Membrane fusion is the key step in the entry of enveloped animal viruses into their host cells. Fusion of vesicular stomatitis virus with membranes occurs at acidic pH and is mediated by its envelope glycoprotein, the G protein. To study the structural transitions induced by acidic pH on G protein, we have extracted the protein from purified virus by incubation with nonionic detergent. At pH 6.0, purified G protein was able to mediate fusion of either phospholipid vesicles or Vero cells in culture. Intrinsic fluorescence studies revealed that changes in the environment of Trp residues occurred as pH decreases. In the absence of lipidic membranes, acidification led to G protein aggregation, whereas protein-protein interactions were substituted by protein-lipid interactions in the presence of liposomes. Bis(4-aniline-5-naphthalene sulfonate) (bis-ANS) binding was utilized to probe the degree of exposure of hydrophobic regions of G protein during acidification. Bis-ANS binding was maximal at pH 6.2, suggesting that a hydrophobic segment is exposed to the medium at this pH. At pH 6.0, a dramatic decrease in bis-ANS binding was observed, probably due to loss of tridimensional structure during the conformational re-arrangement. This hypothesis was confirmed by circular dichroism analysis at different pH values, which showed a great decrease in α-helix content at pH values close to 6.0, suggesting that a reorganization of G protein secondary structure occurs during the fusion reaction. Our results indicate that G protein undergoes dramatic structural changes at acidic pH and acquires a conformational state able to interact with the target membrane.

Viral infection depends on the transfer of viral genetic material into the host cell. After binding to its cellular receptor, the virus must cross the plasma membrane and release its genome into the cellular milieu for subsequent replication. Enveloped viruses always gain entry to the cytoplasm by membrane fusion (reviewed in the Refs. 1–3), whereas nonenveloped viruses must use alternative strategies to cross the membrane. The membrane of some enveloped viruses, such as paramyxoviruses, retroviruses, or herpesviruses, fuses directly with the host cell plasma membrane after virus binding to their cell receptor. Other enveloped viruses, such as influenza, alphaviruses, or rhabdoviruses, enter the cells by the endocytic pathway, and fusion depends on the acidification of the endosomal compartment. In both cases, membrane fusion is mediated by viral envelope glycoproteins, which have already been identified for most enveloped animal viruses (reviewed in Refs. 3 and 4).

The best studied low pH-activated viral fusion protein is the influenza hemagglutinin (HA). This is the only fusion protein for which atomic resolution structure has been obtained in neutral (pre-fusogenic) and in low pH (5). The conformational changes observed suggest that the apolar fusion peptide moves to the tip of the molecule and is delivered toward the target membrane. The low pH-induced conformational changes that enable membrane fusion in Semliki Forest virus (SFV) are also dramatic. Time-resolved cryoelectron microscope studies showed that low pH treatment resulted in movement of E1 subunits to the center of the spike, which initiates the formation of E1 trimer. Subsequently, the fusion sequence is extended toward the target membrane (6).

Vesicular stomatitis virus (VSV) is a member of the Rhabdoviridae family, a group of enveloped single strand RNA viruses. Both VSV binding to the cell surface and fusion between viral envelope and endosomal membrane are mediated by its trimeric surface type I glycoprotein, the G protein. This protein of 67 kDa is anchored to viral and cellular membranes via a single transmembrane anchor sequence close to the C-terminus (7). Unlike other viral fusion proteins, VSV G protein does not contain an obvious hydrophobic peptide sequence that can serve as the fusion peptide. Mutation analysis revealed that an ectodomain segment localized between the amino acids 117 and 136 is essential for fusion (8, 9). This segment is highly conserved among the vesiculoviruses and is believed to contain VSV G protein internal fusion peptide. Another region of G protein, encompassing residues 395–418, has been identified as a segment that affects fusogenic activity of the protein by influencing the low pH-induced conformational changes (10). In addition, it was recently shown that not only the ectodomain segment but also the membrane anchoring domain is required for VSV fusion activity (11, 12).

