Folding, Unfolding, and Refolding of the Vesicular Stomatitis Virus Glycoprotein†

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Received December 12, 1995; Revised Manuscript Received February 2, 1996⊗

ABSTRACT: Folding and refolding of the vesicular stomatitis virus (VSV) glycoprotein (G protein), New Jersey serotype, were studied both in infected cells and after urea denaturation and reduction of isolated protein *in vitro*. To assess the contribution of disulfide bonds to the conformation of this type I membrane glycoprotein, reduced and alkylated forms were compared with unreduced G proteins by their mobility on SDS−polyacrylamide gels and by their reactivity with conformation-dependent monoclonal antibodies (MAbs). Pulse−chase experiments showed that G protein folding in the endoplasmic reticulum (ER) of infected cells occurred rapidly (estimated half-time of 1−2 min) and involved transient association with the ER chaperone calnexin. Inhibition of glycosylation by tunicamycin slowed the folding process and emergence from the ER but did not prevent the appearance of a conformationally mature transport-competent G protein. For *in vitro* refolding studies, native G protein isolated from virus particles was denatured and reduced with urea and β-mercaptoethanol. When rapidly diluted into a denaturant-free buffer containing oxidized glutathione and the nonionic detergent octyl glucoside, the G protein regained considerable native structure, as determined by reactivity with five monoclonal antibodies specific for different conformation-dependent epitopes. Whereas the refolding process was slow and inefficient *in vitro* relative to folding in the cell, this observation nonetheless demonstrated that an integral fully glycosylated membrane protein can be refolded to form a structure similar to that of the original protein processed during *in vivo* synthesis. If, however, unfolded nonglycosylated G protein was the starting material, refolding *in vitro* failed. In summary, we have shown that VSV G protein folding can be analyzed both *in vivo* and *in vitro* and that folding in the cell involves at least one chaperone and can occur *in vivo* even if not glycosylated.

Many of the known principles of protein folding have been deduced from biochemical and/or biophysical characterization of polypeptide refolding *in vitro* [reviewed by Creighton (1990), Jaenicke (1991), and Kim (1990)]. These investigations have largely considered small soluble proteins, with few studies of integral membrane proteins which are insoluble and, therefore, more difficult to study. However, some progress has been made in the analysis of intracellular folding of membrane glycoproteins. It has been shown, for example, that folding of the lumenal ectodomain of type I and II glycoproteins in the endoplasmic reticulum (ER)[1] is mechanistically similar to that of soluble protein folding. The nascent forms of these proteins interact with cellular chaperones and folding enzymes and acquire intrachain disulfide bonds. Proper folding in the cell also generally requires the addition of glycans to appropriate asparagine residues, a favorable redox environment, and the presence of a physiological cellular ATP concentration [recently reviewed by Gething and Sambrook (1992)].

The glycoprotein (G protein) of the vesicular stomatitis virus (VSV), New Jersey serotype, is a 64 kDa type I membrane glycoprotein with two N-linked oligosaccharides and six disulfide bonds in the ectodomain (Gallione & Rose, 1983). In the mature virion, the VSV-New Jersey G protein is likely present as a noncovalent homotrimer, like the G proteins of other rhabdoviruses such as VSV-Indiana and the rabies virus. The general features of VSV-Indiana G protein folding during synthesis and processing in the ER have recently been described (Doms *et al*., 1987, 1988; de Silva *et al*., 1990, 1993; Machamer *et al*., 1990). Direct extrapolation to the VSV-New Jersey G protein may not be appropriate despite 60% amino acid homology (Gallione & Rose, 1983), since the proteins have notable structural and biological differences. For example, there is evidence suggesting that N-linked glycosylation is necessary for the maturation of the VSV-Indiana but not the VSV-New Jersey G protein (Machamer *et al*., 1990; Grigera *et al*., 1991), antigenic domains are diffusely distributed on the VSV-Indiana G protein but largely clustered within a narrow region of the VSV-New Jersey G protein (Keil & Wagner, 1989), and the VSV-Indiana but not the VSV-New Jersey G protein contains an additional cysteine residue which is palmitylated in the Golgi apparatus (Pal *et al*., 1987). Since glycosylation may be less critical for maturation of the VSV-New Jersey G protein and since the molecule likely consists of a major centrally clustered antigenic domain with a minor amino-

† This research was supported by Grants R37 AI-11112, GM38346, and CA46128 from the National Institutes of Health and by Grant 3158 from the Council for Tobacco Research. M.E.M. was supported partly by a grant from the Robert Wood Johnson Foundation under the Minority Medical Faculty Development Program.

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⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1996.

[1] Abbreviations: ER, endoplasmic reticulum; VSV, vesicular stomatitis virus; G protein, glycoprotein; G_s, soluble glycoprotein; F, folded glycoprotein; IT, folding intermediate glycoprotein; G_r, reduced glycoprotein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; β-ME, β-mercaptoethanol; MAb, monoclonal antibody; EDTA, ethylenediaminetetraacetic acid.

0006-2960/96/0435-4084$12.00/0

terminal component (Grigera *et al*., 1992), folding may be under less rigorous control than that for the VSV-Indiana G protein. The results reported in this study support such a hypothesis.

In this paper, we have analyzed folding of newly synthesized VSV-New Jersey G protein in host cells; we have also developed an approach for studying *in vitro* refolding after reduction and denaturation of the isolated G protein. We found that the VSV-New Jersey G protein folds faster intracellularly than does its VSV-Indiana counterpart. Fewer incompletely oxidized intermediates were detected, and deglycosylation slowed the rate of *in vivo* folding and exit from the ER but did not prevent the eventual accumulation of antigenically authentic molecules. The folding process was found to be much slower *in vitro* but nevertheless resulted in a final conformation that was antigenically similar to that of the native protein monomers isolated from virions.

MATERIALS AND METHODS

Virus and Cells. VSV-New Jersey (Ogden strain) was grown in BHK-21 cell monolayers in Dulbecco's minimal essential medium (DMEM) supplemented with fetal calf serum and 2% tryptose−phosphate broth. Virus was released into and harvested from the supernatant medium and purified as previously described (Luo *et al*., 1988). Purified virions were stored at −70 °C in 0.5 M NaCl, 10 mM TRIS-HCl (pH 7.5), and 1 mM EDTA. Nonglycosylated virions were prepared as above except that tunicamycin was added to the culture medium to a final concentration of 3 μg/mL (Grigera *et al*., 1991). For production of [^{35}S]methionine-labeled virus, the media overlay following adsorption of the virus consisted of methionine-free DMEM supplemented with 50 μCi/mL [^{35}S]methionine (New England Nuclear). CHO-15B cells were used for studying the *in vivo* processing of the G protein.

