a trimer; however, these trimers dissociate easily compared to the intact protein (Doms & Helenius, 1986).

We expected Triton X-100 to increase the rate of G protein trimer dissociation on the basis of sedimentation velocity data (Doms et al., 1987; Lyles et al., 1990) and the lack of resonance energy transfer upon mixing of fluorescein- and rhodamine-labeled G protein trimers in the presence of Triton X-100 (Lyles et al., 1990; Table I). However, our present experiments demonstrated the opposite result. When the labeled G proteins were mixed first in octyl glucoside and then diluted in the presence of Triton X-100, resonance energy transfer within these mixed trimers persisted, which indicated that the trimers did not dissociate (Table I). Thus the lack of resonance energy transfer when labeled G proteins were diluted in the presence of Triton X-100 prior to mixing resulted from the lack of subunit exchange between trimers in Triton X-100 and not due to dissociation of trimers to monomers. The dissociation of G protein trimers to monomers in Triton X-100 as shown by sedimentation experiments appears to be due to irreversible changes in the G protein structure which prevent reassociation. Doms et al. (1987) have shown that when monomers isolated by centrifugation at pH 7.4 are then centrifuged at pH 5.8, they still sediment as monomers. Further evidence supporting the existence of an irreversible change in G protein monomers has been obtained by dilution of labeled G protein to concentrations in the range of the trimer dissociation constant (4–12 nM) to induce trimer dissociation (Lyles et al., 1992). When the G protein concentration was raised at various times after dilution, the dissociation was found to be reversible at early time points and increasingly irreversible at longer times (greater than 30 min). Thus Triton X-100 may provide short-term stabilization of G protein trimers by slowing the reversible dissociation; however, irreversible changes in monomers may occur more readily in the presence of Triton X-100 than octyl glucoside.

The effects of changing pH further emphasize the fact that G protein trimer dissociation is a complex mixture between reversible and irreversible processes. G protein undergoes a conformational change at pH values below 6 that is responsible for promoting fusion of the virus envelope with cellular membranes during virus penetration by endocytosis (Bailey et al., 1984; Doms et al., 1987). Treatment of the G protein at pH 5.8 was expected to decrease dissociation of the trimers on the basis of sedimentation experiments; however, this was not observed. The trimers freely exchanged subunits at low pH in the presence of octyl glucoside, which indicates that the low pH did not enhance trimer stability sufficiently to prevent subunit exchange (Table I). However, as in the case of the higher pH, no exchange of subunits was observed when the trimers were treated at pH 5.8 in the presence of Triton X-100. The enhanced stability of the trimer in Triton X-100 at pH 5.8 versus pH 7.4, which has been observed by sedimentation velocity analysis (Doms et al., 1987; Lyles et al., 1990), may be attributed to the prevention of the irreversible changes in G protein monomers at the acid pH. G protein trimers that were treated at pH 5.8 in octyl glucoside exhibited similar

dissociation and subunit exchange kinetics when returned to neutral pH as untreated trimers (Figure 2). This supports the observation by Doms et al. (1987), that the G protein is unique among acid-activated viral glycoproteins in that the conformational changes induced by acid treatment are reversible.

The results presented here provide insight into the mechanisms by which detergent solubilization influences subunit interactions of membrane proteins. Structural differences exist in the membrane-associated sequences when G protein is in detergent micelles versus membrane bilayers. Furthermore, differing micellar environments of Triton X-100 and octyl glucoside have differential effects on the rate of subunit dissociation. The driving forces that determine these differences probably lie in how well the detergent or lipid molecules fit the hydrophobic surfaces of the protein and how readily these contacts can be rearranged upon transition from G protein trimer to monomer. The geometry of packing of fatty acyl chains in lipid bilayers differs markedly from that of hydrophobic constituents in detergent micelles. Adaptation of phospholipids to the surfaces of the G protein may be more readily achieved in a different protein conformation from that preferred in a detergent micelle. Likewise, the differences in the chemical nature of both the hydrophobic and hydrophilic constituents of the detergents may lead to a more favorable packing of Triton molecules around the trimer compared to octyl glucoside (i.e., stabilization of the ground state in the trimer dissociation reaction). Alternatively, there may be a greater energy barrier associated with the rearrangement of Triton molecules than octyl glucoside in a detergent–G protein micelle upon transition from trimer to monomer. In either case, the underlying mechanism probably involves the ability of the detergents to adapt to the irregular surfaces of the membrane-associated regions of the G protein.

REFERENCES

Bailey, C. A., Miller, D. K., & Lenard, J. (1984) *Virology 133*, 111–118.

Cartwright, G. S., Smith, L. M., Heinzelmann, E. W., Ruebush, M. J., Parce, J. W., & McConnell, H. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1506–1510.

Crise, B., Ruusala, A., Zagouras, P., Shaw, A., & Rose, J. K. (1989) *J. Virol. 63*, 5328–5333.

Doms, R. W., & Helenius, A. (1986) *J. Virol. 60*, 833–839.

Doms, R. W., Keller, D. S., Helenius, A., & Balch, W. E. (1987) *J. Cell. Biol. 105*, 1957–1969.

Doms, R. W., Ruusala, A., Machamer, C., Helenius, J., Helenius, A., & Rose, J. K. (1988) *J. Cell Biol. 107*, 89–99.

Dubovi, E. J., & Wagner, R. R. (1977) *J. Virol. 22*, 500–509.

Hurtley, S. M., & Helenius, A. (1989) *Annu. Rev. Cell Biol. 5*, 277–307.

Kreis, T. E., & Lodish, H. F. (1986) *Cell 46*, 929–937.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lyles, D. S., McKinnon, K. P., & Parce, J. W. (1985) *Biochemistry 24*, 8121–8128.

Lyles, D. S., Varela, V. A., & Parce, J. W. (1990) *Biochemistry 29*, 2442–2449.

Lyles, D. S., McKenzie, M. O., & Parce, J. W. (1992) *J. Virol. 66*, 349–358.

Rose, J. K., & Gallione, C. G. (1981) *J. Virol. 39*, 519–528.

Stephens, E. B., & Compans, R. W. (1988) *Annu. Rev. Microbiol. 42*, 489–516.

Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197–203.

Zagouras, P., Ruusala, A., & Rose, J. K. (1990) *J. Virol. 65*, 1976–1984.

Registry No. DMPC, 13699-48-4; Triton X-100, 9002-93-1; octyl glucoside, 6801-93-0.