Identification of the Pore-lining Residues of the BM2 Ion Channel Protein of Influenza B Virus

Chunlong Ma, Cinque S. Soto, Yuki Ohigashi, Albert Taylor, Vasilios Bournas, Brett Glawe, Maria K. Udo, William F. DeGrado, Robert A. Lamb, and Lawrence H. Pinto

From the Department of Neurobiology and Physiologv, School of Medicine, and Physics Department, Loyola University, Chicago, Illinois 60626

The influenza B virus BM2 proton-selective ion channel is essential for virus uncoating, a process that occurs in the acidic environment of the endosome. The BM2 channel causes acidification of the interior of the virus particle, which results in dissociation of the viral membrane protein from the ribonucleoprotein core. The BM2 protein is similar to the A/M2 protein ion channel of influenza A virus (A/M2) in that it contains an HXXXW motif. Unlike the A/M2 protein, the BM2 protein is not inhibited by the antiviral drug amantadine. We used mutagenesis to ascertain the pore-lining residues of the BM2 ion channel. The specific activity (relative to wild type), reversal voltage, and susceptibility to modification by (2-aminoethyl)-methane thiosulfonate and N-ethylmaleimide of cysteine mutant proteins were measured in oocytes. It was found that mutation of transmembrane domain residues Ser9, Ser12, Phe13, Ser16, His19, and Trp23 to cysteine were most disruptive for ion channel function. These cysteine mutants were also most susceptible to (2-aminoethyl)-methane thiosulfonate and N-ethylmaleimide modification. Furthermore, considerable amounts of dimer were formed in the absence of oxidative reagents when cysteine was introduced at positions Ser9, Ser12, Ser16, or Trp23. Based on these experimental data, a BM2 transmembrane domain model is proposed. The presence of polar residues in the pore is a probable explanation for the amantadine insensitivity of the BM2 protein and suggests that related but more polar compounds might serve as useful inhibitors of the protein.

Influenza B virus, which is a distinct virus from influenza A virus and is classified in a separate genus from influenza A virus, has caused infections that have totaled up to 50% of all influenza disease in recent years, making it a “significant human health problem, particularly for those under 5 years old and for those over 50 years old” (see Centers for Disease Control site on the World Wide Web). The BM2 protein of influenza B virus, like the M2 protein of influenza A virus (A/M2), conducts protons and acidifies the virus particles during virion uncoating in endosomes (1–4). The BM2 protein, like the A/M2 protein, is essential for viral replication (5–7). However, unlike influenza A virus, influenza B virus is not inhibited by the antiviral drug amantadine (8). The reason for this is that the BM2 ion channel is not inhibited by the drug (9), whereas the A/M2 ion channel is completely inhibited by the drug (2, 10). It has been suggested that the failure of amantadine to inhibit the BM2 channel is due to differences in the transmembrane (TM) domain pore-lining residues (9), but these residues have not yet been identified.

It has been demonstrated in many ways that the ion channel activity of the A/M2 protein is intrinsic (2, 11, 12). Recent crystallographic (13) and NMR (14) studies of the structure of the A/M2 protein have suggested similar mechanisms for proton conduction and activation but quite different mechanisms for inhibition by amantadine. An essential foundation for both of these studies was prior biochemical information about the A/M2 channel, particularly its pore-lining residues (15–17) and its active oligomeric state (18). Although the active oligomeric state of the BM2 channel is known to be a tetramer (19), the pore-lining residues of the channel have not yet been identified. Identification of the pore-lining residues in the BM2 channel would provide a needed foundation for further studies.

The mature BM2 protein consists of a 6-residue N-terminal extracellular domain, a single internal hydrophobic domain of 19 residues that acts as a TM domain and forms the pore of the channel, and an 83-residue cytoplasmic tail (20). Because protons must pass through the membrane via the pore of an ion channel, knowledge of the pore region of these ion channels is necessary 1) for understanding the conduction of protons and the binding of inhibitors 2), to understand why amantadine does not inhibit the BM2 ion channel, and 3) to identify potential BM2 inhibitors.

This is an open access article under the CC BY license.
The pore-lining residues of the A/M2 ion channel were identified by a series of approaches. The first employed cysteine scanning mutagenesis and the use of water soluble sulfhydryl specific reagents that attach a large hydrophobic adduct to cysteine residues (16). If the current of a mutant channel with a cysteine residue in a particular location is modified by the reagent, then it is concluded that the sulfhydryl moiety of the cysteine faces the aqueous pore at that location. The current of cysteine mutant proteins A/M2-A30C and -G34C were diminished when the reagent was applied to the medium bathing the N-terminal ectodomain. The current of the cysteine mutant protein A/M2-W41C was decreased when the reagent was injected into the cytoplasm but not when applied to the bathing medium. Moreover, the A/M2-G34C mutant protein was not affected by cytoplasmic injection of the reagent. These data are consistent with His$^{37}$ of the A/M2 protein forming a barrier to large molecules. Cysteine-scanning mutagenesis was also used together with oxidative disulfide cross-linking (17) to show that residues 27, 30, 34, 37, and 41 formed dimers most rapidly. Finally, two naturally occurring amantadine-resistant mutations occurred for residues that were identified as pore-lining (A/M2-A30C and -G34E). The secondary structure of the TM domain of the A/M2 and BM2 proteins is aligned, the TM domains of the A/M2 and BM2 proteins. The TM domain has a tilt angle of $25^\circ$. Further hydrogen/deuterium exchange measurements showed that the above residues in the TM helix exchange more rapidly than residues located in the cytoplasmic domain (27), consistent with the presence of an aqueous pore. Most recently, the crystal structure of the TM peptide (residues 22–46) revealed that the aqueous cavity at the N-terminal half of the TM domain was lined by residues Val$^{27}$, Ala$^{30}$, and Gly$^{34}$. At the C-terminal half of the TM domain, His$^{37}$ and Trp$^{41}$ construct the A/M2 channel (13). Thus, the aqueous pore of the A/M2 ion channel is formed by Val$^{27}$, Ala$^{30}$, Gly$^{34}$, His$^{37}$, and Trp$^{41}$; these residues line the pore of the four-helix bundle. The present study employed three of the above approaches to identify the pore-lining residues of the BM2 ion channel protein.

