The Oligomeric State of the Active BM2 Ion Channel Protein of Influenza B Virus*

Victoria Balannik, Robert A. Lamb, and Lawrence H. Pinto

Received for publication, November 16, 2007, and in revised form, December 10, 2007. Published, JBC Papers in Press, December 11, 2007, DOI 10.1074/jbc.M709433200

Influenza A virus and influenza B virus particles both contain small integral membrane proteins (A/M2 and BM2, respectively) that function as a pH-sensitive proton channel and are essential for virus replication. The mechanism of action of the M2 channels is a subject of scientific interest particularly as A/M2 channel was shown to be a target for the action of the antiviral drug amantadine. Unfortunately, an inhibitor of the BM2 channel activity is not known. Thus, knowledge of the structural and functional properties of the BM2 channel is essential for the development of potent antiviral drugs. The characterization of the oligomeric state of the BM2 channel is an essential first step in the understanding of channel function. Here we describe determination of the stoichiometry of the BM2 proton channel by utilizing three different approaches. 1) We demonstrated that BM2 monomers can be chemically cross-linked to yield species consistent with dimers, trimers, and tetramers. 2) We studied electrophysiological and biochemical properties of mixed oligomers consisting of wild-type and mutated BM2 subunits and related these data to predicted binomial distribution models. 3) We used fluorescence resonance energy transfer (FRET) in combination with biochemical measurements to estimate the relationships between BM2 channel subunits expressed in the plasma membrane. Our experimental data are consistent with a tetrameric structure of the BM2 channel. Finally, we demonstrated that BM2 transmembrane domain is responsible for the channel oligomerization.

Influenza A and B viruses are enveloped viruses with a genome consisting of eight segments of negative-strand RNA. RNA segment 7 of both influenza A and B viruses encodes the M2 integral membrane protein (A/M2 and BM2, respectively) that functions as a pH-sensitive proton channel and is essential for virus replication (1–5). A/M2 and BM2 proteins both consist of a small N-terminal ectodomain (24 and 7 residues, respectively), a single transmembrane domain, 19 amino acids long, and a cytoplasmic tail (54 and 83 residues, respectively) (5). Although A/M2 and BM2 proteins have similar structural and functional properties, the only homology between their amino acid sequences is found in the HXXXW motif of the membrane-spanning region. This motif plays a critical role in the ion channel activity (4, 6–8). The A/M2 channel activity is inhibited by the antiviral drug amantadine (9, 10) but unfortunately, amantadine does not inhibit the BM2 channel activity (5, 11).

The oligomeric state of the A/M2 channel had been investigated by several approaches. First, A/M2 was shown to form homo-oligomers, most likely homotetramers, by cross-linking and sedimentation experiments (12, 13). Site-specific mutagenesis studies demonstrated that the oligomeric structure of A/M2 channels is stabilized by disulfide bonds (14). Finally, by functional analysis of mixed oligomers of known composition, the active oligomeric form of A/M2 protein was shown to be a tetramer (15). Most recently the atomic structure of the A/M2 transmembrane region shows it forms a four-helix bundle (16).

Little is known about BM2 structural and functional properties. The only available evidence for the stoichiometry of the BM2 channel comes from chemical cross-linking data that indicates BM2 can be chemically cross-linked to dimers, trimers, tetramers, and higher order complexes, data interpreted to suggest that the most likely oligomeric state of the channel is a homotetramer (5). Unlike for A/M2, where disulfide bonds in the ectodomain stabilize the homotetramer (14) BM2 does not contain cysteines in its ectodomain. Thus, the lack of a BM2 disulfide bond raises the possibility that the BM2 channel assembly differs from that of A/M2 (5).

As knowledge of the active oligomeric state of a channel is an essential first step in understanding the mechanism of action of a channel, we determined the stoichiometry of the influenza B virus proton channel by utilizing three different approaches. In addition, we have demonstrated that A/M2 and BM2 proteins do not form heteromeric channels, when coexpressed and that the BM2 transmembrane domain is responsible for the oligomerization process.

EXPERIMENTAL PROCEDURES

Molecular Biology, in Vitro cRNA Transcription, and Transient Expression of Plasmids in HEK293-cultured Cells—The cDNAs encoding influenza virus A/Udorn/72 A/M2 protein, the B/Lee/40 BM2 protein and chimeric cDNAs were inserted into pGEMHJ (a gift from N. Dascal, Tel-Aviv University, Israel) for the expression in Xenopus oocytes, or the cDNAs were inserted into the pCAGGS (17) for expression in HEK293 cells. Generation of the BM2(H19C) construct was described previously (4). The epitope tags FLAG (DYKDDDDK), HA