Although much progress has been made on the identification and characterization of VSV G protein segments involved in membrane fusion activity using site-directed mutagenesis, information on the structural changes that occur during acidification are still lacking. In this work we investigated the con-
formational changes in G protein during acidification. Intrinsic fluorescence analysis showed that, when the pH was decreased, the environment of the G protein Trp residues changed, allowing interaction of these residues with the target membranes. Binding of a fluorescent probe revealed that the exposure of hydrophobic domains was maximal at pH 6.2. Between pH 6.0 and 5.6 a dramatic conformational change occurred, which includes loss of secondary and tertiary structures.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Virus Propagation**—VSV Indiana was propagated in monolayer cultures of baby hamster kidney-21 cells. The cells were grown at 37 °C in roller bottles containing 150 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil), 100 μg/ml ampicillin, 5 μg/ml gentamicin. When the cells reached confluence, the medium was removed, and the cell monolayer was infected with VSV at a multiplicity of 5 plaque-forming units/ml. The cultures were kept at 37 °C for 16–20 h, and the viruses were harvested and purified by differential centrifugation followed by equilibrium sedimentation in a sucrose gradient as described elsewhere (13). Purified virions were stored at −70 °C.

**G Protein Purification**—G protein extraction from purified native VSV was adapted from Newcomb and Brown (14). Purified suspensions of VSV in 10 mM Tris-HCl (pH 7.4) at a final protein concentration of 1 mg/ml were brought to a concentration of 30 mM CHAPS (Sigma) by the addition of an equal volume of 60 mM CHAPS in Tris-HCl buffer. The suspension was allowed to stand at room temperature for 1 h. The insoluble structures called “skeletons” (nucleocapsids associated with M protein) were pelleted by ultracentrifugation through a 1-ml glycerol cushion at 43,000 rpm for 90 min at 4 °C in a Beckman 50 Ti rotor. The supernatant was collected and dialyzed against Tris-HCl buffer. For circular dichroism analysis, G protein was extracted with 30 mM β-octyl glucoside.

**Preparation of Liposomes**—Equimolar amounts of PC and PS were dissolved in chloroform and evaporated under nitrogen. The lipid film formed was resuspended in 20 mM MES, 30 mM Tris buffer (pH 7.5) at a final concentration of 1 mM. The suspension was vortexed vigorously for 5 min. Small unilamellar vesicles were formed by sonicating the turbid suspension using a Branson Sonifier (Sonic Power Company, Danbury, CT) equipped with a titanium microtip probe. Sonication was performed in an ice bath, alternating cycles of 30 s at 20% full power, with 60-resting intervals until a transparent solution was obtained (~10 cycles).

**Liposome Fusion Assay**—A suspension containing PCPS liposomes in a final concentration of 0.1 mM phospholipids was incubated at room temperature at pH 7.5 or 6.0. The reaction was initiated by addition of purified VSV or G protein, and fusion was monitored by the increase in light scattering.

**Cell Fusion Assay**—Cell-to-cell fusion was assayed as described in White et al. (15), using 5 μl of purified VSV (protein concentration of 100 μg/ml) or 10 μl of G protein (protein concentration of 300 μg/ml).

**Fluorescence and Light Scattering Measurement**—Intrinsic fluorescence, binding of 1,1’-bis(4-aniline-5-naphthalene sulfonate) (bis-ANS) (Molecular Probes, Eugene, OR), and light scattering measurements were recorded using a Hitachi F-4500 Fluorescence Spectrophotometer. Intrinsic fluorescence was measured exciting samples at 280 nm and collecting emission between 300 and 420 nm. Bis-ANS binding was determined by the increase in fluorescence emission between 400 and 600 nm upon excitation at 390 nm. Light scattering was measured at 90° in the spectrophotometer by selecting the same wavelength for both excitation and emission (280 nm).