In contrast to the structure of A/M2 TM domain, very little is known about the TM domain of the BM2 ion channel protein (9). When the conserved histidine and tryptophan residues of the TM domains of the A/M2 and BM2 proteins are aligned, the residues of the BM2 protein that fall opposite to the pore-lining Val$^{27}$, Ala$^{30}$, and Gly$^{34}$ of the A/M2 protein are all serines (Ser$^{9}$, Ser$^{12}$, and Ser$^{16}$), these are the “a” and “d” residues in Sequences 1 and 2). However, alignment of protein sequences is insufficient to demonstrate which residues line the pore, and furthermore, alignment alone does not provide accessibility information (Sequences 1 and 2).

Here, we report findings using cysteine-scanning mutagenesis in combination with the substituted cysteine accessibility method and measurement of key ion channel properties. Our data suggest that the pore of BM2 proton channel is lined with polar residues, which is very different from the A/M2 protein of influenza A virus. A computational model of the BM2 TM domain was proposed that is in good agreement with the experimental data. The pore-lining residues and molecular model were further confirmed by quantification of disulfide-linked dimers. Knowledge of these pore-lining residues will serve as the foundation for detailed biophysical studies and will help to identify inhibitors of the channel.

**EXPERIMENTAL PROCEDURES**

_Cloning and Site-specific Mutagenesis—_Plasmid constructs were based on the cDNA to segment 7 RNA of influenza B/Lee/40 virus. Cloning of BM2-FLAG into plasmid vector, pGEM3, was done as described previously (20). Site-directed mutagenesis using site overlap extension PCR was carried out so that each of the BM2 TM domain residues was mutated to cysteine. Serine to glycine and isoleucine mutations were prepared employing the QuickChange site-directed mutagenesis method. It should be noted that all of the cysteine substitution mutations were made in the wild type (WT) BM2 sequence, and thus all of the mutants also contain a cysteine at residue 11, but as discussed below, this does not affect the interpretation of the data. Sequences of plasmids and details of all cloning steps are available upon request.

_mRNA Synthesis, Culture, and Microinjection of Oocytes—_BM2-pGEM cDNA was linearized using the HindIII restriction site found downstream of the gene, and in vitro transcription reactions were performed on the linearized cDNA using a T7 mMESSAGE mMACHINE® transcription kit (Ambion, Houston, TX). _Xenopus laevis_ ovoidal lobules were surgically removed from females (Nasco, Fort Atkinson, WI), and individual oocytes were dissociated from the follicle cells by digestion with collagenase B (2 mg/ml; Roche Applied Science) as previously described (2). Oocytes of good size and color (as described in Ref. 23) were injected with 50 nl of mRNA ($\sim 1.0 \mu g/\mu l$), using a 20-µm diameter glass pipette 24 h postdefolliculation. Oocytes were maintained at 16 °C in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, 2.5 mM pyruvic acid, 5 mM HEPES-NaOH, 0.1 mg/ml gentamycin (pH 8.5)).

_Membrane Current Recording in Oocytes—_Whole cell current was measured from oocytes 48–72 h after mRNA injection using a two-electrode voltage clamp technique and a Dagan TEV 200A amplifier (Dagan Corp., Minneapolis, MN) at room temperature using electrodes filled with 3 M KCl. Individual oocytes were held at a voltage of $-20$ mV and bathed in normal Barth’s solution (88.0 mM NaCl, 1.0 mM KCl, 2.4 mM NaHCO$_3$, 0.3 mM NaNO$_3$, 0.71 mM CaCl$_2$, 0.82 mM MgSO$_4$, 15 mM HEPES (for pH 8.5) or 15 mM MES (for pH 5.5)) or a modified solution during recording (28). Membrane voltage ramp measurements were made throughout the recordings, spanning a 100-mV range from $-40$ to 60 mV. Data were collected and analyzed using the pCLAMP 10.0 software package.

_Reversal Voltage Measurements—_It has been shown that reversal voltage for proton channels, measured in the absence of a mobile buffer, can be altered rapidly because of local pH changes near the cytoplasmic leaflet of the surface membrane.
(29). In order to minimize this effect, membrane voltage ramp measurements were made at pH 5.5 at the time when the amplitude of the steady inward current was maximal. The voltage ramp spanned a 100-mV range from -40 to 60 mV. For each cell expressing a given protein, the reversal voltage was plotted against the steady inward current for that cell, and a straight line was fitted to the plot. The y-intercept of this plot was taken as the reversal voltage ($V_{rev}$) of the protein. The reversal voltage data here represent the best fit y-intercept ± S.E.

**Immunofluorescence of Living Oocytes**—Individual oocytes, the membrane currents of which had been studied previously, were washed with ND96 solution and fixed in 2% paraformaldehyde in preparation for testing the relative surface expression levels of the mutant BM2 proteins. Oocytes were first incubated with nonfat milk (5% in ND96 solution) containing 0.1% saponin for 1 h, followed by a 1-h incubation with ANTI-FLAG® M2 monoclonal antibody (F 3165; Sigma; 10 μg/ml in ND96 solution containing 2% milk and 0.1% saponin), three washes with ND96 solution (10 min each), incubation with goat anti-mouse IgG1 (y1), and labeling with Alexa Fluor® 546 (A21123; Molecular Probes, Inc., Medford, OR; 20 μg/ml in ND96 solution containing 2% milk, 0.1% saponin) for 1 h, followed by three more washes (10 min each) with ND96 solution. All steps were carried out at 4 °C, and the ND96 solution used lacked CaCl2, gentamycin, and pyruvate. Fluorescence was quantified using a PTI Image Master microfluorometer (Photon Technologies) with a 20 × 0.5 numerical aperture objective, as described previously (30). About one-half of the surface of each oocyte was imaged using the CCD camera. The excitation wavelength for the Alexa Fluor® was 540 nm. Intensity quantifications were made using Image Master version 5.0 software (Photon Technologies). Uninjected oocytes were subjected to the same conditions just described as a control for the autofluorescence from the yolk (31).

**Measurement of the Effects of MTSEA and NEM on the BM2 Cysteine Substitution Mutant Ion Channels**—The effect of Cys-specific reagents on BM2 ion channel activities was determined as follows. For MTSEA, oocyte inward currents were measured first in pH 5.5 Barth’s solution, and then oocytes were incubated in pH 8.5 Barth’s solution with 2.5 mM MTSEA for 5 min followed by a 2-min washout. The oocyte inward currents were measured again in pH 5.5 Barth’s solution and compared with the original current before the MTSEA application. In the case of BM2-F13C protein, at the end of measurement, we further applied 5 mM dithiothreitol for 2 min at pH 8.5 to the oocyte, washed for another 2 min, and measured the maximal inward current again. For NEM, the overall procedure was similar to that for MTSEA, except that we applied 1 mM NEM to the outside of the oocytes and incubated for 10 min.