* This work was supported by National Institutes of Health Research Grant AI-57363 (to L. H. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 An Investigator of the Howard Hughes Medical Institute.
2 To whom correspondence should be addressed: Dept. of Neurobiology and Physiology, Hogan Hall, 2205 Tech Drive, Northwestern University, Evanston, IL 60208-3500. Tel.: 847-491-7915; Fax: 847-491-5211; E-mail: larry-pinto@northwestern.edu.
(YPYDVPDY), and Myc (EQKLISEEDL) were inserted in-frame at the N terminus after the first methionine residue or at the C terminus after the last amino acid of BM2 using the PCR extension method. The tagged subunits were indistinguishable from the respective wild-type subunits based on ion channel activity measured in oocytes. Chimeras between A/M2 and BM2 were generated using the PCR overlap extension method. For the generation of BM2-CFP and BM2-YFP constructs the DNA fragments encoding cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were prepared from pCFP-C1 and pYFP-C1 plasmids (Clontech, Palo Alto, CA) by standard PCR, the amplified fragments were linked to the C terminus of BM2 with the helical linker LAAEAAKEAAA (18) using the PCR extension method and the final products were cloned into the appropriate plasmids. Cloning details are available upon request. The regions amplified by PCR were verified by double-strand DNA sequencing. In all cases numbering of amino acids starts from the first methionine residue of the open reading frame. For expression in oocytes, plasmids were linearized with HindIII, and capped cRNA was transcribed in vitro using T7 RNA polymerase (mMessage mMACHINE; Ambion, Austin, TX). The quality of transcripts was assessed by agarose gel electrophoresis and ethidium bromide staining and analytical UV spectroscopy. HEK293 cells (American Type Culture Collection, Manassas, VA) were grown on 35-mm or 60-mm dishes in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Transfections were done on 30–50% confluent cultures using TransIT transfection reagent (Mirus Bio Corporation, Madison, WI) with 3 μg/dish total plasmid cDNA.

Heterologous Expression and Electrophysiological Recordings—Stage V–VI Xenopus laevis oocytes were prepared as described previously (19). Oocytes were injected with 5–10 ng of cRNA in 50 nl/oocyte, and assayed 2–3 days later. Two electrode voltage clamp recordings were carried out using TEV-200 (Dagan, Minneapolis, MN) connected to Digidata 1440A and pCLAMP10 (Axon Instruments, Foster City, CA). Oocytes were superfused with Barth’s solution containing (in mM) 8 8

For co-immunoprecipitation assays oocytes were homogenized in ice-cold solubilization buffer (15 μl/oocyte) containing (in mM) 50 Tris-HCl pH 8.0, 150 NaCl, 1% Triton X-100, and protease inhibitor mixture. Homogenates were incubated for 30 min on ice and centrifuged for 10 min at 14,000 rpm. The soluble fraction was collected and incubated with 4 μg/ml of anti-Myc polyclonal antibody (Abcam, Cambridge, MA) overnight at 4 °C, loaded on prewashed Protein A-agarose beads (Sigma) and incubated for 4 h at 4 °C with shaking. Samples were washed twice with ice-cold solubilization buffer and bound proteins were released by 5 min of boiling with SDS-PAGE loading buffer, collected after centrifugation, and loaded on a 17% SDS-PAGE gel. Western blot analysis was performed and precipitated proteins were detected with appropriate antibodies. All other protein samples from HEK293 cells or oocytes which were subject to Western blot analysis were homogenized in the appropriate solubilization buffers described above. Primary antibodies and their respective working dilutions were: 14C2 (anti-A/M2) monoclonal antibody 1:1000 (20), anti-BM2 polyclonal antibody 1:600 (21), anti-FLAG monoclonal antibody 1:2000 (Sigma), anti-Myc polyclonal antibody 1:1000 (Abcam, Cambridge, MA). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG (Jackson Laboratories, West Grove, PA) were used at 1:20,000 dilution. Blots were visualized using a Storm 860 BioImager (Molecular Dynamics, Buckinghamshire, UK) and quantified using ImageJ software.

Imaging Techniques, FRET Efficiency Measurements, and Distance Calculation—For FRET experiments HEK293 cells were cultured on coverslips in 35 mm dishes and co-transfected with BM2-CFP- and FLAG-BM2-YFP-expressing plasmids as described above. 24 h after transfection cells were fixed and mounted on slides as described previously (28). A Zeiss LSM510 META ConfoCor3 system with a 63 × 1.4 numerical aperture oil-immersion objective was used for image acquisition. Images were collected at 8 bit resolution over 512 × 512 pixels at a pixel dwell time of 12.8 μs. The argon laser line of 458-nm was used to excite CFP and the line of 514-nm to excite and bleach YFP. To minimize the impact of bleaching due to imaging, the images were taken with both laser lines at 0.15% of full laser power. To prevent cross-talk images were obtained in a configuration described previously (28). FRET was measured by acceptor photobleaching method (donor dequenching), where an increase in CFP fluorescence during an incremental photobleaching of YFP was observed. Total YFP bleaching was achieved by repetitive (3 × 20 iterations) scanning of a region of interest (ROI) using the 514-nm laser line at 100% intensity. Background contamination of both fluorophores was quantified from the cell-free area and subtracted from the overall intensity. The overall intensity was measured from the ROI at the outer perimeter of the cell in order to restrict the analysis to...
the assembled channels at the plasma membrane. Mean fluorescent intensities were measured using FRET Plus (Version 4.0.6, Zeiss) software. FRET efficiencies (E) were calculated from the ratio of the normalized donor (CFP) fluorescence intensity before (I_{D/A}) and after (I_p) the photobleaching of acceptor (YFP) in Equation 1.

\[
E = 1 - \frac{I_{D/A}}{I_p} \quad \text{(Eq. 1)}
\]

