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Structural Characteristics of the M2 Protein of Influenza A Viruses: Evidence That It Forms a Tetrameric Channel[1]

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The evidence presented shows that the M2 protein of influenza A viruses exists in infected cells as a homotetramer composed of two disulfide-linked dimers held together by noncovalent interactions. The amphiphilic nature of the transmembrane α-helical domain is consistent with the protein forming a transmembrane channel with which amantadine, the specific anti-influenza A drug, interacts. Together these features provide a structural basis for the hypothesis that M2 has a proton translocation function capable of regulating the pH of vesicles of the trans-Golgi network, a role important in promoting the correct maturation of the hemagglutinin glycoprotein. © 1991 Academic Press, Inc.

INTRODUCTION

The M2 protein, a minor component of the influenza A virus envelope (Zebedee and Lamb, 1988), is the target of the specific antiviral actions of amantadine hydrochloride and related compounds (Hay et al., 1985). Evidence as to its function has been derived largely from studies of the action of these inhibitors. Characterization of drug-resistant mutants, arising both "*in vitro*" and "*in vivo,*" has indicated that the drugs interact with the membrane-spanning domain of the protein (Hay et al., 1985; Belshe et al., 1988; Bean et al., 1989). The most common inhibitory activity of these agents is directed against a stage in virus uncoating about which there is little detailed information (Kato and Eggers, 1969; Bukrinskaya et al., 1982). With certain strains of viruses, in particular of the H7 subtype, the hemagglutinin (HA) of which is cleaved intracellularly in cell culture, the drugs also act at a later stage in virus infection, preventing virus release apparently as a consequence of an M2-mediated conversion of the HA to its low pH conformation (Hay et al., 1986; Sugrue et al., 1990a; Ruigrok et al., 1990). The very nature of this alteration and its reversibility by, e.g., monensin suggest that M2 plays a role in regulating the pH of vesicular compartments of the trans-Golgi network (TGN).

In this paper we present evidence showing that M2 exists as a homotetramer composed of two disulfide-linked dimers. This provides a structural basis for the proposition that the protein forms a transmembrane channel possibly involved in proton translocation, a function which can be blocked by the direct interaction of amantadine.

MATERIALS AND METHODS

Viruses and cells

Influenza viruses A/chicken/Germany/34 (H7N1, "Rostock") and A/duck/Germany/1215/73 were grown in 11-day-old fertile hen's eggs. Primary chick embryo fibroblasts (CEF) were infected with virus at a multiplicity of approximately 50 plaque forming units (PFU) per cell at room temperature for 30 min, washed, and incubated at 37° in Tris-buffered Gey's medium, medium plus 5 mM sodium pyruvate, or medium minus phosphate.

Labeling of cells

Between 5 and 6 hr after infection cells were incubated with medium containing [^{35}S]cysteine (25 μCi/ml; >1000 Ci/mmol) or [^{35}S]methionine (25 μCi/ml; >1000 Ci/mmol); medium plus 5 mM pyruvate containing [^{3}H]palmitic acid (200 μCi/ml; 50–60 Ci/mmol); or minus-phosphate medium containing [^{32}P]orthophosphate (1 mCi/ml; carrier-free).

Immunoprecipitation

CEF monolayers (6 cm) were extracted at 4° for 10 min with 0.5 ml NP-40 buffer (1% NP-40, 150 mM sodium chloride, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 50 μg/ml soya bean trypsin inhibitor, 20 mM Tris–HCl, pH 7.5). The lysates were incubated at 4° for 60 min with 30 μl pro-

tein A–Sepharose (10% w/v suspension), and protein A-Sepharose removed by centrifugation. One hundred microliters of lysate was then added to 600 μl binding buffer (0.5% NP-40, 150 mM sodium chloride, 1 mM EDTA, 0.25% BSA, 20 mM Tris–HCl, pH 8), 2 μl of anti-M2 rabbit serum added, and all incubated at 4° overnight. The rabbit antiserum was prepared against the N-terminal peptide of M2, MSLLTEVETPIR, coupled to BSA with glutaraldehyde. The immune complexes were isolated by adding 50 μl protein A–Sepharose and incubating at 4° for 2 hr. The protein A–Sepharose was washed 6 times with high salt buffer (1% Triton X-100, 650 mM sodium chloride, 1 mM EDTA, 10 mM sodium phosphate, pH 7.0) and once with low salt buffer (1% Triton X-100, 150 mM sodium chloride, 1 mM EDTA, 10 mM sodium phosphate, pH 7.0). The protein A–Sepharose-bound immune complexes were resuspended in 40 μl sample buffer (1% SDS, 15% glycerol, 60 mM sodium phosphate, pH 6.8) with or without 1.0% β-mercaptoethanol and heated at 100° for 2 min. After removing the protein A–Sepharose by centrifugation the samples were analyzed by electrophoresis on 8 or 16% polyacrylamide gels followed by autoradiography or fluororadiography. Apparent molecular weights were estimated using ^{14}C-methylated proteins (Amersham) in the molecular weight range 14–200 kDa: lysozyme (MW 14,300), carbonic anhydrase (MW 30,000), ovalbumin (MW 46,000), serum albumin (MW 69,000), phosphorylase b (MW 92,500), and myosin (MW 200,000).