**Data Analysis**—The periodicity of various parameters, $Y$ (i.e., specific activity, $V_{rev}$, perturbation index, and oligomer formation percentage), associated with the amino acid side chains versus their positions in the TM sequence, $n$, was analyzed by fitting a sinusoidal function, $Y = A + B\sin(6.28 (C + n)/D)$, to the data using Graphpad Prism (version 5.0), in which $A$ is the midpoint of the curve, $B$ is the amplitude, $C$ is the phase, and $D$ is the period of the sinusoid wave.

The perturbation index was determined as previously described (21). Briefly, the degree of perturbation to the specific activity or reversal voltage with a mutation at a given position $n$ is defined as follows: perturbation index (PI) = 1 - $X(n)/X(WT)$, in which $X(n)$ is the specific activity or reversal voltage at position $n$, and $X(WT)$ is specific activity or reversal voltage of wild type. The PI values increase as the severity of the perturbation increases. The final PI values at each position, $n$, were normalized. The average PI values were calculated separately for specific activity and reversal voltage.

**Cross-linking by Cu(II) (1,10-Phenanthroline)$_3$**—The BM2 proteins expressed in oocytes were cross-linked by Cu(II) (1,10-phenanthroline)$_3$ (CuP) using a modification of the method described previously (17). After 48–60 h of injection, 15–20 oocytes expressing each BM2 protein were incubated in 10 μl/oocyte RSB buffer (10 mM Tris, pH 7.4, 10 mM KCl, 15 mM MgCl2) for 10 min on ice and homogenized by pipetting. Cell lysates were incubated at 37 °C for 10 min in the absence or presence of 300 μM CuP (from 30 mM stock CuP solution composed of 30 mM CuSO$_4$ and 90 mM 1,10-phenanthroline, prepared fresh from 0.15 M CuSO$_4$ in water and 0.45 M 1,10-phenanthroline in DMSO). The reaction was stopped by the addition of 10 mM NEM and 10 mM EDTA. Membranes were dissolved by the addition of radioimmune precipitation buffer (10 mM Tris, pH 7.4, 1% deoxycholate, 1% Triton X-100, 0.1% SDS) in the presence of proteinase inhibitors and 50 mM iodoacacetamide. The cross-linking experiments were done three times with three batches of oocytes taken from different *X. laevis*. 

**SDS-PAGE and Western Blot**—Polypeptides were separated by nonreducing SDS-PAGE and transferred onto nitrocellulose membranes. PageRuler Plus Prestained Protein Ladder (Fermentas Life Sciences, Glen Burnie, MD) was used as a molecular weight marker. The BM2 proteins were probed with ANTI-FLAG® M2 monoclonal antibody (F 3165; Sigma), followed by horseradish peroxide-conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Labeled proteins were detected by ECL Plus Western blotting detection reagent (GE Healthcare). The intensity of bands corresponding to monomers, dimers, trimers, and tetramers of the BM2 proteins was quantified using ImageJ. The uninjected oocyte sample lane was used for the background. The percentage of each form was expressed as a fraction of each form to the total protein (i.e. the sum of monomer, dimer, trimer, and tetramer).

**Constructing TM Bundle Scaffolds**—Both idealized and coiled helical TM bundles were constructed for residues 7–25 of the BM2 protein sequence. TM bundles composed of idealized α-helices were generated using a rigid body search. The rigid body search started with an idealized α-helical backbone structure aligned on the global z axis. The helix was oriented about the global z axis so that the angle formed between the Ca atoms of Ser$^{16}$ and His$^{19}$ would be bisected by the global x axis. The centered helix was then translated along the global z axis ($x_{\text{trans}}$) followed by translation along the global x axis ($x_{\text{rot}}$). After translation, the helix was rotated about the global x axis ($x_{\text{rot}}$). Finally, the helix was allowed to rotate about its central axis ($Axial_{\text{rot}}$) to vary the phase. The parameters were systematically varied as follows: ($a$) $x_{\text{trans}}$ was varied between −15 and
Pore-lining Residues of BM2 Ion Channel

15 Å using a step size of 2.0 Å; (b) \( x_{\text{trans}} \) was varied between 2.5 and 7.5 Å using a step size of 1.0 Å; (c) \( x_{\text{rot}} \) was varied between \(-40\) and \(40°\) using a step size of \(5°\); and (d) \( Axial_{\text{rot}} \) was varied between \(-50\) and \(50°\) using a step size of \(2°\). C4 symmetry was used to generate the symmetry mates.

TM bundles were also constructed using coiled \(\alpha\)-helices based on Crick’s equations (32, 33) with the following parameters kept constant: (a) superhelical pitch set at 194 Å; (b) rise per residue of the minor helix set to 1.5 Å; (c) radius of the minor helix set at 2.25 Å; (d) periodicity of the minor helix set to \(2\pi/3.5\); and (e) periodicity of the major helix set to \(2\pi/(\text{pitch/rise per residue})\). The superhelical radius was varied between 8.5 and 10.5 Å using a step size of 0.2 Å. The phase of the minor helix was varied between \(-180°\) and \(180°\) using a step size of \(2°\). C4 symmetry was used to generate the symmetry mates.

Filtering TM Bundles—After construction of the TM bundles, each model was screened using two filters. The first filter screened for steric clashes between the different monomers. If the distance between two heavy atoms was less than 4.0 Å, the bundle was discarded. A large cut-off distance was used to accommodate the placement of bulky side chains.

The second filter correlated the PI values for the residues (see above) with a normalized expression involving the phase angle. The details of the filter and its implementation will be described more fully elsewhere. Briefly, the phase angle is formed between two vectors after projecting \(C(\alpha)\) coordinates from the bundle onto the \(xy\) plane. The first vector was constructed from the helix axis to a \(C(\alpha)\) atom on the helix. The second vector was constructed from the helix axis to the global origin. Adding a value of 1 to the cosine of this angle and then normalizing the quantity by 2 gives an expression that can range in value from 0 to 1. At an angle of zero, the \(C(\alpha)\) atom is facing directly inward toward the bundle axis. The expression will yield a maximal value of 1. At an angle of \(180°\), the \(C(\alpha)\) atom is facing directly outward, and the expression will yield the minimum value of 0. Bundles with correlation coefficients of less than 0.60 between the PI values and the expression involving the phase angle were discarded. Application of the two filters yielded 2055 bundles composed of straight helices and 107 bundles composed of coiled helices.