**Computation of the FRET Efficiency for Dimeric Channels**

Based on Equation 1, the FRET efficiency for channels consisting of two subunits can be presented as Equation 2,

\[
E = 1 - \frac{I_{(\text{donor-donor})} + (I_{(\text{donor-acceptor})} - I_{\text{transferred}})}{I_{(\text{donor-donor})} + I_{(\text{donor-acceptor})}} \quad \text{(Eq. 2)}
\]

where \(I_{(\text{donor-donor})}\) is the fluorescence intensity of the population of donors that form donor-donor dimeric complexes; \(I_{(\text{donor-acceptor})}\) is the fluorescence intensity of the population of donors that form donor-acceptor dimeric complexes and \(I_{\text{transferred}}\) is the fluorescent intensity transferred from donors to acceptors in the donor-acceptor complexes. Because \(I_{(\text{donor-donor})}\) and \(I_{(\text{donor-acceptor})}\) are defined as intensities of the populations of donors, they can be presented as Equations 3 and 4,

\[
I_{(\text{donor-donor})} = p^2k \quad \text{(Eq. 3)}
\]

\[
I_{(\text{donor-acceptor})} = 2p(1 - p)k \quad \text{(Eq. 4)}
\]

where \(p^2\) is a probability of the formation of dimers consisting of two donor subunits, and \(2p(1 - p)\) is a probability of the formation of dimers consisting of one donor and one acceptor subunit. Those probabilities can be obtained from the binomial distribution models for each donor-acceptor ratio expressed in subunit. Those probabilities can be obtained from the binomial distribution models for each donor-acceptor ratio expressed in subunit. Those probabilities can be obtained from the binomial distribution models for each donor-acceptor ratio expressed in subunit. Those probabilities can be obtained from the binomial distribution models for each donor-acceptor ratio expressed in subunit.

Then \(I_{\text{transferred}}\) can be defined as Equation 5,

\[
I_{\text{transferred}} = 2p(1 - p)x \quad \text{(Eq. 5)}
\]

where \(x\) stands for the amount of energy transferred from the donor to the acceptor in a single donor-acceptor pair. Thus, based on Equation 2, the FRET efficiency can be presented as Equation 6,

\[
E = 1 - \frac{pk + 2k(1 - p) - 2x(1 - p)}{pk + 2k(1 - p)} \quad \text{(Eq. 6)}
\]

according to Equation 6.

The FRET efficiencies (E) were experimentally calculated using Equation 1 for each donor-acceptor coexpressed ratio. If BM2 channel protein consists of two subunits, according to Equation 7

\[
\frac{1}{E} = \left(\frac{p^2}{2p(1 - p)}\right)x + \frac{k}{x} \quad \text{(Eq. 7)}
\]

the relationship between \(1/E\) and the ratio between the probabilities of donors to form homomeric dimers to the probabilities of donors to form heteromeric dimers \(\left(\frac{p^2}{2p(1 - p)}\right)\) will be linear for the coexpressed donor-acceptor ratios.

**Intersubunit Distance Calculations**

FRET efficiencies from the experiment shown in Fig. 4B were used to estimate the distances between the adjacent and diagonally opposite subunits. The FRET efficiency measured for the protein ratio of 1 BM2-CFP and 9 FLAG-BM2-YFP was assumed to represent the FRET efficiency of one donor and three acceptor channel configuration. Consider a single donor molecule in the presence of a number \(k\) of acceptors, located at distances \(r_1, \ldots, r_k\) from the donor, according to the Förster model in Equation 8,

\[
E = 1 - \left\{1 + \sum_{i=1}^{k} \left(\frac{R_0}{r_i}\right)^6\right\}^{-1} \quad \text{(Eq. 8)}
\]

where \(R_0\) is a Förster distance, a physical constant that determines a distance corresponding to the 50% FRET efficiency for the specific donor-acceptor pair. For the CFP/YFP pair \(R_0\) was found to be 4.92 nm, assuming a relative dipole orientation factor \(k^2\) of 2/3 \(\times 10^{12}\). The \(R_0\) range was not estimated experimentally because we were not interested in the measurements of absolute distances between the fluorophores, but rather to support our interpretations concerning the BM2 channel geometry.

Thus the total FRET efficiency for tetrmeric consisting of one donor and three acceptor subunits is given by Equation 9,

\[
E = 1 - \left\{1 + 2\left(\frac{R_0}{R_{\text{adj}}}\right)^6 + \left(\frac{R_0}{R_{\text{diag}}}\right)^6\right\}^{-1} \quad \text{(Eq. 9)}
\]

where \(R_{\text{adj}}\) and \(R_{\text{diag}}\) are distances within adjacent and diagonally opposite donor-acceptor pairs.

If we assume that for this channel configuration energy transfer form the donor to the diagonally opposite acceptor is negligible, \(R_{\text{adj}}\) can be calculated according to Equation 10.

\[
R_{\text{adj}} = R_0\sqrt{2(E^{-1} - 1)} \quad \text{(Eq. 10)}
\]

The value of \(R_{\text{adj}}\) can be used to calculate the FRET efficiency between the adjacent subunits in Equation 11.

\[
E_{\text{adj}} = 1/(1 + (R_{\text{adj}}/R_0)) \quad \text{(Eq. 11)}
\]

If we assume that FRET efficiency measured from cells expressing 9 BM2-CFP to 1 FLAG-BM2-YFP protein ratio is mainly contributed by channels with three donors and one acceptor subunit configuration, the total efficiency for this configuration can be presented as Equation 12.

\[
E = \frac{2}{3}E_{\text{adj}} + \frac{1}{3}E_{\text{diag}} \quad \text{(Eq. 12)}
\]