Two-dimensional SDS–PAGE

Immunoprecipitates were heated at 100° for 2 min in sample buffer without reducing agent and applied to a 16% polyacrylamide gel. After electrophoresis at 200 V for 40 min gel tracks were cut out and incubated at 37° for 50 min in reducing buffer (0.3 M dithiothreitol (DTT), 1% SDS, 4 M urea, 60 mM sodium phosphate, pH 7.0) before placing across the top of a second 16% discontinuous SDS–polyacrylamide gel. Electrophoresis in the second dimension was at 200 V for 40 min.

Cross-linking

A stock solution of dimethyl suberimidate (DMS) (10 mg/ml) was prepared in 10 ml PBS, to which 80 μl sodium hydroxide (10 M) was added. DMS at 1 to 8 mg/ml in PBS was added to labeled CEF monolayers and left overnight at 4°. Stock solutions of dithiobis (succinimidyl propionate) (DSP; 100 mM), ethylene glycolbis (succinimidyl succinate) (EGS; 10 mg/ml) and bis [2-(succinimidooxycarbonyloxy) ethyl] sulfone (BSO-COES; 10 mg/ml) were prepared in DMSO and diluted in PBSA to the required concentration prior to addition

to infected monolayers. After overnight at 4° glycine was added to a final concentration of 2 mM, the monolayers washed in cold PBS and NP-40 lysates prepared.

Sucrose density gradient centrifugation

Rostock-infected CEF monolayers labeled with [^{35}S]cysteine were extracted with octylglucoside buffer (1% octylglucoside, 150 mM sodium chloride, 1 mM EDTA, 2 mM PMSF, 5 μg/ml aprotinin, 50 μg/ml soyabean trypsin inhibitor, 20 mM Tris/HCl, pH 7.5). The lysates were applied to a 5–25% continuous sucrose gradient (in 1% octylglucoside, 150 mM sodium chloride, 20 mM Tris/HCl, pH 7.5) which was then centrifuged at 40,000 rpm in a Beckman SW41 rotor for 16 hr at 17°. The gradients were harvested and individual fractions assayed for M2 by immuneprecipitation followed by SDS–PAGE. The positions of trimeric and monomeric HA were determined by direct electrophoretic analysis of the gradient fractions. The monomeric hemagglutinin was labeled during 5 min incubation with [^{35}S]cysteine.

RESULTS

Multimeric structure

Analyses of immuneprecipitates of M2, dissociated in the presence of β-mercaptoethanol, indicated a single species with an apparent molecular weight of 15 kDa as reported previously by Lamb et al. (1985). In the absence of reduction, however, two additional bands with apparent molecular weights of 33 and 70 kDa were observed (Fig. 1A). Analysis in the second dimension following reduction with DTT showed that both the 33- and 70-kDa species contained only the [^{35}S]-cysteine-labeled M2 monomer (Figs. 1B and 4B), indicating that they are homomultimers of M2 stabilized by disulfide bonds. On the basis of apparent molecular weights the 33-kDa molecule corresponds to a dimer and the 70-kDa molecule to either a tetramer or a pentamer. The absence of any difference in the ratio of the three M2 species following a labeling period of 5 min or longer indicates that multimerization occurs within a few minutes of synthesis.

Sucrose gradient analysis of octylglucoside lysates of virus-infected cells showed that M2 had a sedimentation coefficient of approximately 4 S (Fig. 2), similar to the HA monomer (Doms and Helenius, 1986), which correlated with the electrophoretic mobility of the 70-kDa band. There was no evidence for the association of M2 with any larger complex.