Side Chain Addition and Energy Minimization—After filtering the bundles, side chains were placed onto the helix backbones using the side chain addition program SCAP (34). Since the backbone geometry was kept rigid, side chain addition occasionally resulted in steric “bumps” that could be relieved using energy minimization with a molecular mechanics force field. Bundle geometry was optimized using the CHARMM22 molecular mechanics force field implemented in the XPLOR-NIH package (35). Hydrogen atom addition was carried out using the “hbuild” procedure, followed by 1000 steps of Powell energy minimization using a distance-dependent dielectric set to a value of \(4r\).

Clustering TM Bundles—The 200 top scoring bundles were clustered such that each member in the cluster had a \(C(\alpha)\) root mean square distance of less than 1.5 Å to any other member in the cluster. This produced a total of eight clusters. The bundle with the smallest sum of \(C(\alpha)\) root mean square distance values to all of the other bundles in the cluster was chosen as the centroid model. Parameters used to generate the centroid models are shown in supplemental Table 1.

RESULTS

Relative Specific Activity of Cysteine Substitution Mutant Ion Channels—The goal of these experiments was to identify the amino acid residues that line the pore of the BM2 ion channel. TM domain residues were changed individually to cysteine, since it is thought that changing the residues that line the pore would cause modification of channel properties. The single channel conductance and open probability are the best measures of ion channel activity, but in previous studies, it was found that the single channel conductance of the A/M2 ion channel is too low to be measured (29, 36). Thus, it is likely that the single channel conductance of the BM2 ion channel is also very low. Therefore, for BM2, the relative specific activity was calculated from the ratio of steady current that flowed at pH 5.5 to the relative amount of protein that was expressed at the surface of the oocyte for each cell studied. For each experiment, at least five oocytes expressing the WT BM2 protein and three uninjected oocytes were also measured. For each un.injected cell and for each cell expressing a given mutant protein, the current was plotted against the BM2-specific oocyte surface immunofluorescence for that cell, and a straight line was fitted to the plot. This was also done during each experiment using oocytes expressing the WT protein. The relative specific activity of a given mutant ion channel was calculated as the ratio of the slope for this mutant protein plot to the slope of the WT protein plot. Fig. 1A shows the calculated relative specific activity against residue number. This analysis indicated which residues, when mutated to cysteine, were able to alter
the relative specific activity. Because there are no effective inhibitors for the BM2 proton channel, only oocytes with steady inward currents at pH 5.5 that were larger than 500 nA were used. This was done to minimize the effect of any possible endogenous oocyte currents. The activities of some mutant ion channels (i.e. S9C, F13C, and H19C mutant) were so low that a few oocytes with less than 500 nA of inward current were included. As shown in Fig. 1A all of the mutant M2 ion channels retained a measurable specific activity, except the H19C mutant ion channel. A repeat of \(3.4 \pm 0.1\) residues was observed across the entire TM domain, strongly suggesting the existence of an \(\alpha\)-helical bundle in this region. Mutation of seven residues (i.e. Ser\(^9\), Ile\(^{10}\), Phe\(^{13}\), Leu\(^{15}\), Ser\(^{16}\), His\(^{19}\), and Trp\(^{23}\)) resulted in functional defects such that the mutant ion channel had a relative specific activity that was less than 60% that of WT BM2 ion channel. These observations suggested that these residues are important for BM2 channel activity and that some of them may line the pore of the BM2 channel.

**Reversal Voltage \((V_{rev})\) of Cysteine Substitution Mutant Ion Channels**—Mutation of a pore-lining residue often alters key channel properties. Fig. 1B depicts the changes of one of the key ion channel properties, \(V_{rev}\), upon mutation of the native residue in the BM2 TM domain to Cys. Several points are noteworthy. First, the periodicity of the plot for \(V_{rev}\) was indistinguishable from that of the plot for specific activity (Fig. 1A). Second, the \(V_{rev}\) values for five mutant ion channels (F13C, H19C, F20C, W23C, and H27C) were decreased to 30 mV or less, compared with 55 mV for the WT BM2 ion channel. Interestingly, these mutants, with the exception of F20C, have low specific activity. Moreover, the steady inward current at pH 5.5 of the BM2-H19C mutant channel was so low (i.e. less than 100 nA), that the \(V_{rev}\) could not be determined accurately. Third, a few mutant ion channels (i.e. S9C, I15C, and S16C) only suffered a small decrease in \(V_{rev}\), although their specific activities were greatly affected. We hypothesized that only a mild decrease in \(V_{rev}\) values occurred for mutation of Ser\(^9\) and Ser\(^{16}\) to Cys, because Ser and Cys share very similar chemical characteristics, despite their possible location lining the BM2 channel pore (see below).

Reversal voltage was measured in these experiments with a method intended to minimize the effect of intracellular acidification, and thus for a mutant ion channel, the finding of a reversal voltage that differed from that of the WT channel probably reflects altered ion selectivity of the mutant channel. A prior example of this type of altered ion selectivity was demonstrated using the pore-lining His\(^{37}\) residue of the A/M2 ion channel. Mutation of His to Ala or Gly resulted in less positive reversal voltage, the result of increased K\(^+\) permeation (30).

The results of the cysteine-scanning mutagenesis were expressed as the combined perturbation index (supplemental Fig. 1A). This parameter measures severity of the perturbation in channel properties resulting from a point mutation. The period of the combined perturbation index was the same as the period of the two-component individual data sets (\(3.4 \pm 0.1\) residues). These results strongly suggest the existence of an \(\alpha\)-helical bundle and indicate that the residues underlying the peaks (Ser\(^9\), Ser\(^{12}\), Phe\(^{13}\), His\(^{19}\), and Trp\(^{23}\)) are located in or near the pore lumen. In addition, the perturbation index of the BM2 ion channel had the same value obtained for the A/M2 proton channel (supplemental Fig. 1B) (21). In both cases, the value is significantly less than 3.6 (the period of an ideal helix), which indicates that the helices have left-handed crossing angles relative to the central helical axis (21). There was also a distinct heptad repeat to the data, with the most important residues occupying the “a” and “d” positions (see Sequences 1 and 2). These findings indicate that the bundle is probably a helical bundle or a coiled-coil, so both possibilities were investigated in molecular modeling studies.