Then the distance between the diagonally opposite subunits is given by Equation 13.

\[
R_{\text{diag}} = R_0\sqrt{(3E - 2E_{\text{adj}})^{-1} - 1} \quad \text{(Eq. 13)}
\]

The relation between \(R_{\text{adj}}\) and \(R_{\text{diag}}\) was compared with that expected for a quadratic arrangement of subunits by using the Pythagorean theorem in Equation 14.

\[
\frac{R_{\text{diag}}}{R_{\text{adj}}} = \sqrt{2} \quad \text{(Eq. 14)}
\]
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RESULTS

BM2 Can Be Chemically Cross-Linked to the Tetrameric State—Unlike the A/M2 protein, the BM2 protein migrates on a non-reducing SDS-PAGE gel as a monomer of ∼15 kDa (5, 15) (Fig. 1). As no stable BM2 oligomers are detected under non-reducing conditions, chemical cross-linking of BM2 monomers can be indicative of the oligomeric state of the channel. We examined the cross-linking characteristics of BM2 monomers using three homobifunctional amino-reactive cross-linkers with different spacer arm lengths. BM2 protein was modified such that it contained the FLAG epitope tag at its C terminus (BM2-FLAG). HEK293 cells transiently expressing BM2-FLAG were homogenized in a solubilization buffer as discussed under “Experimental Procedures” and incubated with DTSSP (0–5 mM) for 30 min at room temperature. The reactions were quenched by 10 mM Tris, pH 7.5 for 15 min at room temperature. Cross-linked samples were separated on a 12% non-reducing SDS-PAGE gel, immunoblotted, and detected with an anti-FLAG antibody. Black arrows on the right indicate monomers, dimers, trimers, and tetramers respectively.

Some less well resolved higher molecular mass complexes were detected on the blot. As the majority of these latter species disappeared upon protein dilution (data not shown) we suggest that these complexes can result from a nonspecific intramolecular cross-linking of proteins. Graded formation of tetrameric protein complexes as a function of increasing cross-linker concentrations suggests that the BM2 channel oligomeric state is a tetramer. Although this finding is consistent with our knowledge about the A/M2 oligomeric state (15) and confirms suggestions regarding the BM2 oligomeric state made by Paterson et al. (5), the data do not prove that BM2 channels function as tetramers.

BM2 Wild Type and BM2(H19C) Non-functional Subunits Form Tetramers in Which Two Non-functional Subunits Are Sufficient to Eliminate the Channel Activity—To investigate further the oligomeric state of the functional BM2 channel we used the mixed oligomer strategy (23). BM2 channels allow proton flow upon the acidification of the extracellular medium (4). Substitution of the pore lining residue His-19 with Cys (BM2(H19C)) results in the formation of non-functional channels (4). We expressed BM2 wild-type proteins and BM2(H19C) proteins in Xenopus oocytes and measured the activity of the coexpressed channels by two electrode voltage clamp. Both constructs were tagged with a C-terminal FLAG (F) epitope for immunodetection and the wild-type BM2 construct was also tagged with the N-terminal HA (H) epitope tag (H-BM2-F and BM2(19C)-F, respectively). The channel activity of H-BM2-F construct was indistinguishable from that of the non-tagged BM2 (Fig. 2A, left), whereas BM2(H19C)-F mutant homomeric channels did not conduct current (Fig. 2A, right). The surface expression of both homomeric channels was found to be similar (Fig. 2B).

Previous studies have shown that the subunit stoichiometry of some membrane complexes can be determined by combined measurements of protein function and surface expression for mixed oligomers, consisting of subunits with different phenotypes (15, 23). This approach requires that there is a dominance of one subunit phenotype, while it is assumed that both subunits are equally and independently expressed and assemble stochastically (23). To apply this approach to BM2 channels, we examined the ability of H-BM2-F and BM2(H19C)-F subunits to form mixed oligomers by coexpression in Xenopus oocytes, followed by current measurements and surface expression quantification. The ratio of coexpressed subunits was monitored by the different gel mobilities of H-BM2-F and BM2(H19C)-F subunits (Fig. 2B). If the H-BM2-F and BM2 (H19C)-F subunits co-assemble randomly, the influence of the non-functional BM2(H19C)-F subunit on the heteromeric channel activity will follow a binomial distribution (W(n) = Σ\(p^n(1-p)^{n-1}\)). Fig. 2C (upper part) demonstrates possible permutations of subunit assembly for tetrameric BM2 channels (models a–f). If we assume that each channel expresses either the wild-type or the mutant phenotype (no intermediate phenotypes), then the assembly models can be used to predict the total fraction of channels that express either the mutant or the wild-type phenotype. Fig. 2C (lower part) shows a series of Activity Models (1–4), each of which assigns the mutant phenotype to a different subset of the six possible subunit configurations. Model 1 assumes that the inclusion of one or more BM2(H19C)-F non-functional subunits is sufficient to convert the phenotype of the assembled channels from fully conducting to non-conducting. In Model 2 two or more non-functional subunits are sufficient to convert the phenotype. Models 2A and 2B address the case in which two non-functional subunits influence the heteromeric state of the channel.
channel activity as a function of their relative location in the tetramer. Model 3 assumes that a minimum of three BM2(H19C)-F subunits are required to convert the phenotype, whereas according to the Model 4 only when all four subunits are mutant, the channel fails to conduct current. We recorded currents from oocytes expressing H-BM2-F and BM2(H19C)-F subunits in various ratios. In Fig. 2D the amplitudes of the currents were normalized to the activity of homomeric H-BM2-F channels and plotted against the fraction of H-BM2-F subunits measured from the relative band intensities (Fig. 2B). The experimental data were compared with the predicted Activity Models (Fig. 2D gray curves) and the coexpression results were best described by Model 2 (Fig. 2D). This finding indicates that if the active oligomeric state of BM2 channel is a tetramer, two or more non-functional subunits are required in order to eliminate channel activity. The goodness-of-fit for the various Activity Models was assessed using $\chi^2$ statistics (Fig. 2E).