Pulse-Labeling of Infected Cells. For *in vivo* folding studies, Chinese hamster ovary cells, clone 15B (CHO-15B), which lack the Golgi enzyme *N*-acetylglucosamine transferase, were infected with VSV at a multiplicity of infection of 20−50 pfu/cell in RPMI media, pH 6.8, supplemented with 0.1% bovine serum albumin. At 5−6 h post-infection, cells were incubated at 37 °C in methionine-free media for 30 min. For studies of nonglycosylated G protein, tunicamycin was added to the culture media to a final concentration of 5 μg/mL 45 min before the pulse and maintained at this concentration subsequently. The cells were then pulse-labeled with 100 μCi of [^{35}S]methionine for 3 min and either chased in DMEM, pH 7.4, supplemented with excess cold methionine and 1 mM cycloheximide, or immediately prepared for lysis. The chase (or pulse) was terminated by the addition of ice-cold PBS with 20 mM *N*-ethylmaleimide. Cells were then lysed in 0.5 mL of ice-cold 1% Triton X-100, 20 mM MES, 30 mM TRIS (pH 8.6), 100 mM NaCl, 1.0 mM phenylmethanesulfonyl fluoride (PMSF), and 10 mg/mL each of chymostatin, leupeptin, antipain, and pepstatin. Nuclei were removed by centrifugation in an ultrafuge at 12 000 rpm for 5 min. Either the lysates were quick-frozen in liquid nitrogen, or 0.1−0.2 mL aliquots were immediately subjected to immunoprecipitation with polyclonal or monoclonal antibody coupled to protein A−Sepharose beads (Braakman *et al*., 1991). Preparation of cell lysates for reaction with anti-calnexin antibody utilized the nonionic detergent CHAPS rather than Triton X-100 (Ou *et al*., 1993). The precipitated proteins were liberated from antibody/protein A−Sepharose complexes by boiling for 3 min in 20 μL of sample buffer containing 3% SDS, 0.2 M TRIS (pH 6.8), 10% glycerol, and 1 mM EDTA and were then subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), often in the absence of reducing agents.

Preparation of Glycoprotein. G protein was extracted from VSV-New Jersey virions essentially as previously described (Keil & Wagner, 1989). Briefly, desalted virions in 10 mM TRIS-HCl (pH 7.5) were diluted into 60 mM octyl glucoside (Calbiochem) and 10 mM TRIS-HCl (pH 7.5) at a ratio of 1:1 and a final protein concentration of 1 mg/mL. The suspension was allowed to stand at room temperature for 1 h. Nucleocapsids were removed by centrifugation at 38 000 rpm for 90 min in a Beckman SW 50.1 rotor, and the supernatant was placed on a 15−30% sucrose gradient containing 0.5 M NaCl, 60 mM octyl glucoside, and 10 mM TRIS (pH 7.4) and subjected to centrifugation at 55 000 rpm in a Beckman SW 60 rotor for approximately 16 h. Gradients were manually fractionated into 20 fractions; absorbance was measured at 280 nm, and the initial portion of the protein peak was retained as previously described. The pooled fractions were dialyzed once against 4 L of deionized distilled water and then concentrated by ultrafiltration (10 kDa molecular mass cut-off Amicon filter in a Model 3 Amicon ultrafiltration cell). The water used for dialysis was not replaced since more complete detergent removal resulted in precipitation of the protein. Aliquots of 0.1 mg were quick-frozen, lyophilized, and stored at −70 °C. The same procedure was applied to radiolabeled protein, except that aliquots of 40 μg were stored.

For Western blot analysis, SDS−PAGE separated proteins were electroeluted onto nitrocellulose filters, 0.2 or 0.45 μm pore-size (Schleicher & Schuell, Keene, NH). Nitrocellulose sheets with electroblotted protein were first incubated at 37 °C in 3% bovine serum albumin, 0.15 M NaCl, and 10 mM TRIS-HCl (pH 7.4) for 1 h. After being rinsed, sheets were incubated at room temperature for 1 h in 150 mM NaCl, 5 mM EDTA, 50 mM TRIS-HCl (pH 7.4), 0.25% gelatin, and 0.05% Nonidet P-40 containing a monoclonal antibody at the appropriate dilution. ^{125}I-Protein A was diluted 1:1000 and allowed to react with the nitrocellulose-bound immune complexes and then subjected to autoradiography. For protein dot-blots, antigen was applied directly to nitrocellulose sheets in a BRL (Gaithersburg, MD) Hybridot apparatus under suction. Following brief air-drying, the same protocol as described for electroeluted proteins was followed.

Partial Reduction of Purified G Protein. Lyophilized glycoprotein was resuspended at 1 μg/mL in deionized sterile water. Dithiothreitol (DTT) was added to give the desired concentration of reducing agent in a 50 mM TRIS-HCl buffer (pH 8.0). After a 1 h incubation at 37 °C, free thiols were alkylated by the addition of 0.5 M iodoacetic acid to a final volume of 25%, allowing a 10−15 min incubation at room temperature. The alkylated proteins were then subjected to SDS−PAGE or dot-blot analysis.

Refolding of Reduced Denatured Protein. Unfolded reduced glycoprotein for refolding studies was prepared by resuspending an aliquot of lyophilized protein in 50 mM TRIS-HCl (pH 8.8), 8 M urea, 50 mM β-mercaptoethanol (β-ME), and 80 mM octyl glucoside at a concentration of 1

mg of protein/mL and incubating at 37 °C for 1−2 h. Attainment of complete reduction and unfolding was supported by two lines of evidence: (1) trichloroacetic acid-precipitable counts of [^{14}C]iodoacetate−protein were similar in SDS−PAGE migration to those of protein heat-denatured and reduced excess DTT; and (2) circular dichroism (CD) spectroscopy of alkylated reduced denatured protein showed a nearly total random coil structure (data not shown). An aliquot was removed from the reduced denatured protein at the end of the incubation period and reacted with iodoacetic acid; this sample was designated time 0. To initiate refolding, protein was diluted 100-fold at room temperature with 50 mM TRIS-HCl at pH 8.0, 1 mM EDTA (to inhibit copper-catalyzed air oxidation), 80 mM octyl glucoside, and 0.2 mM oxidized glutathione. Refolding was monitored kinetically by trapping thiol intermediates with iodoacetate alkylation at various time points.

Samples removed from the refolding reaction were blotted on premoistened nitrocellulose sheets under suction as described above and probed with monoclonal antibodies. [^{35}S]Methionine-labeled protein was also alkylated and subjected to nonreducing SDS−PAGE. After electrophoresis, gels were fixed in 50% methanol and 7.5% acetic acid for at least 30 min and then treated with the fluorographic solution Resolution (New England Nuclear), in accordance with the manufacturer's instructions. The gels with precipitated fluorophore were dried and subjected to fluorography with Kodak XRP-1 film at −70 °C.

Monoclonal Antibodies. The eight monoclonal antibodies (MAbs) used in these studies were previously found to react with eight nonoverlapping antigenic determinants (epitopes) on the VSV-New Jersey G protein (Bricker *et al*., 1987). The location of each of these epitopes on the G protein was mapped by their reactivity with deletion mutants and chimeras of the VSV-New Jersey G protein (Keil & Wagner, 1989). MAbs to four epitopes (V, VI, VII, VIII) neutralize the infectivity of VSV-New Jersey; these four epitopes are discontinuous and are abolished by disulfide bond reduction of G protein, as are the nonneutrilization epitopes IV and IX; epitopes I, II, and III are linear and react with their specific MAbs after reduction of G protein (Bricker *et al*., 1987; Keil & Wagner, 1989). The monoclonal antibodies used in these studies were MAb 2 (epitope I), MAb 8 (epitope III), MAb 9 (epitope IV), MAb 11 (epitope V), MAb 13 (epitope VII), MAb 15 (epitope VIII), and MAb 20 (epitope IX). These monoclonal antibodies were used for immunoprecipitation of [^{35}S]methionine-labeled G protein synthesized in VSV-New Jersey infected cells and for their capacity to bind various forms of G protein following SDS−PAGE and electroblotting or dot-blotting onto nitrocellulose paper. These MAbs were recognized by reactivity with ^{125}I-labeled staphylococcal protein A complexed with Sepharose beads.