**Characterization of Ser to Gly and Ile Mutants**—As shown in Fig. 1 and supplemental Fig. 1 and as discussed above, mutation of BM2 Ser\(^{12}\) and Ser\(^{16}\) to cysteine did not substantially perturb channel properties, perhaps due to the similarity in chemical and physical properties between serine and cysteine. Thus, Ser\(^{9}\), Ser\(^{12}\), and Ser\(^{16}\) were mutated to either glycine or isoleucine, and their channel properties were measured. As shown in Fig. 2, the ion channel properties of the Ser \(\rightarrow\) Gly mutant channels were almost unchanged, whereas for the serine to isoleucine mutant channels, channel activity was lost completely in each case. Furthermore, it should be noted that if the mean of the values of the Cys, Gly, and Ile mutations is used in the perturbation index analysis, the periodicity of the fitted sinusoid curve is not changed; similarly, if the Ile substitutions for Ser residues are used and all other TM domain substitutions are cysteine, the periodicity is the same.

**Extracellular Application of MTSEA and NEM to Cysteine Substitution Mutant Ion Channels**—If a Cys is located in the lumen of the BM2 ion channel, its chemical modification by the addition of extra bulk might perturb the properties of the channel. To determine which residues will be affected after modification, we applied extracellularly MTSEA (2.5 mM) or NEM (1
The modification studies suggest that MTSEA is too bulky to access the targeted Cys residues deep in the BM2 pore region. Thus, we applied another lipid-permeable sulfhydryl-specific reagent, NEM, which is smaller than MTSEA (37). As shown in Fig. 3B, the current amplitude of five mutant ion channels (S12C, F13C, S16C, W23C, and H27C) was decreased by more than 10% after NEM treatment. His27 is located at the boundary of the TM helix and the cytoplasmic region. Moreover, all of these residues are located in the trough positions in the plot of specific activity and the plot of reversal voltage against residue number (Fig. 1, A and B). These results are consistent with the interpretation that residues Ser11, Phe13, Ser16, Trp23, and His27 have access to the BM2 channel pore. It is also interesting to note that the activity of the L8C mutant ion channel was elevated by more than 30% after NEM treatment. This residue lies near the N terminus of the TM helix and hence might exert effects that are more difficult to predict due to the intrinsic flexibility of helices and their packing near their termini.

**Molecular Modeling of BM2 Proton Channel TM Domain and Structural Features of the Models**—We used the results of cysteine-scanning mutagenesis to guide the construction of a computational model for BM2. We expect mutations of residues that line the pore of the channel or that lie at the helix-helix interface to cause the greatest perturbation to the electrophysiological properties, and less major effects would occur when the mutations occur at the membrane-exposed positions. In this work we used this information in a new approach to structure prediction. A detailed computation modeling procedure is described under "Experimental Procedures" and in the supplemental materials.

We did not expect to find one unique model for BM2. Functional and spectroscopic measurements of the related channel, A/M2 from influenza A virus, have revealed a number of different conformational states associated with its resting, pH-activated, and inhibited states (38); we expected to observe a similar range of conformational states for BM2 ion channel under our experimental conditions. Thus, given that the experimental measurements reflect the properties of the channel in at least two states (low and high pH), one would expect the perturbation analysis to guide the modeling to an ensemble of relevant conformations rather than one single conformation. It is important to note that our computational approach does not consider the effect of solution conditions or the bilayer in the simulations but instead uses a preliminary filter to select backbones that are consistent with the experimental measurements. Thus, it is intended to elucidate possible families of conformations, but the energetic preferences for these conformations will be a function of

![FIGURE 3. Effect of extracellular MTSEA and NEM on the current of BM2 cysteine substitution mutant proteins expressed in oocytes. A, the changes in current after extracellular application of 2.5 mM MTSEA. The inset shows that the increase in current of the F13C mutant ion channel could be reversed by a reductant. B, the changes in current after extracellular application of 1.0 mM NEM. *, not determined. DTT, dithiothreitol.](image-url)
solution conditions. A representative BM2 TM domain model is shown in Fig. 4. The detailed structural features are described in the supplemental materials. Here we will explain these models only briefly.

These models each have left-handed crossing angles ranging from −10° to −30°. The bundles have different shapes, depending on the helical crossing angles and their points of closest approach to the central axis (supplemental Figs. 2 and 5). In these models, all serine side chains (Ser⁹, Ser¹², and Ser¹⁶) are positioned toward the pore of the channel (supplemental Fig. 5). This makes sense physically, since polarity at the N terminus might facilitate the formation of an aqueous pore in the channel. Moreover, the Phe¹³ residue is positioned approximately at the interface between neighboring helices (supplemental Fig. 3) or toward the interior of the pore (supplemental Fig. 4). His¹⁹ and Trp²³ are structurally analogous to the much-studied His³⁷ and Trp⁴¹ in the A/M2 channel. Together, these residues are critical for proton selectivity and gating. As found previously for the corresponding histidine in the A/M2 protein (His³⁷), we found His¹⁹ of the BM2 protein predominantly directed toward the interior of the pore. Depending on the conformation, this residue can either be tightly packed or more loosely packed, allowing hydration of the pore. Similar transitions might be responsible for gating of the channel. In parallel, we observed linked changes in Trp²³, which adopts two predominant rotamers in our models with either the hydrophobic benzenoid 6-member portion of the bicyclic ring directed in or out of the pore region.

Confirmation of the Identity of Pore-lining Residues by Disulfide Bond Formation—Oxidative cross-linking has been used to understand the arrangement of the TM domains of many membrane proteins and to probe structural rearrangements that occur in proteins. This approach has also been used to study the TM domain of the A/M2 protein (17). The principle of this approach is that cysteine residues will be more likely to cross-link in a disulfide bridge if they are in close proximity than if they are not readily accessible to one another. Thus, in a homomeric protein, two cysteines that line the aqueous pore will be more likely to cross-link than two cysteines facing the membrane lipid (17). The BM2 TM pore domain model was tested further by detecting the formation of disulfide bonds in each of the BM2 TM domain cysteine mutant proteins. To facilitate detection of BM2 and disulfide cross-linked BM2 proteins, the BM2 protein was modified by addition of a C-terminal FLAG epitope tag. The proteins were expressed in oocytes and treated with or without CuP (Fig. 5 and supplemental Fig. 6). The resulting BM2 proteins were visualized by immunoblotting using an antibody against the FLAG epitope tag. The proteins were expressed in oocytes and treated with or without CuP (Fig. 5 and supplemental Fig. 6). The expression levels of these three mutants in oocytes were consistently low even when mRNAs prepared at different times were used. However, the low expression level does not affect the interpretation of the data, because the quantitative method used to compare one mutant with another is independent of expression level. In the absence of CuP, all of the mutants and the WT BM2 protein migrated mostly as monomers. Some of the mutants, however, showed formation of dimers. Approximately 20% of I¹⁴C, A¹⁷C, and L¹⁸C were detected as dimers under nonreducing conditions. A small fraction of BM2-S¹²C mutant protein had an electrophoretic mobility consistent with a
trimeric species, possibly involving Cys\textsuperscript{11} found in WT BM2 protein.