Fig. 2, C and D present the distributions and the Activity Models for the tetrameric configuration only, since based on the preliminary data (5, 15) and chemical cross-linking results (Fig. 1) this is the most likely oligomeric state of BM2 channels. However, we also fitted the experimental data to the Activity Models predicted for some other possible oligomeric states (dimers through octamers). Using $\chi^2$ statistics we rejected all the alternative models ($p < 0.05$), except for the dimeric channel model, consisting of one wild-type and one mutated subunit with dominant negative phenotype ($\chi^2 = 0.7362, p = 0.3909$). The results of the mixed oligomer expression assay strongly support the tetrameric structure of BM2 channels, although they do not exclude the dimeric conformation.

**BM2 Proteins Tagged With CFP or YFP Fluorophores Form Active Oligomeric Channels**—To distinguish rigorously between a dimeric or tetrameric oligomer of BM2 we used the fluorescence resonance energy transfer measurement approach. CFP and YFP were fused to the C terminus of BM2 subunits through a 15 amino acid helical linker yielding BM2-CFP and BM2-YFP subunits. BM2-YFP subunit was also epitope-tagged by addition of an N-terminal FLAG tag, yielding F-BM2-YFP. To verify that fusion with the fluorescent proteins did not affect the functional properties of the channel, modified BM2 proteins were homomERICally expressed in Xenopus oocytes and whole cell
currents were recorded. Fig. 3A demonstrates that both fusion proteins formed active homomeric channels. The expression of BM2-CFP and F-BM2-YFP proteins in HEK293 cells was confirmed by Western blot analysis under reducing and non-reducing conditions (Fig. 3, B and C). As shown in Fig. 3B under reducing conditions both fused constructs had gel mobilities consistent with their predicted mass (42–45 kDa). Under non-reducing conditions bands corresponding to monomers and SDS-resistant dimers, trimers and tetramers of BM2-CFP and F-BM2-YFP were observed (Fig. 3C, white triangles), suggesting that both constructs were successfully expressed in HEK293 cells and form oligomers.

FRET Measurements Indicate 4-Fold Symmetry of BM2 Channels—According to the binomial distribution, coexpression of BM2-CFP and FLAG-BM2-YFP subunits in various ratios will produce channels with mixed fluorescent labeling. Those channels can be a subjected to a FRET assay. Because FRET efficiency (E) tapers off by the sixth power of the distance separating the donor and the acceptor, relative to a characteristic distance R₀ (see below) (24–26), and depends on the donor/acceptor ratio within the oligomeric channel, the efficiency would be higher for a donor that can transfer energy to multiple equidistant acceptors compared with a single acceptor (25, 27–29). Then, by comparing the FRET efficiencies from cells expressing various ratios of CFP- and YFP-labeled BM2 subunits, it would be possible to distinguish between dimeric and tetrameric channel configurations.

Fluorescent signals were measured using confocal microscopy. The fluorescent signals from BM2-CFP and FLAG-BM2-YFP co-expressing cells, measured either by CFP or YFP excitation, were limited to the outer perimeter of the cell indicating a localization of assembled channels on the plasma membrane (Fig. 4A). FRET efficiency (E) was calculated from the ROI at the outer perimeter of the cell, using the acceptor photobleaching technique. In this approach the acceptor signal is bleached in the YFP channel by a repetitive scanning of the ROI at high laser intensity (“Experimental Procedures”) and donor fluorescent intensity is measured before and after the acceptor bleaching. The increase in donor fluorescent intensity as a result of acceptor bleaching can be directly related to the FRET efficiency (30–32).

If BM2 channels function as dimers, FRET will occur only in complexes consisting of one donor-labeled and one acceptor-
labeled subunit. However FRET efficiencies calculated from donor quenching experiments would be influenced by the fluorescence signals coming from channels consisting of two donor-labeled subunits (Equation 2). As the distance between the labeled subunits within a dimer is constant regardless of the protein expression ratios, the dependence of the FRET efficiencies on the subunit composition of dimers can be theoretically predicted (Equations 2–7). According to Equation 7, $1/E$ values relate linearly to the ratios between the probabilities of donor-labeled subunits to form homomeric dimers to the probabilities of the donors to form heteromeric dimers for all coexpressed protein ratios tested. However, this will not hold if the BM2 channel protein is a tetramer, since, for cells co-expressing various donor/acceptor ratios, both the fraction of channels with maximal number of acceptors per donor molecule and the distribution of distances between donors and acceptors will change. Fig. 4B shows that FRET efficiency calculated for cells expressing various ratios of CFP- and YFP-labeled subunits increased from 19.7 ± 1.3% ($n = 23$) to 43.3 ± 2.7% ($n = 26$) as a function of the amount of acceptor-labeled subunits expressed. The ratios between the BM2-CFP and FLAG-BM2-YFP proteins expressed in cells were derived from quantifying the amount of each protein after Western blot analysis (data not shown). When the values of $1/FRET$ efficiencies ($1/E$) were plotted against the ratios between the probabilities of donors to form homomeric dimers to the probabilities of donor to form heteromeric dimers (according to Equation 7), the relationship between those variables was not linear, contrary to the expectation for dimers (Fig. 4C, $R$-square = 0.610).