A number of cross-linking reagents were used to stabilize the multimeric structure of M2 present in the
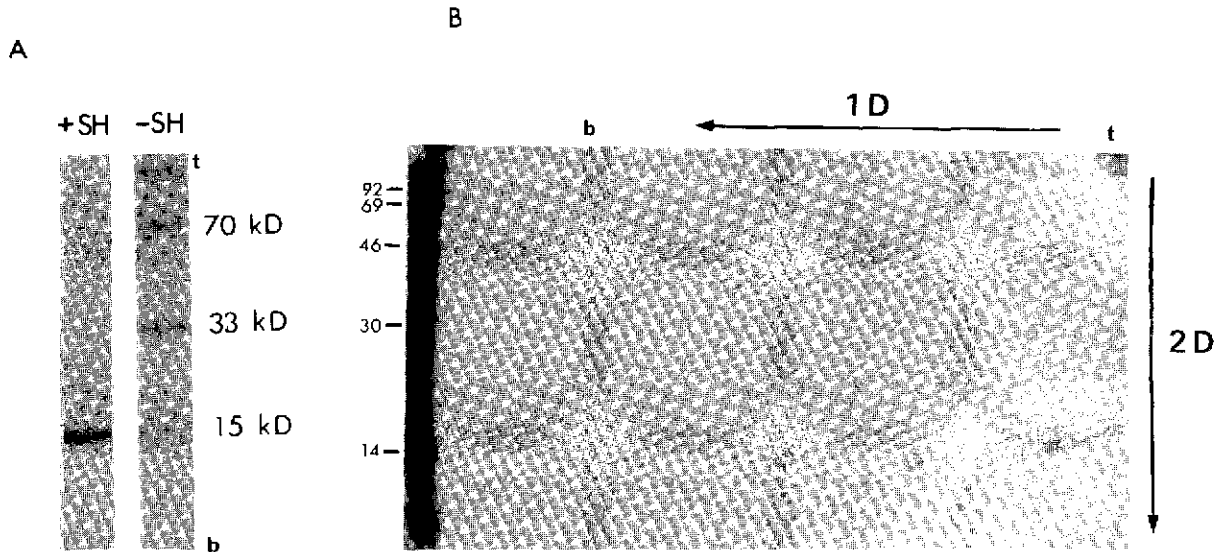
FIG. 1. Multimers of M2. (A) Immunoprecipitates of [³⁵S]cysteine labeled M2 were analyzed by SDS–PAGE following dissociation in the presence (+SH) or absence (−SH) of 1% β-mercaptoethanol. (B) A track of the nonreduced sample equivalent to that in (A) was subjected to electrophoresis on a second-dimension 16% gel following reduction. Molecular weight markers range from 92 to 14 kDa.

plasma membrane of infected cells. For example, following incubation of virus-infected cells with DMS at a concentration greater than 2 mg/ml, the 70K species was the most prominent in immunoprecipitates of M2 (Fig. 3), some of which was resistant to dissociation by β-mercaptoethanol. Slower-migrating bands of 95 kDa or greater were also evident in samples obtained following incubation of infected cells with DSP (Fig. 4), EGS or BSOCOES (Fig. 5). Two-dimensional electro-

phoretic analysis of DSP-cross-linked M2 confirmed that both the dimeric and 70-kDa species are composed solely of M2 (Fig. 4). In contrast the 95-kDa band was not dissociated into smaller components by reduction with 300 mM DTT for 50 min at 37°. The identity of this component is unresolved. Nonreduced M2 present near the top of the first dimension gel in these
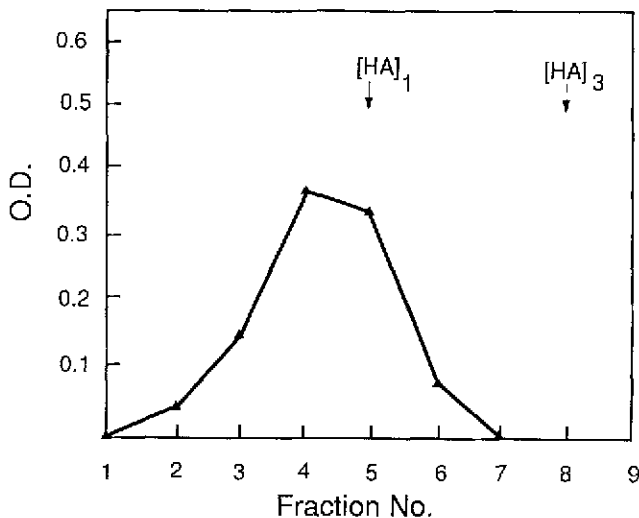

FIG. 2. Sedimentation analysis of M2. Octylglucoside lysates of [³⁵S]cysteine-labeled Rostock-infected CEF cells were analyzed on a 5–25% sucrose gradient, as described under Materials and Methods. Relative radioactivity of M2 was determined by microdensitometry of autoradiograms. [HA]1 and [HA]3 indicate the peak fractions of monomeric (5-min label) and trimeric HA, respectively.
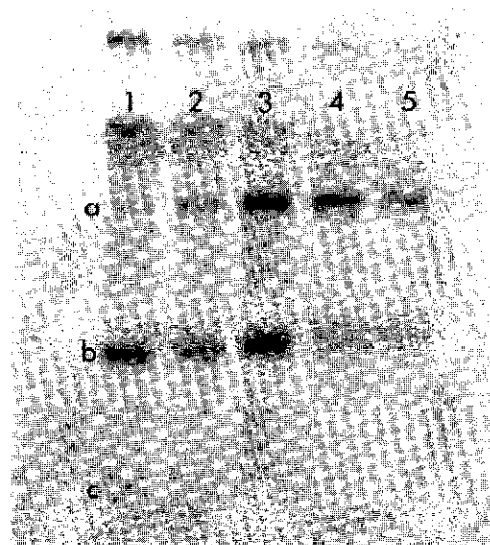