When the oocytes were subjected to oxidizing conditions (300 \muM CuP for 10 min at 37 °C) (supplemental Fig. 6), in addition to the anticipated dimeric species, many trimer and higher order oligomeric species were observed, possibly due to the natural cysteine (residue 11), which existed in the TM domain of each mutant protein. Nevertheless, examination of the dimer band of the gels showed that there was considerable dimer formation for BM2-I7C, -S9C, -S12C, -I14C, -S16C, and -W23C before the treatment with CuP (Fig. 5) and the dimer bands increased in intensity for six mutant proteins: BM2-S12C, BM2-F13C, BM2-S16C, BM2-H19C, BM2-W23C, and BM2-H27C after CuP treatment (supplemental Fig. 6). Moreover, when a sinusoidal curve was fitted to the plots of the dimer formation percentage versus residue number and the sum of the oligomer formation percentage versus residue number, the periodicity of each of these plotted sinusoid curves was 3.48 ± 0.1 (supplemental Fig. 7), which is very similar to the periodicity in perturbation index analysis (supplemental Fig. 1). Thus, the results of cross-linking support the experimental conclusions as well as those from molecular modeling.

**DISCUSSION**

Recently, it was shown that the oligomeric state of the active BM2 ion channel protein of influenza B virus is a homotetramer (19). This finding is consistent with the pore of the BM2 channel occupying a space in the middle of the coiled-coil TM domain, a conclusion that is supported by the present work. To have a more complete knowledge of the mechanism for ion channel activity and to form a basis for designing inhibitors for the channel, it is also necessary to identify the residues that line the TM pore. The present investigation employed three experimental approaches to identify these pore-lining residues. Together, these results support the conclusion that three polar residues (Ser\textsuperscript{9}, Ser\textsuperscript{12}, and Ser\textsuperscript{16}) have access to the aqueous solution in the outer region of the pore. One of these residues, Ser\textsuperscript{12}, is adjoined by aromatic residue (Phe\textsuperscript{13}) that might lie in the interface between two subunits of the tetrameric protein, and thus Phe\textsuperscript{13} might have access to the aqueous pore. In addition, one residue lying on the exterior of the pore (Leu\textsuperscript{8}) is accessible to the aqueous phase on the exterior of the virion. Thus, the outer portion of the BM2 TM pore differs greatly from that of the A/M2, which is lined with hydrophobic residues (16, 17, 21). This difference between the A/M2 and BM2 channels is sufficient to explain the inability of amantadine to inhibit BM2 ion channel activity. The present results are also consistent with His\textsuperscript{19} and Trp\textsuperscript{23} occupying pore-lining positions in the BM2 protein. Trp\textsuperscript{23} is not accessible to the water-soluble sulfhydryl reagent MTSEA, when applied extracellularly, possibly because His\textsuperscript{19} occludes the pore. The locations and functions of His\textsuperscript{19} and Trp\textsuperscript{23} of the BM2 ion channel are therefore probably similar to those of the homologous His\textsuperscript{37} and Trp\textsuperscript{41} of the A/M2 ion channel. The evidence supporting the conclusions that the above residues line the pore or have access to the pore of the BM2 ion channel is summarized below.

*Ser\textsuperscript{9}—Three results support the conclusion that this residue is pore-lining: the reversal potential and specific activity of the BM2-S9C mutant ion channel differ from those of the WT ion channel, and the reversal potential and specific activity of the BM2-S9C mutant ion channel differ from those of the WT ion channel. The present results are also consistent with His\textsuperscript{19} and Trp\textsuperscript{23} occupying pore-lining positions in the BM2 protein. Trp\textsuperscript{23} is not accessible to the water-soluble sulfhydryl reagent MTSEA, when applied extracellularly, possibly because His\textsuperscript{19} occludes the pore. The locations and functions of His\textsuperscript{19} and Trp\textsuperscript{23} of the BM2 ion channel are therefore probably similar to those of the homologous His\textsuperscript{37} and Trp\textsuperscript{41} of the A/M2 ion channel. The evidence supporting the conclusions that the above residues line the pore or have access to the pore of the BM2 ion channel is summarized below.

*Ser\textsuperscript{9}—Three results support the conclusion that this residue is pore-lining: the reversal potential and specific activity of the BM2-S9C mutant ion channel differ from those of the WT ion
channel (results 1 and 2), and the BM2-S9C mutant protein forms disulfide-linked dimers (result 3).

Ser$^{12}$—Four results support the conclusion that this residue is pore-lining: applications of MESEA and NEM to the BM2-S12C mutant ion channel result in reduced current amplitude (results 1 and 2); the BM2-S12C mutant protein forms disulfide-linked dimers (result 3); and although replacement of Ser with Cys at position 12 did not alter channel properties, replacement of Ser with Ile resulted in abolition of channel activity (result 4). One possible explanation for the complete inhibition of the BM2 ion channel S12I mutant is that the size of BM2 channel pore is relatively small and cannot accommodate the bulky isoleucine residue (but see “Phe$^{13}$”).

Phe$^{13}$—Four results support the conclusion that this residue has access to the pore: the reversal potential and specific activity of the BM2-F13C mutant protein differ from those of the WT ion channel (results 1 and 2), and the BM2-F13C mutant channel is modified by MTSEA and NEM (results 3 and 4).

Ser$^{16}$—Four results support the conclusion that this residue is pore-lining: the specific activity of the BM2-S16C and BM2-S16I mutant channels are less than that of the WT channel (results 1 and 2); the BM2-S16C mutant channel is modified by NEM (result 3); and the BM2-S16C mutant protein forms disulfide-linked dimers (result 4). It is possible that the fact that the reversal potential of the BMB-S16C mutant ion channel does not differ from that of the WT ion channel is due to the somewhat similar nature of the Ser and Cys side chains.