If BM2 channels are tetramers with a 4-fold rotational symmetry, the distance between adjacent and diagonally opposite donor-acceptor pairs should follow the Pythagorean theorem. Distances between adjacent and diagonally opposite subunits can be estimated from FRET efficiencies measurements (27, 28, 33–35). When the ratio between coexpressed CFP- and YFP-labeled proteins is 1:9 (this ratio was the lowest that allowed donor protein quantification from immunoblots), according to the binomial distribution 85% of FRET contributing tetramers are arranged in the one donor to three acceptors configuration. Thus, we assume that for the 1 BM2-CFP: 9 FLAG-BM2-YFP protein ratio the FRET efficiency is mainly contributed by channels consisting of one donor and three acceptors. Because FRET signals decrease by the sixth power of distance, total FRET efficiency is dominated by energy transfer between adjacent subunits. Based on this property, we assumed that for tetramers consisting of one donor and three acceptors the contribution of the diagonally opposite acceptor to the apparent FRET efficiency would be negligible (27, 28, 34, 35). Thus, the distance between adjacent fluorophores ($R_{adj}$) was calculated to be ~5.8 nm (Equation 10).

Distance estimation between the diagonally opposite subunits of the tetramer becomes possible if the efficiency of energy transfer for the tetramers consisting of three donors and one acceptor is known. 85% of FRET contributing complexes in cells with a 9 BM2-CFP: 1 FLAG-BM2-YFP protein ratio are arranged in a three donors and one acceptor configuration. Although the contribution of this configuration to the total FRET efficiency is lower then the contribution of other mixed tetramers, channels with three donors and one acceptor will be prevalent on the membrane. We assumed, though, that the FRET signal measured for the 9:1 protein ratio was mainly contributed by channels with three donors and one acceptor. Total FRET efficiency for this configuration is defined as a sum of the relative efficiencies of $E_{adj}$ and $E_{diag}$ (Equation 12). Using the above value of $R_{adj}$, $E_{adj}$ was calculated (Equation 11). Then the distance between the diagonally opposite subunits ($R_{diag}$) was derived from Equation 5 and was calculated to be ~8.4 nm (Equation 13).

The relationship between the calculated adjacent and diagonal distances within a tetramer followed the Pythagorean equation (Equation 14), indicating a 4-fold symmetry of BM2 channels.

*The BM2 Transmembrane Domain Is Involved in the Channel Oligomerization Process*—The above experiments demonstrated that the BM2 channel functions as a tetramer. To permit later identification of regions of the BM2 protein that mediate channel oligomerization we first tested the ability of BM2 subunits to form functional oligomers with A/M2 proteins. We coexpressed the non-functional BM2(H19C)-F mutant subunits and the wild-type A/M2 subunits in various ratios in *Xenopus* oocytes and measured the channel activity by two electrode voltage clamp. As BM2(H19C) homomeric channels do not conduct current, whereas A/M2 homomers are fully functional, if co-oligomerization did occur, the activity of heteromeric channels would follow the same Activity Models presented for the coexpression of the BM2(H19C) and the wild-type BM2 proteins (Fig. 2). Fig. 5A shows that both proteins (A/M2 and BM2(H19C)-F) were efficiently expressed in oocytes. The current amplitudes obtained from oocytes that coexpressed the non-functional BM2 subunits and the A/M2 subunits were directly proportional to the amount of A/M2 proteins expressed. The relationship between the normalized currents and A/M2 protein amounts was well-fitted with a linear fit ($R$-square = 0.989) (Fig. 5B). The currents were efficiently inhibited by the A/M2 channel-specific inhibitor amantadine, regardless the coexpressed protein ratio. However, Sakaguchi *et al.* (15) demonstrated that only the channels consisting of four amantadine sensitive A/M2 subunits are inhibited by amantadine. Thus, the linear dependence of the channel activity in the amount of A/M2 protein together with the full amantadine sensitivity of recorded currents indicates that BM2 subunits do not co-oligomerize with A/M2 subunits.

Having established that A/M2 and BM2 proteins do not co-oligomerize, we further divided the A/M2 and BM2 proteins into N-terminal (NTD), transmembrane (TMD) and C-terminal (CTD) domains and generated two chimeric proteins (ABB and ABA) between the A/M2 and BM2 subunits (Fig. 6, left). When the chimeric proteins ABB or ABA were expressed alone or coexpressed with wild-type BM2 subunits in *Xenopus* oocytes, functional channel activity was observed.