FIG. 3. Cross-linking of M2 with DMS. Rostock-infected CEF monolayers labeled with [³⁵S]cysteine were incubated at 4° overnight with PBS (1) or PBS containing 1 mg/ml (2), 2 mg/ml (3), 4 mg/ml (4) or 8 mg/ml (5) DMS. Nonreduced immunoprecipitates of M2 were analyzed on 16% gels. a, b, and c indicate the 70-, 33-, and 15-kDa M2 bands, respectively.
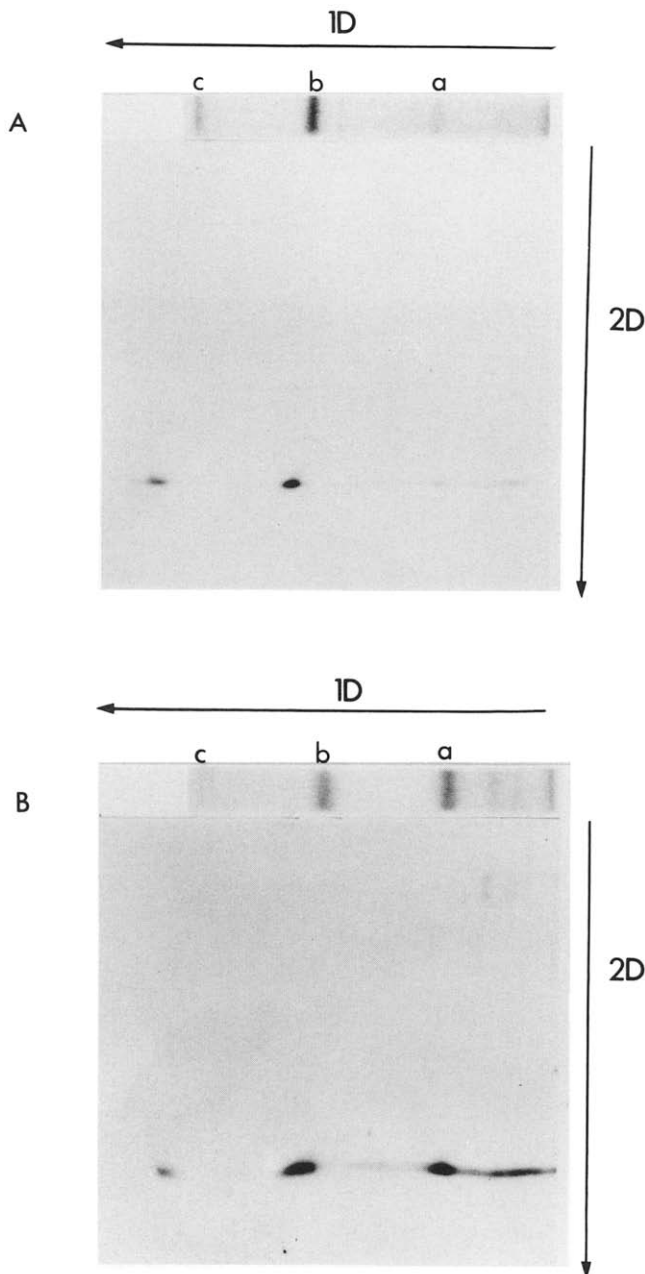
FIG. 4. Two-dimensional gel electrophoresis of DSP-cross-linked M2. Rostock-infected CEF monolayers, labeled with [^{35}S]cysteine, were incubated at 4° overnight in PBSA (A) or PBSA containing 1 mM DSP (B). a, b, and c indicate the 70-, 33-, and 15-kDa M2 bands, respectively.

and other experiments may in part reflect aggregation of the protein. No convincing evidence was obtained for its presence in any specific higher-molecular-weight complex, alone or in association with other cellular or viral proteins.

M2 is a phosphoprotein, all multimeric species being phosphorylated to a similar degree (R. J. Sugrue and

A. J. Hay, in preparation). ^{32}P-labeling readily produces M2 with a higher specific radioactivity and facilitates the detection of minor M2 species. Incubation of ^{32}P-labeled infected cells with 0.4 mg/ml BSOCOES or EGS yielded, in addition to a substantial increase in the 70-kDa form, a minor band with an apparent molecular weight of 46 kDa (Fig. 5), corresponding to that expected for an M2 trimer. No other M2 band between 46 and 70 kDa was detected. The three forms of M2 (33, 46, and 70 kDa) were also detected after reduction of the immuneprecipitates of cross-linked M2 prior to gel electrophoresis.

Further evidence for the trimeric "intermediate" was provided by studies of deacylated M2. The M2 proteins of a variety of virus strains including Rostock have been shown to contain palmitate moieties most likely attached to cysteine 50 on the cytoplasmic domain (Sugrue et al., 1990b). Its removal by treatment with 1 M hydroxylamine resulted in the formation of additional intersubunit disulfide linkages as shown by the reduction in the proportion of 15-kDa monomer and the increase in the 33-kDa and "70-kDa" bands as well as the appearance of a 46-kDa band (Fig. 6A). Two dimensional analysis showed that the 46-kDa species was composed exclusively of M2. Removal of palmitate also increased the electrophoretic mobility of the 70-kDa species, which migrated with an apparent molecular weight of approximately 65 kDa. Again, the absence of any M2 species of molecular weight interme-
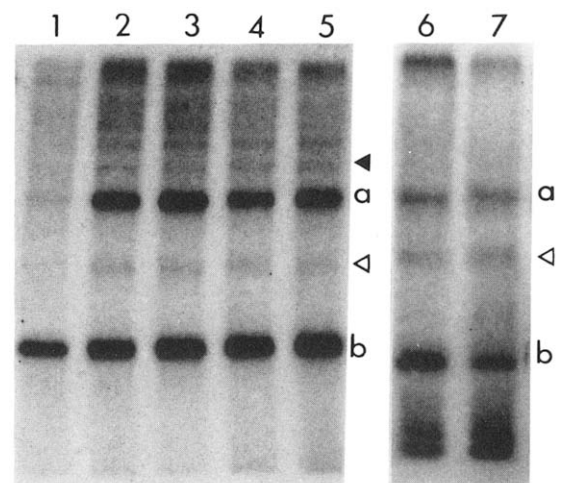