His$^{19}$ and Trp$^{23}$—In previous studies on the A/M2 ion channel, when MTS reagents were applied from the outside of the oocytes, His$^{37}$ prevented the passage of MTS reagents to the cytoplasmic side of the membrane (16), suggesting that His$^{37}$ acts to obstruct the passage of large molecules through the pore of the A/M2 ion channel. Our finding that BM2 mutant proteins with cysteine internal to His$^{19}$ were unaffected by water-soluble MTSEA is consistent with the interpretation that His$^{19}$ in the BM2 protein is at the position analogous to that of His$^{37}$ in the A/M2 protein. However, it is also possible for modification to occur without effect. Thus, the finding that two mutated amino acids that lie interior to His$^{19}$ were affected by application of smaller, lipid-soluble NEM suggests the interpretation that these residues (Trp$^{23}$ and His$^{37}$) have access to the pore but that MTSEA did not have access to these residues. Two findings suggest that the BM2 Trp$^{23}$ residue is pore-lining: 1) the BM2-W23C mutant protein formed disulfide-linked dimers in the absence of oxidative reagents, and 2) the specific activity and reversal potential of the BM2-W23C mutant ion channel differ from those of the WT ion channel. Since the BM2-H19C mutant ion channel was inactive, its properties could not be studied; however, two observations suggest that this residue is pore-lining: 1) the BM2-H19C mutant protein formed a high percentage of disulfide-linked dimers under oxidative conditions (supplemental Fig. 6), and 2) cysteine mutant proteins internal to His$^{19}$ were not affected by MTSEA but were affected by NEM.

The findings for Ser$^{9}$, Ser$^{12}$, Ser$^{16}$, His$^{19}$, and Trp$^{23}$ agree with the molecular model proposed in that these same residues are predicted by the model to be pore-lining. Finally, these experiments have demonstrated that the predictions of the pore-lining residues made from the sequence alignment of the TM domains of the two channels (see Sequences 1 and 2) is correct.

We attempted to test the above conclusions by constructing a BM2 mutant ion channel that was inhibited by amantadine. In the first construct, Ser$^{9}$, Ser$^{12}$, and Ser$^{16}$ were replaced by the corresponding residues found in the A/M2 ion channel (valine, alanine, and glycine). This mutant protein was expressed at the cell surface but did not have sufficient current to permit characterization (data not shown). We also constructed a chimera in which residues 6–18 of the BM2 protein were replaced by the corresponding residues of the A/M2 protein (Asp$^{74}$ to Leu$^{360}$). This chimeric protein was expressed at the cell surface but had lower specific activity than the WT BM2 ion channel. The currents were partially inhibited by amantadine (data not shown). It is not surprising that neither of these manipulations of the BM2 protein yielded an ion channel with properties identical to the A/M2 ion channel, because A/M2 and BM2 are different proteins, and large perturbations would not be expected to preserve function precisely.

The graph of each ion channel property against residue number was informed by the helical nature of the TM domain of the BM2 protein. Each graph was fitted with a sinusoid having a periodicity of $\sim$3.4 residues. This periodicity is consistent with the TM domain having a left-handed helical coiled-coil structure (21). The finding that Ile replacement of Cys in each of three residues (for Ser$^{9}$, Ser$^{12}$, and Ser$^{16}$) did not alter the periodicity of the fitted sinusoid further supports our expectation that the periodicity analysis is not sensitive to small changes in the individual parameters or even the method used.

We are not able to interpret the results of mutation of Ile$^{7}$, Leu$^{8}$, or His$^{27}$ clearly, because these residues occur near the ends of the TM domain. Previous studies have shown that anomalous results of cysteine-specific chemical modification studies are often observed when the modified cysteine is located at the end of TM helices near the membrane/aqueous compartment interface (39, 40).

Comparison with A/M2 and Other Proton Channels—It is interesting to compare the sequence of BM2 with that of A/M2 and LS2 (41–43), the latter being a four-helix bundle proton channel peptide that was designed from first principles and characterized before the sequence of BM2 had been elucidated (Sequences 3–5). The primary determinants of the pore in each of these peptides occur in a heptad repeat at pore-facing positions “a” and “d”. In LS2, each of these residues is Ser, precisely as in the first half of BM2 (Ser$^{9}$, Ser$^{12}$, and Ser$^{16}$). In LS2, which is a BM2 mutant ion channel that was inhibited by amantadine. In LS2, each of these residues is Ser, precisely as in the first half of BM2 (Ser$^{9}$, Ser$^{12}$, and Ser$^{16}$). In LS2, each of these residues is Ser, precisely as in the first half of BM2 (Ser$^{9}$, Ser$^{12}$, and Ser$^{16}$). In LS2, each of these residues is Ser, precisely as in the first half of BM2 (Ser$^{9}$, Ser$^{12}$, and Ser$^{16}$).
Val27, Ala30, and Gly34 (Fig. 6). Fig. 6 compares the crystallographic model for the closed form of A/M2 with BM2 centroid 5, which is closed at both the top and bottom of the bundle. The presence of small residues, Ala30 and Gly34, near the center of the bilayer gives rise to a large aqueous pore in models of A/M2. Drugs such as amantadine bind in this large apolar cavity. By contrast, the larger Ser side chains decrease the size of the polar cavity of BM2, preventing binding of these channel-blocking drugs.

Although LS2 is a highly selective channel, it is not gated by pH as is observed for A/M2 and BM2. This appears to be achieved in A/M2 and BM2 by exchanging the pore-lining Ser residues of LS2 with the pH-sensing His and Trp pair in the second half of the TM bundle. These residues undergo pH-dependent transitions that appear to block the C-terminal end of the channel in the low pH form and allow opening at lower pH. Additionally, it is interesting to note that in the recently determined crystal structure of the A/M2 TM four-helix bundle, Val27 forms a hydrophobic sphincter that might contribute to selectivity and/or help prevent entry of protons into the N-terminal mouth of the channel. The corresponding residue in BM2 is Ser9, which is not as hydrophobic; instead, the critical residue, Phe13, may serve a similar role. As discussed above, this residue can pack either at the helix–helix interface or within the pore. In conclusion, molecular modeling of the BM2 pore also predicts that the diameter of the aqueous pore at the center of the bilayer is small. The presence of many polar residues lining the BM2 pore, together with its smaller cavity, will present a challenge for the identification of inhibitory molecules.

Conclusion—The results from three different approaches indicated that the following residues are pore-lining or lie near a pore-lining residue: Ser9, Ser12, Phe13, Ser16, His37, and Trp23. The identification of the pore-lining residues of the BM2 ion channel protein required several approaches to be employed, rather than a single approach, as has been sufficient for a number of other ion channels. One reason that several approaches had to be used is that there are no known inhibitors of the BM2 ion channel activity, and thus differences in inhibitor action could not be exploited. Perhaps the knowledge of the pore-lining residues that these experiments have provided will permit an inhibitor to be designed. Such an inhibitor would facilitate the study of the BM2 protein and might lead the way to development of an antiviral drug.