To map the BM2 protein domains that participate in its oligomerization, ABB and ABA chimeras were coexpressed with Myc-tagged BM2 (myc-BM2) and examined by coprecipitation assays (Fig. 6). As a control coprecipitation of myc-BM2 either with BM2-FLAG or with A/M2 was tested. The coexpressed proteins were first immunoprecipitated with anti-Myc anti-
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FIGURE 5. Coexpression of A/M2 wt subunits with BM2(H19C)-F non-functional subunits does not result in the formation of active hetero-oligomers. A, relative protein expression of A/M2 and BM2(H19C)-F. Xenopus oocytes were injected with A/M2 and BM2(H19C)-F cRNAs in various ratios. Following current measurements, oocytes were homogenized, separated on a 17% SDS-PAGE gel, immunoblotted, and reacted with anti-A/M2 or anti-FLAG antibodies as indicated. The signal intensity was measured for each blot and normalized to the intensity of the homomerically expressed protein. B, normalized channel activity (black circles) was plotted against the relative A/M2 protein amounts calculated in A. The experimental data are the average of two independent experiments. Each point (±S.D.) is a mean from 12 to 15 oocytes. The channel activity for each subunit composition was normalized to the activity of the A/M2 homomeric channels and presented as % Activity. The experimental data were fitted with a linear fit (R-square = 0.989).

FIGURE 6. Coexpression of BM2 wt with A/M2/BM2 chimeras suggests the involvement of BM2 transmembrane domain in channel oligomerization. Left, schematic composition of coexpressed subunits. Gray bars correspond to BM2 sequences and white bars to A/M2 sequences. NTD represents the first 7 amino acids for BM2 and the first 24 amino acids for A/M2. TM corresponds to the 19 amino acid long transmembrane domain for BM2 and A/M2, CTD corresponds to the cytoplasmic tail, 83 amino acids long for BM2 and 54 amino acids long for A/M2. The numbers above the chimeras indicate the residues forming the respective junction. Epitope tags are shown at the appropriate position for each construct. The bars are grouped in pairs according to the coexpression pattern. Right, the results of the coimmunoprecipitation assays for each coexpressed pair. Following current measurements Xenopus oocytes expressing mixed oligomers were homogenized in solubilization buffer and immunoprecipitated with anti-Myc antibody. T, the total protein fraction loaded on the beads; P, the precipitated fraction. Equal amounts of these fractions were separated on 17% SDS-PAGE gels, immunoblotted, and reacted with anti-Myc, anti-A/M2, or anti-FLAG antibodies as indicated.

body followed by immunoblotting either using anti-FLAG or anti-A/M2 antibody. As expected, BM2-FLAG proteins efficiently coprecipitated with Myc-BM2, whereas no coimmunoprecipitation was observed for A/M2 protein (Fig. 6, right). Under the same conditions both ABB and ABA chimeras coprecipitated with Myc-BM2 (Fig. 6, right). The coprecipitation efficiencies were not estimated, because different antibodies were used for the detection of coprecipitated proteins. Coprecipitation of the chimeric proteins with Myc-BM2 indicates their ability to interact with the wild-type BM2 and possibly to form functional oligomers though interactions between the BM2 transmembrane domains.

DISCUSSION

Determination of the subunit stoichiometry of influenza B virus BM2 channel protein is critical to the understanding of its structural and functional properties. Despite the similar functional characteristics of the influenza A and B proton channels the two proteins are encoded in the influenza virus RNA genomes by very different strategies and thus it cannot be assumed that the two channels have the same oligomeric state. In this study we employed three independent experimental approaches to provide evidence that the oligomeric state of the BM2 protein is a tetramer. First, we showed that BM2 monomers can be efficiently cross-linked by the amino-reactive cross-linker DTSSP to dimers, trimers and tetramers. Second, by applying the mixed oligomer approach we demonstrated that functional BM2 channels are most likely arranged as tetramers, although the dimeric configuration cannot be excluded with this approach alone. Third, FRET efficiency measurements between BM2 subunits labeled with CFP and YFP fluorophores excluded the possibility of dimeric BM2 arrangement and provided additional evidence for the tetrameric structure of BM2 channel. In addition we demonstrated that although A/M2 and BM2 proteins have similar structural and functional properties they cannot form a heteromeric channel and that the oligomerization of homomeric BM2 channel is mediated by its transmembrane region.

Unlike in A/M2 proteins (14), BM2 oligomers are not stabilized by disulfide bonds (Fig. 1 and Ref. 5). Therefore, the detection of BM2 oligomers on SDS-PAGE gels was possible only when the protein was treated with an amino-reactive chemical cross-linker. It was found that DTSSP cross-linked efficiently the BM2 protein (Fig. 1) the largest cross-linked complex being consistent with the presence of a BM2 tetramer. However this observation does not reveal the oligomeric state of the functional channel complex.
The second approach for the determination of the BM2 protein oligomeric state took advantage of the mixed oligomer assay. In contrast to the previous approach, the mixed oligomer strategy allows subunit stoichiometry determination of functional channels, expressed on the cell membrane (15, 23). For this approach, BM2 functional (wild-type BM2) and non-functional (BM2(H19C)) subunits were coexpressed in various ratios, and the channel activity of coexpressed complexes was measured and compared with theoretical activity models. The experimental data were best fitted to a tetrameric model in which two non-functional subunits are sufficient to eliminate channel activity (Fig. 2). The coexpression data were also compared with the phenotype expression models for other possible channel oligomeric states. Because the non-functional BM2 subunit did not show the dominant negative phenotype for tetrameric models (Fig. 2D), the interpretation of the experimental data could not exclude the possibility of a dimer channel configuration, in which one mutant subunit converts the channel phenotype. Taking into consideration that BM2 monomers have a single transmembrane region, the dimeric architecture would be unfavorable for the pore formation. However, to provide the evidence that the channel is not a dimer we measured FRET efficiency between BM2 subunits labeled by either CFP or YFP fluorophores.