FIG. 5. Cross linking of M2 with BSOCOES and EGS. Rostock-infected CEF monolayers were labeled with [^{32}P]orthophosphate and the monolayers incubated at 4° overnight in PBS (1) or PBS containing 0.4 mg/ml (2, 6) or 0.6 mg/ml (3) BSOCOES, or 0.4 mg/ml (4, 7) or 0.6 mg/ml (5) EGS. Immuneprecipitates of M2 were analyzed on 8% polyacrylamide gels without (1–5) or with prior reduction by 1% β-mercaptoethanol (6, 7). a and b indicate the 70- and 33-kDa M2 bands, respectively. ◁, 46 kDa; ◀, approximately 95 kDa.
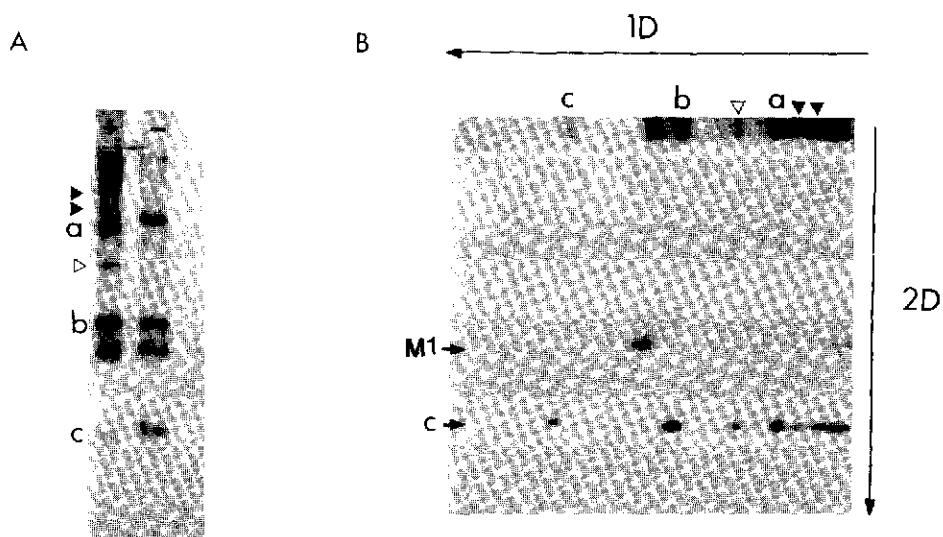
FIG. 6. Appearance of the trimer M2 after deacylation. Rostock infected CEF monolayers were labeled with [^{35}S]methionine and incubated at 25° for 1 hr in the absence (−) or presence (+) of 1 M hydroxylamine in PBS, pH 8.0. (A) Analysis of nonreduced immunoprecipitates of M2. (B) 2D electrophoresis of M2 immunoprecipitated from hydroxylamine-treated cells. Removal of palmitate caused increased mobility of M2, in particular the "70-kDa" multimer, which migrated with an apparent molecular weight of 65-kDa. The use of [^{35}S]methionine accentuated the presence of nonspecifically immunoprecipitated matrix (M1) protein. Filled triangles, M2 species with apparent molecular weights greater than 70 kDa. Open triangles, 46 kDa. a, b, and c indicate 70-, 33-, and 15-kDa M2 species, respectively.

diate between 46 and 70 kDa suggests that the latter most likely represents a tetramer. The significance of the M2-containing bands of greater than 70 kDa is yet again difficult to establish, e.g., whether they result from disulfide formation between M2 tetramers or between M2 and some other protein.

Nature of intersubunit associations

The results shown in Fig. 1 indicate that both the tetrameric and dimeric forms of M2 are stabilized by disulfide bonds. This is also evident from the results of *in situ* reduction of M2. Treatment of infected cells with increasing concentrations of DTT and subsequent quenching with iodoacetamide caused a progressive conversion of the 70-kDa tetramer to the dimeric and monomeric species (Fig. 7). The concomitant appearance of the 46-kDa trimer would appear to result from partial reduction. The tetramer is relatively unstable during heating in the presence of SDS as shown by the fourfold decrease in the proportion of the 70-kDa band following 4 min as compared to 0.5 min boiling in 1% SDS (Fig. 8). There is a corresponding increase in the proportion of dimer with little change in monomer. This instability accounts for the variation in the ratio of 70:33-kDa bands observed in different experiments. Thus the cumulative data indicate that the M2 tetramer is composed of two disulfide-linked dimers held together by noncovalent interactions.