RESULTS

SDS−PAGE Migration and Western Blots To Detect Conformational Forms of Progressively Reduced and Alkylated G Protein. A previously prepared panel of monoclonal antibodies (MAbs) against nine distinct epitopes (I−IX; Bricker *et al*., 1987) was used to follow the folding and refolding of the VSV-New Jersey G protein. The major discontinuous G protein epitopes reactive with MAbs directed to epitopes IV−IX are dependent on disulfide bond conformation (Grigera *et al*., 1992). Our preliminary observations suggested that prior reduction of VSV-New Jersey G protein significantly slowed its migration by SDS−PAGE in the absence of reducing agents. To examine this effect in greater detail, we exposed purified G protein in solutions to increasing concentrations of DTT. Half of the reduced protein at each concentration was alkylated with iodoacetic acid; each sample was then divided three ways and subjected to electrophoresis on three separate 12.5% SDS−polyacrylamide slab gels free of additional reducing agents. One gel was stained with Coomassie brilliant blue, and the two remaining gels were electroeluted onto nitrocellulose sheets for Western blot analysis.

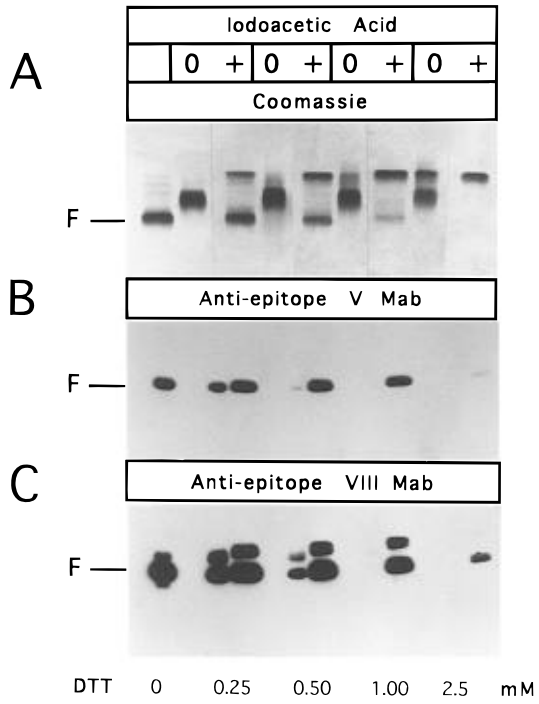

FIGURE 1: Effect of progressive reduction by increasing concentrations of dithiothreitol (DTT) on SDS−PAGE migration and MAb recognition of VSV-New Jersey G protein alkylated or not alkylated with iodoacetic acid. Isolated G protein exposed to the concentrations of dithiothreitol (DTT) shown was divided into two samples, one of which was exposed to iodoacetic acid and the other to buffer alone. These samples were simultaneously subjected to electrophoresis under nonreducing conditions on three parallel SDS−PAGE slab gels, along with a marker virion G protein designated F (for fully folded). After electrophoresis, one slab gel was stained with Coomassie brilliant blue (panel A). The two other slab gels were electroeluted onto nitrocellulose sheets, exposed to monoclonal antibodies directed to epitope V (panel B) or to epitope VIII (panel C) before probing with ^{125}I-labeled protein A−Sepharose and autoradiography.

Figure 1 compares the SDS−PAGE migration of G protein exposed to increasing concentrations of DTT, alkylated or not with iodoacetic acid, and stained with Coomassie blue or immunoblotted with MAbs specific for epitopes V or VIII, which gave the most characteristic results. The Coomassie-stained gel (Figure 1A) shows that DTT induced the appearance of G protein bands with electrophoretic mobility increasingly slower than that of the nonreduced control. Slower SDS−PAGE mobility is typical for reduced, and therefore less compact, proteins (Braakman *et al*., 1991). The amount of G protein in the slower moving band increased with increasing concentration of DTT. When excess

iodoacetic acid was used to alkylate the protein after DTT treatment, preventing reoxidation of native disulfide bonds or their rearrangement, the alkylated bands showed even slower mobility. Alkylation evidently prevented reoxidation of free cysteine thiols and/or disulfide rearrangement of the partially reduced protein. The heterogeneous mobility of unalkylated and alkylated G protein exposed to lower concentrations of DTT suggests that certain disulfide bonds are more readily reduced and less readily reoxidized than others.

Western blot analysis revealed that conformational epitope V was very susceptible to inactivation even by low concentrations of DTT, particularly in the absence of iodoacetic acid (Figure 1B). Quite striking was the failure of MAb to recognize epitope V in the slowly migrating DTT-reduced forms of the G protein, even after alkylation. At a high concentration of DTT, epitope V had essentially disappeared from the G protein even after exposure to iodoacetic acid. Conformational epitope VIII, by contrast, was much more tolerant to G-protein reduction, and the slower-migrating, partially-reduced forms were readily recognized by the epitope VIII-specific monoclonal antibody (Figure 1C).

Taken together, these results demonstrate that the isolated G protein is sensitive to reduction *in vitro* as shown by SDS−PAGE, but conversion to more slowly migrating forms is dependent on DTT concentration and prevention of reoxidation by alkylation of free cysteine thiols. The conformational epitopes vary in their sensitivity to reduction presumably based on their dependence on different disulfide bonds varying in their sensitivity to reduction. Monoclonal antibody binding and SDS−PAGE were thus established as potential methods for assaying the conformational structure of folded and unfolded G proteins related to disulfide bonds.

It was also of interest to determine the degree of susceptibility to reduction of virion membrane-inserted G protein compared to that of the isolated G protein. Although present as a trimer in the membrane of VSV-Indiana (and presumably in VSV-New Jersey), the G protein is known to exist as monomers in equilibrium with dimers and trimers when isolated in detergent solution (Lyles *et al*., 1990). Trimerization of VSV-Indiana G protein in infected cells coincides with a significant increase in resistance to reduction by exogenously added DTT, suggesting that trimerization results in significantly more resistance to reduction (Tatu *et al*., 1993).

To test the reducibility of G protein in its native state as a virion membrane-inserted trimer, suspensions of intact VSV-New Jersey virions were exposed to increasing concentrations of DTT and alkylated with iodoacetate, and the extracted virion proteins were subjected to SDS−PAGE and stained with Coomassie blue. As shown in Figure 2 by relative SDS−PAGE migration, the concentrations of DTT required for reduction of G protein in intact VSV-New Jersey virions were at least 10−20-fold higher than that required for reduction of the detergent-isolated purified G protein. The virion G protein was only slightly affected at 5.0 mM DTT, requiring 50 mM to induce full reduction. Western blotting also confirmed higher resistance of representative conformational epitopes to inactivation by DTT reduction (data not shown). These results indicated that the changes induced in the virion G protein during solubilization and purification included increased susceptibility to reduction by DTT.

