Influenza A M2 Channel Oligomerization is Sensitive to its Chemical Environment

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This PDF file includes:

Main Text
Figures 1 to 5
Abstract

Viroporins are small viral ion channels that play important roles in the viral infection cycle and are proven antiviral drug targets. Matrix protein 2 from influenza A (AM2) is the best characterized viroporin, and the current paradigm is that AM2 forms monodisperse tetramers. Here, we used native mass spectrometry, ion mobility spectrometry, and size-exclusion chromatography to characterize the oligomeric state of full-length AM2 in a variety of different pH and detergent conditions. Unexpectedly, we discovered that AM2 formed a range of different oligomeric complexes that were strongly influenced by its local chemical environment. The monodisperse tetramer was only observed in select conditions when the antiviral drug, amantadine, was added. Native mass spectrometry of AM2 in lipid nanodiscs with different lipids showed that lipids also affected the oligomeric states of AM2. Finally, nanodiscs uniquely enabled measurement of amantadine binding stoichiometries to AM2 in the intact lipid bilayer. These unexpected results reveal that AM2 forms a wider range of oligomeric states than previously thought possible, which provides new potential mechanisms of influenza pathology and pharmacology.

Significance Statement

Many viruses contain small ion channels called viroporins that play diverse roles in viral infections. Influenza A M2 (AM2) is the best characterized viroporin and the target of the antivirals amantadine and rimantadine. Although past structural studies showed AM2 was a monodisperse tetramer, we discovered that AM2 forms polydisperse and dynamic oligomers that are highly sensitive to their local chemical environment. Our findings provide a new perspective on the structure and mechanisms of AM2 that may extend to other viroporins.

Main Text

Introduction

Viroporins are a class of small transmembrane proteins that oligomerize to form channels in membranes. Found in a range of different viruses, they are involved at multiples stages of infection, including budding, replication, assembly, and uncoating. Matrix protein 2 from influenza A (AM2) is a multifunctional viroporin and a clinically approved drug target for amantadine and rimantadine. AM2 is made up of three regions, the extracellular domain, the transmembrane (TM) domain, and the cytosolic tail (Figure 1A). The 20-residue single-pass TM domain of AM2 is necessary and sufficient for oligomerization and formation of a pH-mediated ion channel. There are several dozen X-ray or NMR structures of the AM2 TM domain in a variety of membrane mimetics, all depicting monodisperse homotetramers. Despite the uniform oligomeric state, there are significant differences among many of the AM2 structures, and the membrane mimetic used to solubilize AM2 can have major influences on its structure. However, traditional structural biology techniques are limited in their ability to study oligomeric polydispersity, so these existing structures may not capture the full range of structures possible.
for AM2. Indeed, earlier fluorescence resonance energy transfer studies suggested that dimer might be the minimal proton-conducting unit for the full length AM2 in living cells.\textsuperscript{12}

Native mass spectrometry (MS) has emerged as a powerful technique for studying the oligomerization of membrane proteins.\textsuperscript{13-15} For conventional native MS of membrane proteins, the entire protein-micelle complex is ionized with electrospray ionization (ESI).\textsuperscript{13} The detergent adducts are then removed from the protein using collision induced dissociation (CID), and the mass of the bare membrane protein complex reveals the protein stoichiometry and noncovalent ligands that remain bound (Figure 1). Other membrane mimetics, such as nanodiscs, allow membrane proteins to be solubilized in lipid bilayers during native MS.\textsuperscript{13,16,17} Thus, native MS provides rich information and can capture the polydispersity of membrane proteins in different lipid and detergent environments.

Here, we performed native MS on full-length AM2 in both detergents and nanodiscs. Based upon the existing structures, we predicted that AM2 would form robust tetramers. However, we discovered that AM2 assembled into a range of oligomeric states from dimer to hexamer. Further investigation showed that the oligomeric state of AM2 was heavily influenced by the membrane environment, solution pH, and drug binding. Together, these results reveal that AM2 is likely more polydisperse than previously suggested, and it is highly sensitive to its chemical environment.

**Results**

**AM2 Oligomerization is Sensitive to Detergent and pH**
Our initial goal was to investigate drug binding to AM2 using native MS. Based on prior studies, we expected to find a monodisperse AM2 tetramer. However, initial results immediately revealed a more complex oligomeric state distribution. To identify conditions that would promote the formation of a monodisperse tetramer, we performed native MS on full length AM2 to quantify the oligomeric state distribution (Figure 1) in a range of different conditions. We screened different detergents by exchanging AM2 into solution containing tetraethylene glycol monoocetyl ether (C8E4), lauryldimethylamine oxide (LDAO), n-octyl-β-D-glucopyranoside (OG), n-dodecyl-phosphocholine (DPC), n-dodecyl-β-maltoside (DDM), and lauryl maltose neopentyl glycol (LMNG). We selected detergents that have been previously used for AM2 structural biology studies, including OG and DPC, as well as detergents that are commonly-used for native MS, such as C8E4, LDAO, and DDM. LMNG was selected for additional structural diversity. For each detergent, we tested pH 5, 7, and 9, which encompass the pH conditions that have been previously investigated with AM2. Selected spectra are shown in Figure 2 with oligomeric state distributions for all plotted in Figure S1.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Representative native mass spectra with the deconvolved mass spectra in the inset of AM2 solubilized in (A) C8E4 at pH 5, (B) LDAO at pH 7, (C) OG at pH 9, (D) LDAO at pH 5, (E) DDM at pH 7, and (F) LMNG at pH 9. Each detergent is shown above the spectrum. Average oligomeric state distributions collected in triplicate are shown in Figure S1.
We began by investigating C8E4, which is commonly used for native MS because it is easy to dissociate from membrane proteins.\textsuperscript{27,28} At all pH conditions tested for C8E4, AM2 showed a polydisperse mixture of oligomers that ranged from dimers to hexamers (Figures 2A and S1). The precise oligomeric state distribution varied substantially between replicate measurements, indicating variable and relatively nonspecific oligomerization. When it was diluted at pH 5, AM2 shifted to lower oligomeric states, but it retained higher order oligomers upon dilutions at pH 9 (Figure S2). Overall, AM2 in C8E4 was relatively polydisperse and not heavily influenced by the pH.