The M2 polypeptide has three cysteines, residues 17, 19, and 50, only one of which, cysteine 17, is con-

served in the sequences of the proteins examined to date (Zebedee and Lamb, 1988; Sugrue *et al.*, 1990b). Cysteine 50 on the cytoplasmic side and the site of attachment of palmitate is unlikely to be involved in
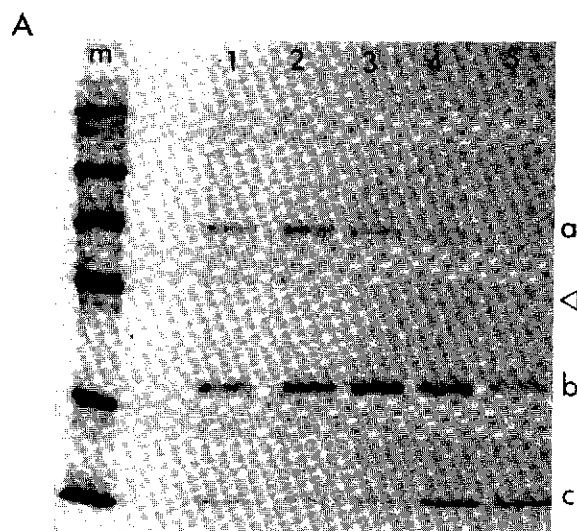

FIG. 7. Reductive dissociation of M2 *in situ*. Rostock-infected CEF monolayers were labeled with [^{35}S]cysteine and incubated at 37° with PBS (1) or PBS + 0.01 mM DTT (2), 0.05 mM DTT (3), 0.10 mM DTT (4), or 1.0 mM DTT (5). After 30 min incubation iodoacetamide was added to a final concentration of 10 mM. M2 immunoprecipitates were dissociated in the absence of β-mercaptoethanol and analyzed on 8% polyacrylamide gels. a, b, and c indicate the 70-, 33-, and 15-kDa M2 bands, respectively. Triangle, 46 kDa. The left-hand track, m, shows molecular weight markers.
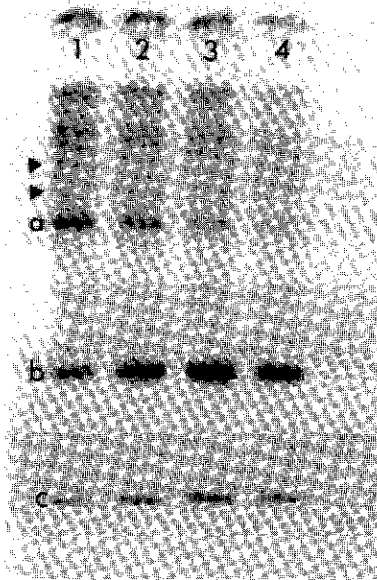
FIG. 8. Stability of the M2 tetramer. Rostock-infected CEF mono-layers were labeled with [^{32}P]orthophosphate. The M2 immunepre-cipitates were incubated at 100° in sample buffer without β-mercap-toethanol for 0.5 min (1), 1 min (2), 2 min (3), or 4 min (4), prior to analysis on 8% gels. a, b, and c indicate the 70-, 33-, and 15-kDa M2 bands, respectively.
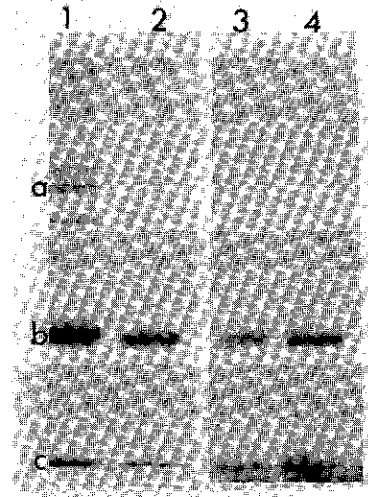

FIG. 9. Oligomerization of M2 with a single external cysteine resi-due. CEF monolayers were infected with either Rostock (1, 3) or A/Duck/Germany/1215/73 (2, 4) and labeled with either [^{35}S]-cysteine (1, 2) or [^{3}H]palmitate (3, 4). M2 immuneprecipitates were analyzed without reduction on 8% polyacrylamide gels.

intersubunit disulfide linkages (Sugrue *et al.*, 1990b). The association of palmitate with dimers of the protein of A/duck/Germany/1215/73, which has tyrosine in place of cysteine 19, limits the disulfide linkage to cys-teine 17 (Fig. 9). Furthermore, the formation of the tetra-meric protein indicates that the interactions between the two dimers do not require additional disulfide link-ages. The residues important for maintaining the stabil-ity of the M2 tetramer are currently being investigated by site-directed mutagenesis.