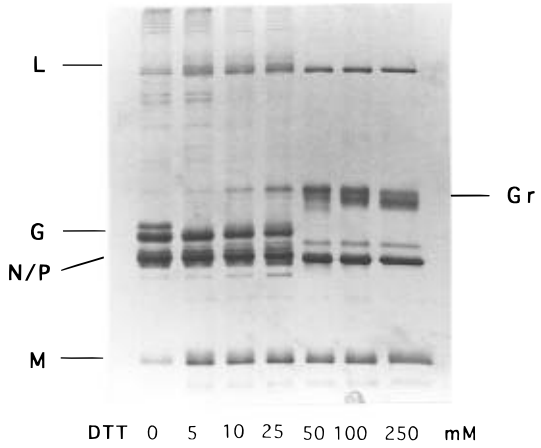

FIGURE 2: Comparative electrophoretic mobility of G protein in intact VSV-New Jersey virions exposed to increasing concentrations of dithiothreitol (DTT). Purified virions were incubated in the concentrations of DTT shown, alkylated with iodoacetic acid, disrupted in Laemmli buffer with SDS, subjected to SDS−PAGE under nonreducing conditions, and stained with Coomassie brilliant blue. Letters to show the positions of proteins extracted from unreduced virions (DTT = 0) are designated L for large (polymerase), G for glycoprotein (ordinarily a split band), N/P for the nucleoprotein and phosphoprotein (not separated in this gel system), and M for matrix protein. G_r indicates the position of reduced G protein.

G Protein Folding in the Infected Cell. To monitor the folding of nascent G protein during synthesis in infected cells, we employed a pulse−chase experimental approach previously described for influenza virus HA and the G protein of VSV-Indiana (Braakman *et al*., 1991; de Silva *et al*., 1993). CHO-15B cells infected with VSV-New Jersey for 5 h were pulse-labeled for 3 min with [^{35}S]methionine and chased for various times in the presence of excess cold methionine and cycloheximide to inhibit additional G protein synthesis. Prior to cell lysis, free thiols were alkylated by exposing the infected cell monolayers to exogenous *N*-ethylmaleimide (NEM). Cell membranes were solubilized in Triton X-100, and the postnuclear supernatants were immunoprecipitated with polyclonal or monoclonal anti-G protein antibodies and subjected to SDS−PAGE under nonreducing and reducing conditions.

Figure 3 shows the electrophoretic migration under nonreducing or reducing conditions of intracellular G protein pulse-labeled with [^{35}S]methionine and chased for varying times before cell lysis and immunoprecipitation with polyclonal antibody or MAb directed to linear epitope I or MAbs directed to nonlinear epitopes V and VIII. As noted, disulfide-coupled folding of the G protein was found to be largely completed by the end of the pulse since most of the labeled unreduced G protein recognized by polyclonal antibody was already present in a band with the same mobility as the fully folded oxidized ER form of the protein (designated F in Figure 3A). After a chase interval of 5 min, this band was gradually replaced by a slightly faster migrating band (Figure 3A, lanes 3−5) corresponding to the mature oligosaccharide-trimmed Golgi form of G protein. A single incompletely folded intermediate (IT), with slower mobility than F, was detected transiently as a retarded band during the initial portion of the chase (Figure 3A, lanes 1 and 2). That this slower band represented an incompletely folded intermediate was shown in the reduced gels where all the G protein comigrated in a single band with a mobility

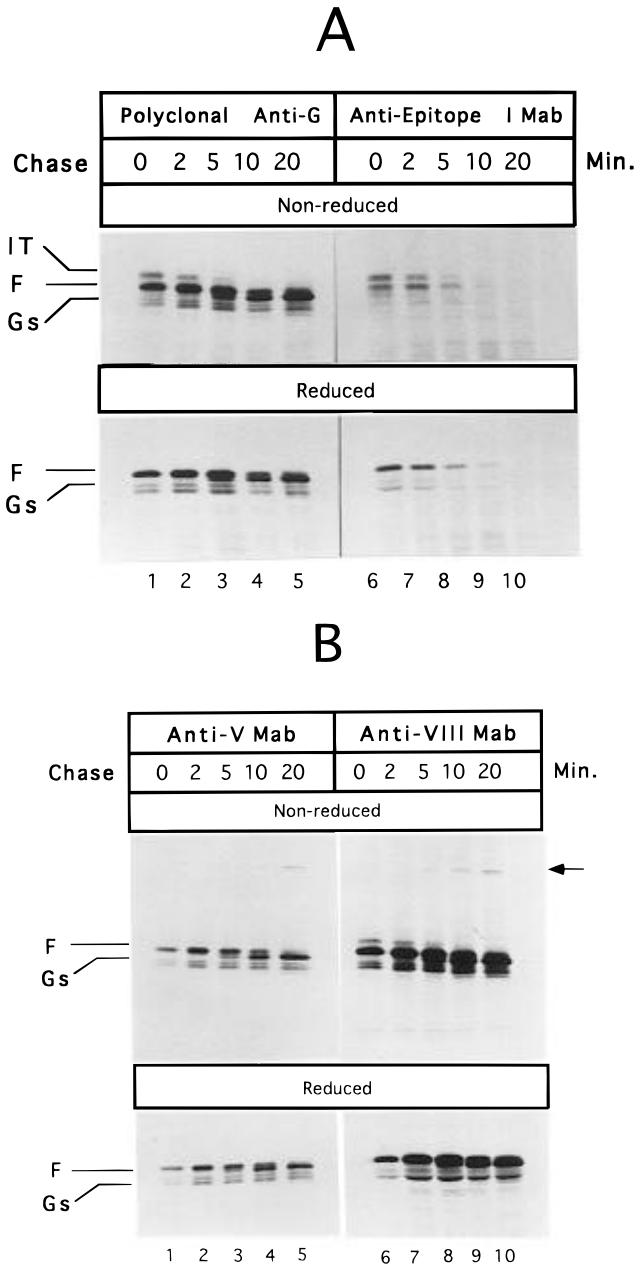

FIGURE 3: [^{35}S]Methionine pulse−chase experiments to demonstrate the forms of G protein during synthesis in cells infected with VSV-New Jersey and analyzed by SDS−PAGE after cytosol immunoprecipitation with polyclonal and monoclonal antibody directed to epitope I (anti-epitope I MAb) of the G protein (panel A) or anti-epitope V MAb or anti-epitope VIII MAb (panel B). CHO-15B cells infected for 6 h with VSV-New Jersey were pulse-labeled for 3 min with [^{32}S]methionine and chased in cold methionine for various periods of time up to 20 min, as indicated (see Materials and Methods for details). Pulsed (0 time) and chased cell aliquots were lysed and immunoprecipitated with polyclonal antibody or MAbs directed to epitope V, or epitope VIII coupled to protein A−Sepharose beads. The immunoprecipitated [^{35}S]methionine-labeled proteins were subjected to SDS−PAGE under nonreducing or reducing (in the presence of β-mercaptoethanol) conditions, followed by autoradiography. The intracellular forms of the G protein are designated IT for the putative folding intermediate, F for the fully folded form in the ER, and G_s for the processed soluble G protein devoid of the carboxyl-terminal anchor.

slower than that of either F or IT. Irrespective of the reduction status of the sample, a pair of faster-migrating bands (designated G_s) below F or G_r was also consistently observed, corresponding to anchor-free cleavage or prematurely terminated products of the G protein (Grünberg *et al*., 1991; Chen *et al*., 1987). The half-time for folding to the F form was estimated to be 1−2 min, which was slightly faster than that described for both VSV-Indiana G protein (1−3 min) (de Silva *et al*., 1993; Doms *et al*., 1987; Machamer *et al*., 1990) and the hemagglutinin of influenza A X31 (3 min) (Braakman *et al*., 1991).