**Circular Dichroism**—Circular dichroism studies were performed with an Jasco model J-715-1505 spectropolarimeter. Samples were prepared in phosphate buffer 20 mM, pH 7.4, at a concentration of 10 μl. Spectra were recorded using a 0.2 nm bandwidth, a 0.2 nm step in quartz cells of 0.1 cm, and a time constant of 8 s. A total of 10 scans was averaged.

**RESULTS**

**G Protein Purification**

G protein was extracted from purified VSV by treatment with the nonionic detergent CHAPS. This detergent does not interfere in fluorescence measurements at the UV range, allowing G protein structural studies using Trp fluorescence. After VSV incubation with 30 mM CHAPS for 1 h, the protein could be completely removed from the virus, and a purified sample was obtained after ultracentrifugation, as shown by SDS-polyacrylamide gel electrophoresis (Fig. 1).

**Purified G Protein Maintains Its Biological Activity**

Two different methods were utilized to test the ability of purified G protein to mediate membrane fusion. First, we developed a spectroscopic method to follow the kinetics of liposome fusion induced by the protein. Fusion of vesicles containing equimolar amounts of PC and PS (PCPS liposomes) was quantified by the increase of the light scattering of the suspension after protein addition (Fig. 2A). Light scattering directly correlates with the size of particles in solution (16) and has already been used to quantify protein aggregation (17, 18). At pH 7.5, no increase in light scattering was observed either in the absence or in the presence of G protein. Although a small increase in light scattering occurred when PCPS liposomes were incubated at pH 6.0 without the protein, a much greater increase in vesicle size was observed in the presence of purified G protein. After ~30 min of incubation, fusion was completed. This result suggests that, at low pH, isolated G protein promotes vesicle fusion. An experiment performed with the whole virus led to similar results, except that fusion occurred more rapidly (Fig. 2B). Because cell-to-cell fusion has been extensively used for the demonstration of fusion activity in several virus families (15, 19), we used cell fusion assay to confirm the ability of purified G protein to mediate membrane fusion (Fig. 3). This assay has already been used to analyze low pH fusion activity of whole VSV (15) or G protein expressed on the plasmatic membrane of transfected cells (8). Formation of large polykarios was observed when either purified G protein or whole virus was incubated at pH 5.8 with monolayers of Vero cell cultures (Fig. 3, B and C).

**pH Induced Conformational Changes**

**Changes in Trp Environment**—Intrinsic fluorescence spectrum of purified G protein showed a maximal emission in 334 nm. Fig. 4A shows that G protein intrinsic fluorescence emission decreased as pH was diminished, indicating that changes in Trp residues environment occurred during acidification. This fluorescence quenching was not a direct effect of pH decrease on Trp residues, since no significant changes in N-acetyl-l-tryptophanamide (NATA) fluorescence was observed at this pH range (Fig. 4A). G protein fluorescence spectrum is more blue-shifted than NATA spectrum, suggesting that Trp residues are not completely exposed to the aqueous medium.
However, no red shift in the intrinsic fluorescence spectrum was observed when the pH was lowered, indicating that Trp exposure to the polar solvent did not occur. Conversely, G protein intrinsic fluorescence greatly increases upon acidification in the presence of PCPS vesicles. Trp fluorescence quantum yield reaches a maximal value at pH 6.2, suggesting that the interaction with the quencher was substituted by interaction with phospholipid molecules in the membranes (Fig. 4C).

Aggregation—Acidification promotes G protein aggregation, as observed by light scattering measurements (Fig. 5). Aggregation and the conformational changes probed by Trp fluorescence were not simultaneous processes. The greater changes in intrinsic fluorescence occurred between pH 7.5 and 6.4, whereas significant increase in light scattering initiates at pH 6.5, where quenching phenomenon is almost completed. G protein aggregation probably occurred due to exposure of hydrophobic regions, which results in intermolecule interactions.