DISCUSSION

The data presented indicate that in the plasma membrane of infected cells the M2 protein of influenza A viruses exists as a homotetramer made up of two disulfide-linked dimers held together by noncovalent interactions. The conservation of a single cysteine at position 17 in the proteins of all viruses examined (Ze-bedee and Lamb, 1988; Sugrue *et al.*, 1990b) indicates that it is the principle candidate for an essential disul-fide linkage. The random secondary structure in this region of the molecule between residues 17 and 23, indicated by computer prediction (Chou and Fasman, 1978) and NMR studies of the N-terminal 24-amino-acid peptide (M. Carr, personal communication), would facilitate the formation of a second disulfide bridge be-tween the cysteines at position 19 (Thornton, 1981), as depicted in Fig. 10. The localization of available cys-teines on only one side of the membrane indicates that the monomers are in a parallel orientation. This sup-

ports previous evidence, from the specific trypsin sus-ceptibility of the external N-terminal segment of M2 and the selective recognition by N- and C-terminal spe-cific antisera (Lamb *et al.*, 1985; Sugrue *et al.*, 1990b), that M2 is exclusively oriented with its N-terminus ex-ternal to the cell and virus and therefore that the four subunits form a parallel array in the tetramer.
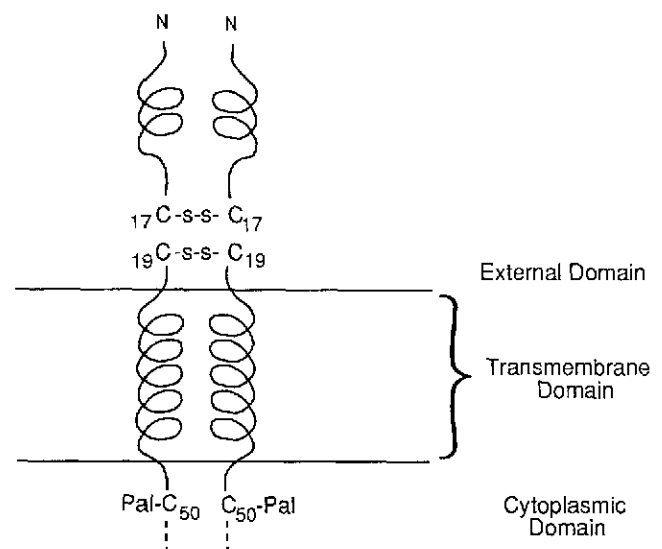

FIG. 10. Diagrammatic representation of structural features of the N-terminal half of the disulfide-linked M2 dimer. The formation of disulphide linkages in proteins lacking cysteine 19 suggests the sym-metric bonds indicated. α-Helical regions encompassing residues 2–9 and the membrane-spanning sequence 25 43 are shown. Pal-mitate is shown in association with cysteine 50 (Sugrue *et al.*, 1990b).
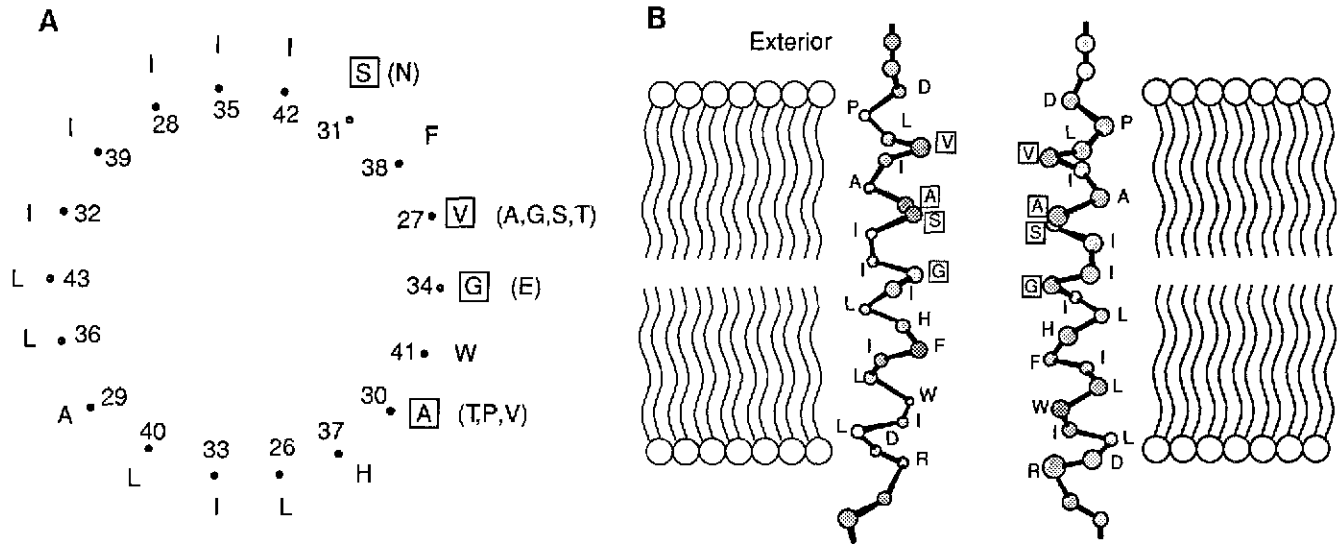
FIG. 11. Diagrammatic representation of an α-helix of the transmembrane domain of M2. The sequence in single-letter code is of the protein of A/chicken/Germany/27 (H7N7) since amantadine-resistant mutants with single substitutions at all four positions (boxed) have been isolated (Hay et al., 1985). (A) Helical wheel representation showing the lateral distribution of the polar (serine, threonine, asparagine) and charged (histidine, glutamic acid) amino acids on one face of the helix (to the right); single amino acids altered in different amantadine-resistant mutants are boxed and the substitutions most commonly observed are in the parentheses. (B) Perceived arrangement of two diagonally located α-helices of the tetramer with the hydrophobic faces towards the lipid bilayer and the polar faces forming the interior of the proposed channel.