REFERENCES
1. Martin, K., and Helenius, A. (1991) Cell 67, 117–130
2. Pinto, L. H., Holsinger, L. J., and Lamb, R. A. (1992) Cell 69, 517–528
3. Hay, A. J. (1992) Semin. Virol. 3, 21–30
4. Lamb, R. A., Holsinger, L. J., and Pinto, L. H. (1994) in Receptor-mediated Virus Entry into Cells (Wimmer, E., ed) 303–321, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
5. Takeda, M., Pekosz, A., Shuck, K., Pinto, L. H., and Lamb, R. A. (2002) J. Virol. 76, 1391–1399
6. Castrucci, M. R., and Kawaoka, Y. (1995) J. Virol. 69, 2725–2728
7. Hatta, M., Goto, H., and Kawaoka, Y. (2004) J. Virol. 78, 5576–5583
8. Davies, W. L., Grunert, R. R., Haff, R. F., McGahan, J. W., Neumayer, E. M., Paulshock, M., Watts, J. C., Wood, T. R., Herman, E. C., and Hoffman, C. E. (1994) Science 264, 862–863
9. Mould, J. A., Paterson, R. G., Takeda, M., Ohigashi, Y., Venkataraman, P., Lamb, R. A., and Pinto, L. H. (2003) Dev. Cell 5, 175–184
10. Wang, C., Takeuchi, K., Pinto, L. H., and Lamb, R. A. (1993) J. Virol. 67, 5585–5594
11. Wang, C., Lamb, R. A., and Pinto, L. H. (1994) Virolology 205, 133–140
12. Chizhmakov, I. V., Geraghty, F. M., Ogden, D. C., Hayhurst, A., Antoniou, M., and Hay, A. J. (1996) J. Physiol. 494, 329–336
13. Schnell, J. R., and Chou, J. J. (2008) Nature 451, 596–599
14. Schnell, J. R., and Chou, J. J. (2008) Nature 451, 591–595
15. Gandhi, C. S., Shuck, K., Lear, J. D., Dieckmann, G. R., DeGrado, W. F., Lamb, R. A., and Pinto, L. H. (1999) J. Biol. Chem. 274, 5474–5482
16. Shuck, K., Lamb, R. A., and Pinto, L. H. (2000) J. Virol. 74, 7755–7761
17. Bazer, C. M., Pinto, L. H., Cross, T. A., and Lamb, R. A. (1999) Virolology 254, 196–209
18. Sakaguchi, T., Tu, Q., Pinto, L. H., and Lamb, R. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5000–5005
19. Balannik, V., Lamb, R. A., and Pinto, L. H. (2008) J. Biol. Chem. 283, 4895–4904
20. Paterson, R. G., Takeda, M., Ohigashi, Y., Pinto, L. H., and Lamb, R. A. (2003) Virolology 306, 7–17
21. Pinto, L. H., Dieckmann, G. R., Gandhi, C. S., Papworth, C. G., Braman, J., Shaughnessy, M. A., Lear, J. D., Lamb, R. A., and DeGrado, W. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11301–11306
22. Kovacs, F. A., Denny, J. K., Song, Z., Quine, J. R., and Cross, T. A. (2000) J. Mol. Biol. 295, 117–125
23. Song, Z., Kovacs, F. A., Wang, J., Denny, J. K., Shekar, S. C., Quine, J. R., and Cross, T. A. (2000) Biophys. J. 79, 767–775
24. Wang, J., Kim, S., Kovacs, F., and Cross, T. A. (2001) Protein Sci. 10, 2241–2250
25. Nishimura, K., Kim, S., Zhang, L., and Cross, T. A. (2002) Biochemistry 41, 13170–13177
26. Tian, C., Tobler, K., Lamb, R. A., Pinto, L. H., and Cross, T. A. (2002) Biochemistry 41, 11294–11300
27. Tian, C., Gao, P. F., Pinto, L. H., Lamb, R. A., and Cross, T. A. (2003) Protein Sci. 12, 2597–2605
28. Shimbo, K., Brassard, D. L., Lamb, R. A., and Pinto, L. H. (1996) Biophys. J. 70, 1336–1346
29. Mould, J. A., Drury, I. E., Frings, S. M., Kaupp, U. B., Pekosz, A., Lamb, R. A., and Pinto, L. H. (2000) J. Biol. Chem. 275, 31038–31050
30. Venkataraman, P., Lamb, R. A., and Pinto, L. H. (2005) J. Biol. Chem. 280, 21463–21473
31. Beumer, T. L., Veenstra, G. J., Hage, W. J., and Destree, O. H. (1995) Trends Genet. 11, 9
32. Crick, F. H. (1953) Acta Crystallogr. 6, 685–689
33. North, B., Summa, C. M., Ghirlanda, G., and DeGrado, W. F. (2001) J. Mol. Biol. 311, 1081–1090
34. Xiang, Z., and Honig, B. (2001) J. Mol. Biol. 311, 421–430
35. Schwiters, C. D., Kuszewski, J. J., Tjandra, N., and Clore, G. M. (2003) J.
36. Lin, T. I., and Schroeder, C. (2001) *J. Virol.* 75, 3647–3656
37. Holmgren, M., Liu, Y., Xu, Y., and Yellen, G. (1996) *Neuropharmacology* 35, 797–804
38. Pinto, L. H., and Lamb, R. A. (2006) *J. Biol. Chem.* 281, 8997–9000
39. Kaplan, R. S., Mayor, J. A., Brauer, D., Kotaria, R., Walters, D. E., and Dean, A. M. (2000) *J. Biol. Chem.* 275, 12009–12016
40. Ma, C., Kotaria, R., Mayor, J. A., Eriks, L. R., Dean, A. M., Walters, D. E., and Kaplan, R. S. (2004) *J. Biol. Chem.* 279, 1533–1540
41. DeGrado, W. F., Wasserman, Z. R., and Lear, J. D. (1989) *Science* 243, 622–628
42. Lear, J. D., Wasserman, Z. R., and DeGrado, W. F. (1988) *Science* 240, 1177–1181
43. Lear, J. D., Wasserman, Z. R., and DeGrado, W. F. (1994) in *Membrane Protein Structure* (White, S. H., ed) 335–354, Oxford University Press, New York