Since the *in vitro* observations shown in Figure 1 suggested that the anti-epitope VIII and V monoclonal antibodies might be useful for discriminating disulfide-linked structure, we tested these same antibodies and a representative MAb directed to a linear (nonconformational) antigenic determinant (epitope I) for their ability to immunoprecipitate G protein folding intermediates from the same pulse−chased cell lysates. Anti-epitope I MAb, which has been shown to recognize both reduced and nonreduced forms of the G protein (Keil & Wagner, 1989), precipitated IT and F but not the Golgi-trimmed form or G_s (Figure 3A, lanes 6, 7). IT was, in fact, precipitated as well or better than F after the initial pulse, precipitation being generally more efficient during the chase than immediately after the pulse. Most striking was the complete disappearance of anti-epitope I precipitable G protein after a 5−10 min chase and the consistent failure to recover trimmed forms, which would have signified arrival in the Golgi. Epitope I was therefore exposed only on early pre-Golgi forms of the G protein. It is important to recognize that F does not necessarily represent a homogeneous population of G protein species; differences such as the presence or absence of disulfide-stabilized loops or trimers versus monomers are likely within the F species.

In contrast to epitope I, the monoclonal antibody directed to epitope V precipitated preferentially only late forms of processed G protein; anti-epitope V MAb recognized F and G_s forms only (Figure 3B, lanes 1−5). Finally, MAbs directed to epitope VIII recognized IT, F, and G_s, yielding results similar to those obtained with the polyclonal antiserum (Figure 3B, lanes 6−10). The failure of MAbs directed to epitope V to recognize the early pulsed intermediate form of G protein and its greater affinity for the more highly processed fully glycosylated F form indicates that full expression of epitope V requires a more highly structured conformational form of the G protein than does expression of epitope VIII.

G Protein Folding in Cells Deficient in N-Linked Glycosylation. Tunicamycin blocks N-linked glycosylation in the ER (Elbein, 1991) and causes misfolding and aggregation of the VSV-Indiana G protein (Machamer & Rose, 1988). VSV-New Jersey infected cells are, however, known to produce transport-competent G protein and virions in the presence of this inhibitor (Grigera *et al*., 1991). To analyze the *in vivo* folding of nonglycosylated VSV-New Jersey G protein, CHO-15B cells were analyzed using the [^{35}S]-methionine pulse−chase protocol. Tunicamycin was added to the media of infected cell cultures approximately 45 min prior to the pulse and remained there throughout the experiment; matched control cultures were not exposed to tunicamycin. Immediately after the [^{35}S]methionine pulse or at interval after the chase, the infected cells were lysed, and the labeled G protein was immunoprecipitated with polyclonal anti-G antibody or monoclonal antibodies directed to epitopes I or V prior to analysis by SDS−PAGE and autoradiography as described under Materials and Methods.

Figure 4 shows the SDS−PAGE profiles of immunoprecipitated pulsed−chased G proteins synthesized in VSV-New

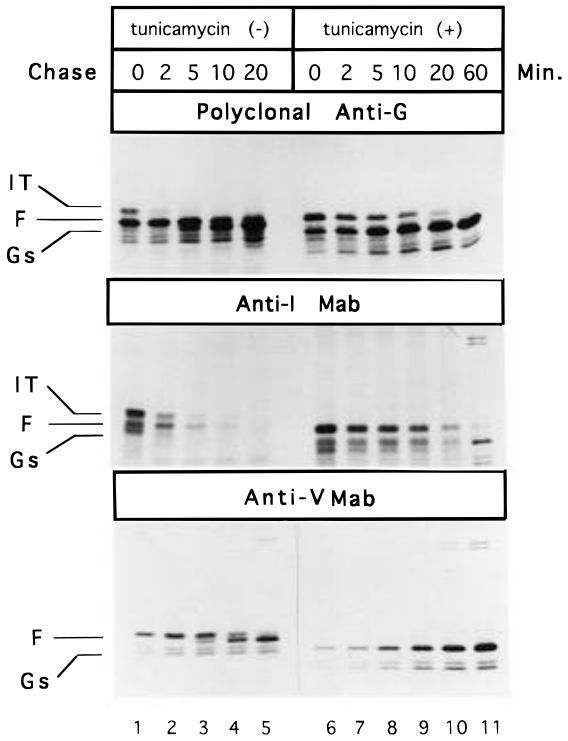

FIGURE 4: Pulse−chase experiments comparing the intracellular immunoprecipitated nascent forms of G protein in VSV-New Jersey infected cells in the presence (+) or absence (−) of tunicamycin. CHO-15B cells were infected and pulse-labeled with [^{35}S]methionine in a manner identical to that described in the legend for Figure 3 except that tunicamycin (5 μg/mL) was added to half the culture medium 45 min before the pulse. The control tunicamycin (−) cells were chased in cold methionine for intervals up to 20 min, whereas the tunicamycin (+) cells were chased for intervals up to 60 min. At the end of the pulse (time 0) or chase, cell lysates were exposed to polyclonal antibody, or to monoclonal antibodies directed to nonconformational linear epitope I (anti-I MAb) or conformational discontinuous epitope V (anti-V MAb). ^{35}S-Labeled immunoprecipitated forms of G protein were subjected to SDS−PAGE under nonreducing conditions followed by autoradiography. The G-protein forms are designated IT for the putative folding intermediate, F for the fully folded ER form, and G_s for the soluble G protein devoid of its carboxyl-terminal anchor. Note the faster migration of the nonglycosylated G protein from cells treated with tunicamycin.

Jersey infected cells in the presence or absence of tunicamycin. The immunoprecipitated intermediates (IT) obtained using the polyclonal anti-G protein antibody showed that the folding of unglycosylated G was considerably slower than that for the glycosylated species, in which $t_{1/2}$ was <2 min for the formation of F (Figure 4, polyclonal anti-G). The folding intermediate IT persisted beyond a 10-min chase. When the anti-epitope I MAb, which precipitated only early folding forms of the glycosylated G protein, was used, a significant signal from F was still apparent following a 60-min chase, confirming either delayed or incomplete folding and probable ER retention of the unglycosylated G protein (Figure 4, anti-I MAb). As already discussed, this antibody recognizes an early subpopulation of F, which persisted much longer in the unglycosylated protein than in its glycosylated counterpart.

In further tests on the effects of glycosylation on folding and conformation of VSV-New Jersey G protein, we compared by pulse−chase experiments the precipitability of glycosylated and unglycosylated G protein by MAb directed to epitope V, since it was found to be specific for more mature forms of G protein. Results shown in Figure 4, anti-V MAb, lanes 1−5, confirmed that only F and subsequent forms were recognized in control cells not exposed to tunicamycin. Processing in the Golgi began after approximately 5 min (lane 3) and was completed between 10 and 20 min (lanes 4 and 5). The results of this experiment reveal that defective glycosylation causes a clear delay in the appearance of conformation-dependent epitope V ordinarily present during the first few minutes of G protein folding into the F form. The result confirms that the F band contains a heterogeneous population of G protein, and that the unglycosylated protein folds more slowly than does the glycosylated form.

Taken together, these observations show that the lack of glycosylation delayed but did not abolish folding of the VSV-New Jersey G protein. Interestingly, although a species that resembled the fully folded form by electrophoretic criteria appeared just after the pulse, this form did not contain epitope V.