The putative transmembrane sequences of the M2 proteins of most natural isolates contain one polar (serine 31) and one charged (histidine 37) amino acid in a sequence of otherwise predominantly hydrophobic amino acids. Additional polar or charged residues at position 27 (serine, threonine, or aspartic acid), 30 (threonine), or 34 (glutamic acid) in the sequences of the proteins of amantadine-resistant and rimantadine-resistant viruses (Hay et al., 1985; Belshe et al., 1988; Bean et al., 1989) emphasizes the sidedness and amphiphilic nature of an α-helical configuration of this segment (Fig. 11). Although there is no definitive evidence regarding the secondary structure of this domain or the relative orientation of the subunits in the tetramer, it is likely that the polar faces are directed towards the interior of the structure and away from the lipid environment of the bilayer. This view would fit the suggestion that amantadine interacts directly with a channel formed by the four subunits of the tetramer and that the amino acid changes, which abolish amantadine-sensitivity, directly influence binding. There is a precedent for this in that amantadine and various N-alkyl derivatives inhibit neuromuscular transmission by interacting with the ion channel of the nicotinic acetylcholine receptor and competitively inhibit the binding of other channel blockers, phencyclidine and histrionicotoxin, to the receptor (Warnick et al., 1982; Eldefrawi et al., 1982).

The parallels apparent between the mechanisms of the antiviral and anticholinergic actions of amantadine also extend to the functions of the target proteins. Evidence indicates that the M2 protein is capable of regulating the pH of vesicles of the TGN and may form a selective ion channel. In the absence of any direct evidence for this function the conclusion is based on observations that amantadine causes an M2-mediated conversion of HA to its low-pH conformation apparently as a consequence of exposure to a reduced pH in the TGN during its transport to the cell surface (Sugrue et al., 1990a; F. Ciampor and A. Hay, unpublished data). It is not known whether the amino acid changes in M2 which abolish this action do so by altering its function or the interaction of amantadine with M2. In the latter regard, reciprocal changes of polar and apolar amino acids at two successive turns of the helix of the "M2" membrane-spanning region of the subunits of the nAchR alter the binding affinity of the quaternary ammonium anesthetic QX-222 (Leonard et al., 1988; Charnet et al., 1990). A disposition of amantadine similar to that suggested for QX-222, with the amino group interacting with the hydroxyl group of serine 31 and the hydrophobic ring moiety directed towards the previous turn of the helix (valine/isoleucine, 27) (Fig. 11), would be consistent with the amantadine resistance changes in the viral protein. On the basis of their effect on HA maturation, it is apparent that certain substitutions, e.g., valine (27) by serine or threonine may enhance while others, e.g., glycine (34) by glutamic acid, clearly compromise the functional activity of the Rostock M2 protein in infected cells (S. Grambas and A. Hay, unpublished data).

In conclusion, therefore, the tetrameric nature of M2

and the proposed orientation of the membrane-spanning domains of the subunits provide a structural basis for the proposed function of the protein in proton translocation, and its inhibition by amantadine and related compounds. This is a novel function for a virus protein which in the case of influenza A viruses has a role in both virus uncoating and glycoprotein (HA) maturation (Hay, 1989; Belshe and Hay, 1989). In view of the similarities in the modes of replication of influenza A and influenza B viruses it is likely that an analogous protein, probably the 100-amino-acid NB protein of B viruses (Williams and Lamb, 1986) performs a similar function. In considering the wider implications for other enveloped viruses, a number of small membrane-associated proteins of undefined function have been identified. These include, for example, the SH and 1A proteins of paramyxoviruses (Hiebert et al., 1985; Olmsted and Collins, 1989), the D3 protein of the coronavirus, infectious bronchitis virus (Smith et al., 1990), and the vpu protein of HIV-1 shown to have an important role in virus maturation (Terwilliger et al., 1989; Klimkait et al., 1990). Finally, the mechanistic similarities between the antiviral and anticholinergic activities of amantadine may well encompass its pharmacological efficacy both in preventing influenza A infection and in alleviating symptoms of Parkinson's disease.

ACKNOWLEDGMENTS

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