Exposure of Hydrophobic Domains—Exposure of hydrophobic segments of G protein at acidic pH was probed by measuring binding of bis-ANS. This fluorescent compound binds noncovalently to nonpolar segments in proteins, especially in proximity to positive charges (20). Bis-ANS binding is accompanied by a large increase in its fluorescence quantum yield, and it has been used to probe protein structural changes (21, 22). Purified G protein was incubated with bis-ANS at pH 7.5, and spectra were obtained as the solution was acidified (Fig. 6). Bis-ANS binding increased when the pH was lowered from 7.5 to 6.2, suggesting the exposure of hydrophobic segments. No change in bis-ANS fluorescence maximum was observed as pH decreased. Intriguingly, a great decrease in bis-ANS binding occurred at pH 6.0. Bis-ANS binding requires not only the presence of nonpolar amino acids but a hydrophobic environment. Denatured proteins or very nonorganized structures do not bind this probe (21, 23). The pH threshold for membrane fusion mediated by VSV is close to 8.0 (15), and the fusogenic activity of expressed wild-type G protein initiates at pH 6.3, reaching the maximum activity between pH 6.0 and 5.6 (8, 9). Conceivably, the major conformational changes in G protein occur at this pH range. Titration of bis-ANS binding to purified G protein was performed at different pH values (Fig. 7A). Fluorescence emission of bis-ANS increased gradually as the probe was added to the protein sample (final protein concentration of 0.1 μM). Binding was saturated at 0.8 μM bis-ANS, suggesting the presence of ~8 binding sites. When the pH decreased from 7.5 to 6.2, less bis-ANS was necessary for maximal emission increase, confirming that at mildly acidic pH values hydrophobic regions became more exposed to the solvent (Fig. 7B). Our results suggest that G protein undergoes a dramatic conformational change, which involves loss of the hydrophobic domain structure before its reorganization in the

**Fig. 2.** G protein-mediated liposome fusion. A, fusion of liposomes induced by purified G protein or B, whole VSV. Small unilamellar vesicles composed by equimolar amounts of phosphatidylcholine and phosphatidylserine in a final concentration of 0.1 mM were incubated in the presence (● and ▲) or in the absence (○ and △) of purified G protein or whole VSV at pH 6.0 (● and ○) or 7.5 (▲ and △). Fusion was determined by measuring the increase in light scattering in the spectrofluorometer. Final protein concentration was 30 μg/ml for G protein and 100 μg/ml for VSV.

**Fig. 3.** pH dependence of G protein-induced cell fusion. Vero cells were washed twice with the binding medium (pH 6.8) and then incubated with 10 μl of medium (A), G protein (0.3 mg/ml) (B), or purified VSV (0.1 mg/ml) (C) for 1 h in ice-cold medium. Fresh medium (pH 5.8) was added for 60 s, and the cells were postincubated with medium (pH 7.2) for 1 h at 37 °C. After this time, the cells were observed under the light microscope and photographed. Observation of polykaryons (arrows) when Vero cells was exposed to G protein or whole VSV at low pH indicates that the isolated purified G protein maintained its ability to promote membrane fusion.
fusion-inducing conformation. To confirm that the conformational changes observed for purified G protein also occur when it is integrated in virus envelope, we performed titration of bis-ANS binding with purified VSV (Fig. 7, C and D). Accordingly, the results were similar to those obtained for purified protein, except by the absence of a defined plateau at higher concentrations of the probe, probably due to nonspecific binding of bis-ANS to viral membrane.

Changes in the Secondary Structure—To examine the alterations in G protein secondary structure during acidification, we analyzed circular dichroism spectra of the protein at different pH values (Fig. 8). In these experiments, the protein was extracted using β-octyl glucoside, to avoid CHAPS interference in the circular dichroism spectra. The secondary structure content decreased as pH was lowered from 7.5 to 6.4 (Fig. 8B). Further acidification led to an increase in the secondary structure content, but protein probably acquires a different conformation, as seen by the different spectra at pH 7.5 and 5.6. These results confirm that a G protein undergoes structure reorganization during the fusion reaction.