FRET efficiency is strongly dependent on the distance between the donor and the acceptor proteins and on the donor/acceptor ratio within an oligomeric channel (25, 27–29). Thus, if the oligomeric state of the BM2 channel is a dimer, the distance between the donor-labeled and the acceptor-labeled subunits will remain constant regardless the donor-acceptor ratios coexpressed in the cells, and the dependence of the FRET efficiencies in the subunit compositions of the channels could be easily predicted (Equations 2–7). The experimentally measured FRET efficiencies significantly increased as a function of the fraction of acceptor-labeled subunits expressed (Fig. 4B), but when presented as (1/E) those efficiencies failed to follow the linear dependence model predicted for dimers (Fig. 4C). Based on the results of the FRET assay, the tetrameric configuration, which agrees with the results of the other two assays presented, was accepted as the BM2 configuration.

To test our conclusion regarding the BM2 channel stoichiometry, we used FRET efficiency measurements presented above for the estimation of the distances between the adjacent and the diagonally opposite donor-acceptor pairs attached to BM2 subunits. The application of FRET to the distance measurements between donor/acceptor pairs is widely used both for the determination of the intersubunit conformational rearrangements within membrane proteins (27, 33, 34) and for the estimation of the subunit stoichiometry of membrane proteins (28, 35). The distance within the adjacent donor/acceptor pair in the BM2 channel was estimated to be ~5.8 nm, while for the diagonally opposite pair the estimated distance was ~8.4 nm. The relationship between the calculated adjacent and diagonal distances within the BM2 tetramer followed the Pythagorean equation (Equation 14), indicating a 4-fold symmetry of BM2 channels. This finding is consistent with the tetrameric arrangement of the functional BM2 protein.

For the estimation of the distances between the fluorophore labeled BM2 subunits we assumed that, based on the strong distance dependence of the FRET efficiency, the contribution of the diagonally located acceptor to the apparent FRET for channels with multiple acceptors and a single donor fluorophores is negligible (27, 28, 34, 35). In those studies that required accurate measurements of intersubunit distances to monitor conformational rearrangements, the experimental verification of this assumption is critical for the correct interpretation of the results. This verification is usually done by constructing tandem protein complexes of restricted subunit composition and comparing of the FRET signals between complexes with linked and non-linked subunits (27, 34). Unfortunately we could not apply this strategy with BM2 channels, as tandem formation is not possible for type III membrane proteins (36). However, it is important to mention that our main interest in the intersubunit distance estimation was not to measure the absolute distances between the channel subunits, but rather to support our interpretations concerning the square architecture of the BM2 channels.

It is also important to emphasize, that the above estimated distances do not apply to the wild-type BM2 channel dimensions, since the measurements refer to the distances between the fluorophore molecules attached to the C-terminal domain of the BM2 subunits. We also assumed that the helical linker that connects the ectodomain of BM2 protein to the fluorophore has restricted flexibility, so that the 4-fold symmetry observed for the fusion proteins does indeed reflect the conformation of the BM2 channel.

In this study we also assessed the ability of the A/M2 and the BM2 channels subunits to form hetero-oligomeric channels. Using the mixed oligomer coexpression assay once again, we demonstrated that despite structural and functional similarities between the A/M2 and BM2 channels, no functional heteromeric complexes were formed (Fig. 5). We were interested, though, to identify the domains of the BM2 protein that mediate the selective assembly of the BM2 channels. For this purpose two chimeric proteins in which either the N-terminal domain or both the N-terminal and the C-terminal domain of the BM2 protein were changed to the corresponding domains from the A/M2 protein were constructed and their channel properties were examined. In electrophysiological assays both chimeras were found to be functional either as homo-oligomers or when coexpressed with wild-type BM2 proteins. The ion channel properties of both chimeric channels were identical to those of the wild-type BM2 channel, indicating that, as the BM2 transmembrane domain was the minimal preserved region between the two chimeras, this domain alone is sufficient for the determination of the channel properties. Coexpression of the chimeras with the wild-type BM2 protein followed by co-immunoprecipitation assays, demonstrated that the BM2 transmembrane domain also mediates the preferential assembly of BM2 proteins (Fig. 6). These findings are consistent with previously published data that show that the synthetic A/M2 peptides corresponding to the A/M2 protein transmembrane domain are capable of forming functional channels in planar lipid bilayers and liposomes (37, 38), although the same A/M2 peptide fails to form functional A/M2 channels in the hetero-
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Oligomeric expression systems (39). Taken together these observations led us to conclude that BM2 channel properties, like the A/M2 channel properties, are strongly dictated by the membrane spanning region of the BM2 protein.

The tetrameric structure of the BM2 channel proteins suggests a channel architecture in which the channel pore is surrounded by four protein bundles. Such architecture is consistent with the findings about the A/M2 channel pore structure (40, 41). As the most striking functional difference between A/M2 and BM2 proteins lies in their sensitivity to amantadine, the investigation of the BM2 protein pore structure is essential for the understanding of this difference and may provide insights for the rational anti-viral drugs design.

Acknowledgments—We thank Dr. Y. Ohigashi and Dr. J. Widom for critical reading of the manuscript and we thank the Biological Imaging Facility staff for help with the FRET imagery.

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