Chaperoning of VSV-New Jersey G Protein by Calnexin. Calnexin is an ER integral membrane protein with lectin-like properties and plays a possible chaperone role in the folding of a variety of glycosylated proteins (Helenius, 1994). The VSV-Indiana G protein has been shown to bind to calnexin transiently during folding (Hammond *et al*., 1994). We tested whether the VSV-New Jersey G protein, which does not require glycosylation for proper folding, would also bind to calnexin during folding. This was tested by an experiment in which [^{35}S]methionine pulse-labeled VSV-New Jersey infected CHO-15B cells, chased in cold methionine for varying times, were lysed and their cytoplasmic contents incubated with an anti-calnexin polyclonal antibody; the immunoprecipitated material was analyzed by SDS−PAGE and autoradiography. The results were similar to what has been described for the VSV-Indiana G protein. VSV-New Jersey ^{35}S-G protein in association with calnexin was evident by coprecipitation immediately after a 3-min pulse and peaked at approximately 5 min of chase time; thereafter, the concentration of calnexin-coprecipitated G protein declined and was not detectable at the 20-min chase (data not shown). The folding intermediate IT and the soluble G_s protein forms were detectable by anti-calnexin antibody coprecipitation but only to a limited extent. VSV-New Jersey G protein synthesized in tunicamycin-treated cells showed barely detectable binding to calnexin (data not shown). Despite differences in the requirement for glycosylation between the VSV-Indiana and VSV-New Jersey G proteins, transient association with calnexin of both G proteins was similar.

In Vitro Refolding of Isolated G Protein. Anfinsen and colleagues demonstrated over 30 years ago that reduced and denatured ribonuclease, a disulfide-containing protein, can be refolded to an enzymatically-active form by removal of the denaturant and most of the reducing agent. The presence of a low concentration of β-mercaptoethanol promoted the formation of native disulfide bonds, presumably by limiting the formation of unnatural disulfide bonds with closely accessible cysteines (Anfinsen, 1973). To refold the G protein, we followed a similar approach except with the inclusion of limited octyl glucoside to help maintain the G protein in a soluble form.

As described in detail under Materials and Methods, lyophilized ^{35}S-labeled G protein preparations were resuspended in 8 M urea and 50 mM β-mercaptoethanol.

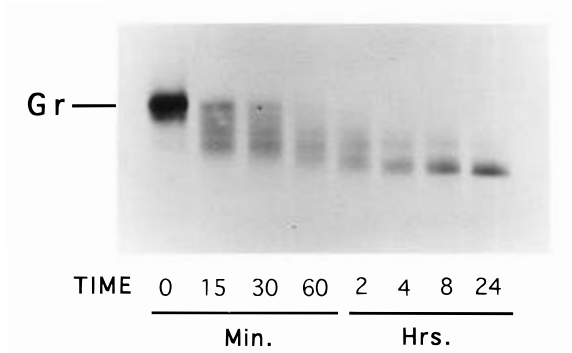

FIGURE 5: Increasing electrophoretic mobility during refolding of denatured and reduced G protein. [^{35}S]Methionine-labeled G protein extracted from VSV-New Jersey virions was lyophilized, and then denatured and reduced by resuspension and incubation in a buffered solution containing 8 M urea, 50 mM β-mercaptoethanol, and 80 mM octyl glucoside, as described under Materials and Methods. Completely unfolded G protein was rapidly diluted into 100-fold greater refolding solution containing 0.2 mM oxidized glutathione and 80 mM octyl glucoside and incubated at room temperature. At the intervals shown, samples were withdrawn, free thiols alkylated with iodoacetic acid, and then subjected to SDS−PAGE under nonreducing conditions followed by autoradiography. The marker G_r indicates the location of fully reduced G protein.

Refolding was initiated by rapid 1:100 dilution of the unfolded protein into a mildly oxidizing glutathione-containing buffered solution containing 80 mM octyl glucoside. Aliquots were withdrawn at 15 min, 30 min, 60 min, 2 h, 4 h, 8 h, and 24 h and incubated with excess iodoacetic acid to block free cysteine thiols and then subjected to SDS−PAGE and fluorography.

Figure 5 demonstrates the appearance of several intermediates soon after initiating refolding and the gradual formation of a single faster-migrating species equivalent to the F form observed in infected cells (see Figure 3). Although apparently successful in terms of recovery of a predominant more-rapidly migrating species, the *in vitro* folding process displayed differences in comparison with folding of G protein in infected cells. First, the rate of folding *in vitro* was extremely slow compared to that in cells. Second, the incompletely folded intermediates observed in the *in vitro* refolding experiment were far more numerous than those in infected cells. In addition, diffuse higher molecular weight species were seen between the stacking gel and the separation gel (not shown). These aggregates presumably represented misfolded and possibly interchain disulfide-bonded complexes, thus accounting for most of the apparent loss in the radioactive signal observed in soluble G protein during the refolding process.

We next compared the rate at which six different epitopes reappeared during refolding *in vitro* of glycosylated and unglycosylated reduced G proteins. In these, studies we examined the conformational characteristics of the refolding and refolded G proteins by testing their reactivity with monoclonal antibodies in an immunodot-blot analysis. Unfolded G protein in various stages of refolding was alkylated and applied to nitrocellulose filters under suction for Western dot-blotting and autoradiography. The samples blotted were neither reduced nor denatured with SDS or heat. Monoclonal antibodies directed to six different epitopes were tested, some of which were not used in the preceding experiments but were previously described in detail (Keil & Wagner, 1989). Epitope III is linear (conformation-independent), maps between amino acids 299 and 317, and