FIG. 4. Effects of pH in G protein intrinsic fluorescence. A, G protein was diluted in 30 mM MES, 10 mM Tris-HCl (pH 7.5), to a final concentration of 30 μg/ml. Tryptophan fluorescence emission at 334 nm (●) was recorded, whereas pH was gradually acidified by addition of HCl. As a control, fluorescence spectra of NATA (▲) were also recorded upon acidification. NATA fluorescence emission was collected at 350 nm. The excitation wavelength was 280 nm in both cases. B, fluorescence spectra of G protein (● and ○) and NATA (▲ and △) at pH 7.5 (● and ▲) and 5.4 (○ and △). The excitation wavelength was 280 nm. C, interaction of G protein with liposomes. G protein was diluted to a final concentration of 30 μg/ml in 30 mM MES, 10 mM Tris-HCl (pH 7.5), in the presence of 0.1 mM PCPS vesicles. Tryptophan fluorescence emission at 334 nm was recorded, and pH was gradually acidified by addition of HCl. The excitation wavelength was 280 nm.

FIG. 5. Effects of pH on G protein aggregation. Aggregation was measured by the increase in light scattering, and pH was gradually acidified by addition of HCl. G protein was diluted in 30 mM MES, 10 mM Tris-HCl (pH 7.5), to a final concentration of 30 μg/ml. The dotted line corresponds to the intrinsic fluorescence quenching at the same pH range.

FIG. 6. Binding of bis-ANS to G protein. A G protein was diluted in 30 mM MES, 10 mM Tris-HCl (pH 7.5), to a final concentration of 6 μg/ml, and incubated with bis-ANS (final concentration of 1.0 μM). Fluorescence emission at 485 nm was recorded when pH was gradually acidified by addition of HCl. The excitation wavelength was 360 nm.

DISCUSSION

G protein mediates membrane fusion at the acidic environment of the endosomal compartment after virus entry into the cell by endocytosis. In this work we described conformational changes of purified VSV G protein during acidification, using light scattering and intrinsic fluorescence measurements, binding of the fluorescent probe bis-ANS, and circular dichroism.

To measure the kinetics of G protein-induced fusion, we developed an assay based on the increase in the light scattering of a suspension containing small unilamellar vesicles in the presence of the protein. The kinetics of whole VSV-induced fusion measured by the light scattering assay presented a half-time of 2.5 min, similar to the 2.0-min half-time measured in vitro by fluorescence energy transfer, using either G protein reconstituted into phospholipid vesicles (24) or whole VSV (25). The kinetics of in situ fusion of VSV and the endosomal membranes during infection has also been determined, showing a half-time of ~25 min (26). In this case, the longer half-time of fusion might be due to the gradual acidification of the endosomal compartment. The light scattering assay revealed that the kinetics of fusion mediated by purified G protein was slower than VSV fusion (Fig. 2). Fusion between the PCPS liposomes...
mediated by G protein at pH 6.0 presented a half-time of 10 min and was virtually completed within 25 min. This result may be explained by the fact that G protein in the intact virus is clustered, therefore increasing the cooperativity of protein membrane-interaction, since ectodomain insertion into the target membrane has been shown to be reversible (27).

In the absence of membranes, intrinsic fluorescence was quenched as the pH decreased, although no red shift in the fluorescence spectrum was observed (Fig. 4, A and B). This result suggests that the pH-induced conformational change probably involves approximation between the Trp and a His or an Arg residue, rather than exposure of the Trp to the aqueous medium. Alternatively, the absence of red shift might be a consequence of simultaneous increase and decrease of different Trp residues exposure to the solvent, since G protein presents 13 Trp residues (7).