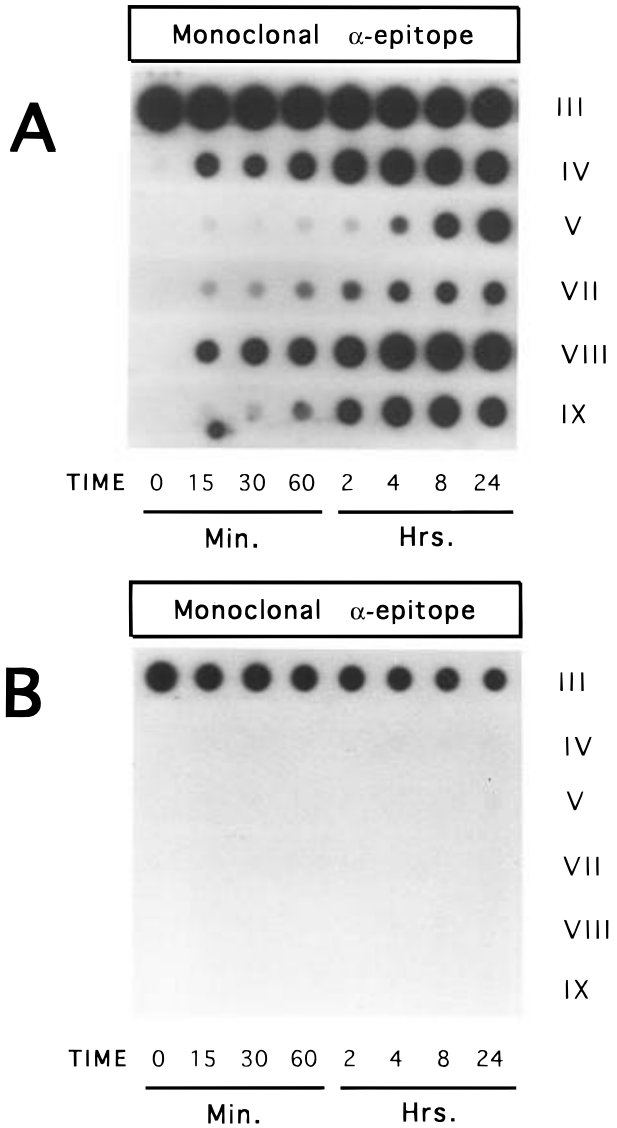

FIGURE 6: Immunodot-blot analysis during increasing refolding of denatured reduced (A) fully glycosylated and (B) unglycosylated G protein to compare their reactivity with six monoclonal antibodies that recognize unique epitopes III, IV, V, VII, VIII, and IX. As detailed under Materials and Methods and the legend for Figure 5, unlabeled G protein isolated from VSV-New Jersey virions grown in the absence or presence of tunicamycin was denatured and reduced with 8 M urea and 50 mM β-mercaptoethanol and then renatured in a refolding solution containing oxidized glutathione and octyl glucoside. Aliquots removed at the time indicated were alkylated with iodoacetic acid and blotted onto nitrocellulose sheets. Each row of dot-blotted protein was exposed to one of six epitope-specific monoclonal antibodies and ^{125}I-labeled protein followed by autoradiography.

reacts with its MAb when G protein is completely reduced, like epitope I (maps to amino acids 193−267). Epitope IV is located between amino acids 80 and 183 and is disulfide bond-dependent, but its MAb is not capable of neutralizing viral infectivity (Grigera *et al.*, 1992). Epitopes V, VII, and VIII map distal to amino acid 214 and are also conformation-dependent, but their respective monoclonal antibodies strongly neutralize infectivity of the virus (Grigera *et al.*, 1992). Epitope IX is rather diffusely distributed, is conformation-dependent, and reacts with MAbs that vary in their capacity to neutralize viral infectivity (Keil & Wagner, 1989).

Figure 6 compares the capacity of six epitope-specific MAbs to bind immobilized reduced glycosylated and non-

glycosylated G proteins progressively undergoing renaturation. The results show that all six epitopes were present at 24 h in the final refolded stage of the glycosylated G protein (Figure 6A). Only the linear epitope III could be recognized by its MAb prior to exposure in the reoxidation solution of the unfolded G protein. Each of the other five discontinuous antigenic determinants reappeared progressively with characteristic kinetics beginning with epitopes IV and VIII, which reacted strongly with their respective MAbs within 15 min of initiating the refolding process. Epitope V was the last to appear, beginning approximately 4 h after dilution into the oxidizing solution. The kinetics of reappearance of antigenic determinants during reoxidation appeared to be intermediate for epitopes VII and IX.

As shown in Figure 6B, we also tested whether a reduced G protein preparation devoid of N-linked oligosaccharides would refold *in vitro*. Nonglycosylated G protein was isolated from viruses obtained from tunicamycin-treated infected cell cultures as previously described (Grigera *et al.*, 1991). When such reduced G protein was subjected to refolding by dilution into oxidized glutathione, the conformation-dependent epitopes IV, V, VII, VIII, and IX failed to recognize specific MAbs even after reoxidation for 24 h. Only the linear conformation-independent epitope III reacted with its MAb even in the fully reduced state. We conclude that glycosylation is critical for refolding *in vitro* of G protein to a functional configuration.

Taken together with the changes in gel mobility, the complete loss after reduction and regeneration after oxidation of conformational epitopes confirm the presence of considerable secondary and tertiary structure in the VSV-New Jersey G protein. The regeneration of epitope V, characteristic of mature late forms of G protein in infected cells, was particularly supportive of this conclusion. The results indicated that the VSV-New Jersey G protein can be refolded *in vitro* from the reduced denatured state to a form similar to that found inserted in the plasma membrane of the infected cell and in the budded mature virion. Clearly, the most conformation-dependent antigenic determinant is epitope V, the least epitopes IV and VIII, and intermediate in conformation dependence are epitopes VII and IX.

DISCUSSION

This study represents the first direct comparison between the folding of a membrane glycoprotein *in vivo* and its refolding *in vitro*. Such an approach may be helpful for evaluating the biological relevance of many *in vitro* observations on which most of our information about protein folding is based. The protein used in this study, the VSV-New Jersey G protein, is a complex homotrimeric protein with most of its mass in the ectodomain. This domain folds in the lumen of the ER, which is also the site for trimeric assembly. While in many respects ideal for *in vivo* folding studies due to its high cellular expression level, extensive disulfide bonding, and efficient folding, the VSV G protein is not well suited for *in vitro* refolding studies. Not only is it large and oligomeric, but it is also an integral membrane protein and therefore inherently insoluble in aqueous solution without detergent. We were encouraged, however, by an earlier report (Petri & Wagner, 1979) which showed that VSV G protein micelles could partition into lipid bilayers to form lipoprotein vesicles.

For *in vitro* refolding studies reported here, we used a modification of the classical approach of Anfinsen (1973) adapted so that it would accommodate an integral membrane protein by inclusion in the refolding mixture of 80 mM octyl glucoside, a mild nonionic detergent with a relatively small micelle size. By binding to the hydrophobic domain, the surfactant was expected to keep the protein in solution during and after folding. However, the octyl glucoside may have had additional favorable effects due to the known fact that nonionic detergents increase the refolding efficiency of soluble proteins such as mitochondrial rhodanese by preventing the aggregation of folding intermediates which are often much less water-soluble than the mature folded proteins (Tandon *et al*., 1986). Detergents thus have an effect similar to that provided in the living cell by chaperones that help the proteins stay in solution during folding by inhibiting inappropriate hydrophobic side chain interactions (Mendoza *et al.*, 1991).

A shift from slower to faster electrophoretic mobility of the G protein was observed both in folding during synthesis in cells and in *in vitro* refolding. In cells, oxidation was quite rapid, with more than half of the labeled full-length protein migrating with a mobility equivalent to that of the native, fully oxidized G protein at the end of the 3 min pulse. While this did not necessarily mean that all disulfides were in place at this time, it did suggest that the disulfides responsible for much of the increased mobility formed rapidly. The fact that the only incompletely oxidized folding intermediate (IT) observed migrated well ahead of fully reduced G protein indicated, moreover, that some disulfides were already formed on the nascent chain. A similar conclusion has been reached for influenza HA, which also begins to fold and acquire disulfides during synthesis (Braakman *et al*., 1991; Huth *et al*., 1992). The *in vitro* folding process of reduced G protein was orders of magnitude slower than that for folding in the living cell. The efficiency of refolding was also lower, with 50% or more of the G protein remaining in non-cross-linked or misfolded aggregates. Among the molecules that folded without aggregation, the stepwise folding and oxidation of cysteine thiols occurred through many more detectable intermediates than was observed during folding of G protein in cells. The order of disulfide bond formation and the mechanism by which folding or refolding occurs for the VSV G protein may be quite different *in vivo* and *in vitro*, but, for at least one protein, the β subunit of human chorionic gonadotropin, the same intermediates have been isolated *in vitro* and *in vivo* (Huth *et al*., 1992, 1993).

In the cell, folding and disulfide bond formation are known to be assisted by protein disulfide isomerase and chaperones, the former showing the kinetic properties of a true foldase (Freedman *et al*., 1994). At least two cellular factors have been experimentally shown to interact with the VSV-Indiana G protein *in vivo*, calnexin and BiP/GRP78 (Hammond & Helenius, 1994; Melnick *et al*., 1994). Likewise, a sequential interaction with BiP/GRP78 followed by GRP94 has recently been shown for processing of immunoglobulin chains (Melnick *et al*., 1994). Folding of the influenza A X31 HA is dependent not only on the appropriate redox environment but also on the presence of metabolic energy (Braakman *et al*., 1992a), which also suggests extensive reliance on chaperones.

There is a striking difference between the rate of folding of VSV-New Jersey G protein in the ER lumen of infected cells and the rate of refolding *in vitro*. We speculate that a number of factors may account for the contrasting kinetics. Interaction with BiP in the cell might increase folding efficiency by minimizing unfavorable hydrophobic side-chain interactions in nascent molecules, especially at physiological and supraphysiological temperature (Rothman, 1989). Unfolded G protein in solution as presented here must rely on low protein concentration and detergent solubilization of at least the membrane-spanning region in order to minimize unfavorable hydrophobic interactions. The exceedingly slow rate of G-protein refolding *in vitro* compared to the rapid rate of folding in the cell strongly suggests *in vivo* catalytic events. Like BiP, protein disulfide isomerase (PDI) is a KDEL-bearing ER resident protein. PDI has the critical function of catalyzing thiol−disulfide exchange, including reduction of non-native cysteines *and* oxidation of natively-paired cysteines (Freedman *et al*., 1994). The enhanced *in vitro* refolding efficiency of BPTI when ER lumenal proteins had been added exogenously has been shown to be predominantly due to PDI. One might expect such an effect to represent a general paradigm for disulfide-containing globular proteins, such as the ectodomain of the VSV G protein (Zapun *et al*., 1992). Cotranslational vectoral folding of domains and subdomains during translocation into the ER lumen may, in itself, favor native over non-native side-chain interactions; however, correct cysteine pairing has been shown to be unaffected by delaying oxidation until after completion of translocation of influenza HA protein (Braakman *et al*., 1992b).

Another way of analyzing the conformation of VSV-New Jersey G proteins relies on recognition of exposed antigenic sites by monoclonal antibodies, an approach pioneered by Teale and Benjamin (1976) in their studies on the conformation of serum albumins; this technique has been utilized for analyzing the structure of tryptophan synthase (Blond *et al*., 1987), β-lactoglobulin (Hattori *et al*., 1993), and other proteins (Goldberg, 1991). In living cells, conformation-specific antibodies have found extensive use in the folding studies of influenza HA, MHC class I antigens, sucrase isomaltase, and other glycoproteins (Braakman *et al*., 1991; Degen & Williams, 1991; Matter & Hauri, 1991).

Immunoprecipitation with epitope-specific MAbs of G protein from VSV-infected cell lysates provided some details about the G-protein maturation process in the ER. The incompletely folded intermediate (IT) could be precipitated by MAbs directed to epitopes I and VIII but not by MAb directed to labile epitope V. Further reoxidation resulted in G protein that acquired gel mobility identical to that of the mature fully-folded (F) form which lost epitope I but acquired epitope V. This antigenic conversion probably reflects conformational maturity of the processed G protein resulting in the formation of a homotrimer with consequent burying of epitope I within the oligomer. In this context, it is significant that epitope I, which maps to the cysteine-rich region between amino acids 193and 267 (Keil & Wagner, 1989), is not exposed on the intact virus particle where the G proteins are trimeric but is present in the isolated nonreduced or reduced monomers in Western blot analysis (Bricker *et al*., 1987; Keil & Wagner, 1989). This suggests that the conversion that eliminated epitope I, in fact, involved or coincided with trimer formation. The appearance of epitope V, on the other hand, is likely to reflect an intramolecular disulfide bond formation event because epitope V can be detected by Western blotting and thus does not require trimeric G protein. Most likely, epitope V, which also maps to the cysteine-rich region amino acids 214−267 (Keil & Wagner, 1989), is formed when a large disulfide-bonded loop is created late in the folding pathway, a disulfide bond responsible for the conversion from IT to F and the attendant electrophoretic mobility shift. Grigera *et al*. (1992) described such a loop in the VSV-New Jersey G protein probably anchored by a disulfide bond between cysteine 108 and cysteine 169. Conversely, reduction of the isolated protein sufficient to induce loss of epitope V on Western blot analysis was also associated with a major shift in electrophoretic mobility.

In conjunction with the *in vivo* studies, our results showed that refolding *in vitro* of denatured and reduced G protein resulted in the formation of presumably native disulfide bonds required for displaying the topography of at least five of the conformation-dependent epitopes. Refolding was orders of magnitude slower than *de novo* folding, yet epitopes appeared with distinct kinetics. Epitope V was the last to be formed, as is the case in living cells. A large fraction of the G protein reached a conformation similar to that of mature G protein monomers solubilized from virions as judged by SDS−PAGE mobility criteria. While this solubilized form was similar to the mature G oligomers present in the virus particles, it differed by being more DTT sensitive as might be expected for the unshielded monomer. Solubilization has been shown by others to result in trimer disassembly, and this may be the main reason for the differences observed (Lyle *et al*., 1990).

Like other glycoproteins, viral spike glycoproteins differ from nonglycosylated proteins in their dependence on N-linked glycosylation for proper folding during synthesis and transport competence (Hammond *et al*., 1994). Those with an absolute requirement for glycosylation include the VSV-Indiana glycoprotein (Hammond *et al*., 1994), the D glycoprotein of herpes simplex virus (Sodora *et al*., 1989), the Sendai virus glycoprotein (Vidal *et al*., 1989), the lymphocytic choriomeningitis virus glycoprotein (Wright *et al*., 1989), the hemagglutinin of influenza X31 (Gallagher *et al*., 1992), and the gp120 of human immunodeficiency virus type-1 (Li *et al*., 1993). The VSV-New Jersey G protein differs from its VSV-Indiana counterpart in that proper folding is possible without the addition of the two N-linked glycans (Grigera *et al*., 1991). However, when analyzed in more detail, we observed that both folding and transport of the unglycosylated VSV-New Jersey G protein were delayed. The slower folding was revealed by the prolonged expression of epitope I and the late appearance of epitope V.

In summary, we have shown that the folding of the VSV-New Jersey G protein occurs more rapidly *in vivo* than for other comparable viral spike proteins and with less dependence on glycosylation. *In vitro* refolding was also possible though the process was slow and dependent on the presence of N-linked carbohydrate chains. The characterization of a panel of monoclonal antibodies with respect to disulfide-linked conformation was useful for this analysis. Future work should seek to explain the discrepancy between de novo folding that occurs in the cell and refolding of the isolated

reduced G protein by attempts to mimic the cellular folding environment *in vitro*.

ACKNOWLEDGMENT

We thank Drs. Jay Fox, David Benjamin, Jay Brown, and William Newcomb of the University of Virginia School of Medicine for many helpful discussions as well as members of the Helenius and Mellman laboratories of the Yale University School of Medicine. Ms. Ruth Snyder, Oneida Mason, and Melanie Willets provided essential technical assistance. Excellent photographic service was provided by Mr. Henry Tan.

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BI952924I