Light scattering results showed that G protein aggregated between pH 6.6 and 5.6. Acid-induced aggregation has already been observed for rabies virus glycoprotein (28). In both cases the aggregation could be explained by exposure of hydrophobic domains at mildly acidic conditions. Indeed, we have demonstrated that hydrophobic domains were gradually exposed as pH decreased from 7.5 to 6.2 (Figs. 6 and 7). Although an obvious hydrophobic fusion peptide was not identified in rhabdovirus glycoproteins, the increase in bis-ANS binding is a direct evidence of the exposure of a hydrophobic domain.

The exposed hydrophobic domain is probably involved in the interaction of G protein and the target membrane. G protein Trp fluorescence greatly increased upon acidification when the protein was incubated in the presence of membranes (Fig. 4C). The increase in fluorescence quantum yield indicated that Trp residues moved from the proximity of a quencher to interact with the membrane environment. The segment between amino acids 117 and 136, which is believed to interact with the target membrane during acidification, contains one Trp residue (Trp 119). The increase in intrinsic fluorescence might be explained by the insertion of this residue into the liposome as pH decreased. NMR and fluorescence studies with a synthetic peptide corresponding to the sequence between amino acids 118 and 136 of G protein indicated that Trp-119 penetrates into PS-containing model membrane (29). These experiments were done at pH 7.4, showing that the fragment is able to interact with the membrane even at neutral pH. The pH dependence of Trp-membrane interaction observed here suggests that the segment is hidden in the three-dimensional structure of the protein and becomes exposed during acidification. Indeed, Durrer et al. (30) showed that interaction of a segment in the ectodomain containing the amino acids 59–221 with membranes increased when the pH was lowered from 7.0 to 6.0.

An even more interesting observation was the dramatic conformational rearrangement at pH 6.0. At this pH, a great decrease in bis-ANS binding occurred, indicating loss of the hydrophobic domain structure. In addition, secondary structure was also lost at this pH, as shown by circular dichroism results. The conformational changes that occur when influenza virus HA and SFV spike glycoprotein are exposed to low pH are equally dramatic (5, 6). A comparison of the three-dimensional structure of the soluble trimers of HA and the trimeric fragment obtained by acid treatment and proteolysis of the trimers revealed a great secondary structure rearrangement resulting in the molecule of the fusion peptide toward the tip of the molecule. In the case of SFV, the spike oligomer was completely reorganized. The stable heterodimers formed by $E_1$ and $E_2$ glycoprotein dissociates to form an $E_3$ homotrimer (31, 32). The pH thresholds of membrane fusion mediated by several enveloped virus are sharp (15, 28). VSV fusion has a threshold between pH 6.2 and 5.8 (15), exactly the pH range where structural reorganization was observed by intrinsic bis-ANS fluorescence and circular dichroism.

Although at lower pH values, the protein recovered the ability to bind bis-ANS and acquire secondary structure, it is likely that structural rearrangements result in a different conformation, as indicated by the circular dichroism spectra obtained at pH 7.4 and 5.6 (Fig. 8A). Taken together, the results presented herein suggest that G protein undergoes extensive confor-

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**Fig. 7. Titration of bis-ANS binding.** Binding of bis-ANS to G protein (A and B) or whole VSV (C and D) was measured. A and C, fluorescence intensity at 485 nm was collected upon addition of increasing concentrations of the probe at pH 7.5 ( ), 6.2 ( ), 6.0 ( ), and 5.6 ( ). B and D, binding of bis-ANS (in a final concentration of 0.8 μM) to G protein or whole VSV at different pH values. The protein final concentration was 6 μg/ml for G protein and 18 μg/ml for whole VSV.

**Fig. 8. Effect of pH on the secondary structure of G protein.** Circular dichroism of G protein. G protein was diluted in 20 mM phosphate buffer (pH 7.5). pH was gradually acidified by addition of HCl. A, CD spectra at pH values of 7.5 ( ), 6.2 ( ), 6.0 ( ), and 5.6 ( ). B, effect of pH in the α-helix content, expressed by the inverse of differential absorption at 222 nm. Final protein concentration was 300 μg/ml.
tional changes that lead to the formation of a hydrophobic domain that might play the role of the fusion peptide.

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