In contrast, the oligomeric state of AM2 was more monodisperse and highly dependent on pH when it was solubilized in LDAO. At pH 6 and below, AM2 in LDAO was almost exclusively hexameric, with a small amount of pentamer present (Figures 3A and S3). However, at pH 7, AM2 in LDAO formed a polydisperse mixture from dimers to hexamers (Figures 2 and 3C). At pH 8 and 9, AM2 was less polydisperse than at neutral pH, forming primarily tetramer with a significant amount of trimer (Figure 3D and E). In contrast with C8E4, these more monodisperse oligomers at pH 5 and 9 remained intact upon dilution, further confirming their specificity (Figure S4). Overall, AM2 formed more specific complexes in LDAO detergent, and the oligomeric states were strongly influenced by the pH.

The pH also had strong influences on the oligomerization of AM2 in DDM (Figure S3 and S5). At pH 7 and below, AM2 was primarily a mixture of tetramers and pentamers. At pH 9, AM2 in DDM was predominantly trimer with significant amounts of dimer and tetramer (Figure S3). These oligomers also remained intact upon dilution (Figure S5). In contrast, the solution pH did
not appear to have a strong influence on the oligomerization of AM2 in OG and DPC detergents (Figure S1). In LMNG, AM2 preferred dimer and trimer at both pH 5 and 9 but was not stable at pH 7 (Figures S1P–Q).

Overall, there were no conditions where we found exclusively tetramer. Instead, we discovered that AM2 oligomerization is influenced by both its detergent environment and solution pH. Depending on the conditions, AM2 can form either highly variable and polydisperse oligomers or relatively specific oligomers with a range of different sizes. Interestingly, the most stable and monodispese oligomer we found was the hexamer in LDAO under acidic conditions (Figure 3A).

Orthogonal Measurements Confirm Oligomeric Sensitivity

Native MS gives accurate relative quantitation for similar species across narrow m/z ranges, but differences in ionization, transmission, and detector efficiency make quantitation across wide m/z ranges difficult. To help rule out instrumental biases, we repeated select measurements using a mass spectrometer with a different type of detector. Both the Orbitrap and time-of-flight (ToF) detectors gave similar results (Figures S6 and S7), which support our qualitative conclusions.

Although both instruments showed similar oligomeric state distributions, we cannot rule out differences in ionization efficiency that could skew the distribution measured by native MS. To further confirm our results, we performed size-exclusion chromatography (SEC) with AM2 in select conditions. It is challenging to directly compare the elution times between different detergents because the micelle sizes can vary. However, qualitative comparisons of the chromatograms of AM2 in different conditions agreed with native MS results. Conditions with a wide range of oligomers in native MS, such as C8E4 at pH 9, had broader SEC peaks and more variability between replicate injections (Figure S8). In conditions where AM2 was more monodisperse, such as LDAO at pH 5, we saw narrower and more reproducible peaks. Thus, although there may be some quantitative differences between the native MS results and the solution concentrations, these data help confirm the qualitative descriptions of the oligomeric state distributions and how they change in response to the chemical environment.

Finally, we used ion mobility-mass spectrometry to measure the collisional cross section (CCS) of some of the complexes (Figure S6). We modeled potential structures assuming oligomerization of the transmembrane domain and disordered soluble domains. Our experimental CCS values agreed with modeled gas-phase structures, where the disordered regions collapse. Our results also matched predicted CCS values for globular proteins of a similar size, and the observed charge states are also consistent with a compact structure. Together, these results point to compact oligomers consistent with oligomerization in the transmembrane domain. Based on the observed charge states and CCS values, we can rule out highly extended oligomeric structures.

Drug Binding Can Remodel AM2 Oligomers
We next measured drug binding with amantadine, a clinically-approved inhibitor of AM2, by adding amantadine at different concentrations in all the detergent and pH conditions. Interestingly, we discovered a shift in the oligomerization when amantadine was added to AM2 in C8E4 at pH 9. At low concentrations of amantadine, AM2 formed a range of variable oligomers. At higher concentrations of amantadine, AM2 shifted towards relatively monodisperse tetramers (Figure 4). A similar trend was observed on the ToF platform (Figure S9). We also compared binding with the drug-resistant S31N mutant of AM2 under the same conditions. Even at high concentrations of amantadine, there were no major changes in the oligomeric state of AM2 S31N. Interestingly, the S31N mutant also had a different oligomeric state distribution without added drug, suggesting the mutant may be unable to bind the drug because it is locked more tightly into a non-binding pentameric state.

![Figure 4](image)

**Figure 4** The average oligomeric state of AM2 wild type (A-E) and drug-resistant S31N (F-J) with 0 µM (A, F), 19 µM (B, G), 37 µM (C, H), 75 µM (D, I), and 150 µM (E, J) amantadine added. Both AM2 WT and S31N were solubilized in C8E4 at pH 9.

One important limitation of these experiments is that we only observed shifts in the oligomeric state distribution in C8E4 detergent at pH 9. It has been previously found that amantadine preferentially binds under basic conditions, so it is not surprising that we only measured changes at higher pH. The lack of response in other detergents may be because these detergents cause AM2 to form oligomers with lower drug binding affinity or oligomers with stronger protein-protein interactions that are not easily altered by drug binding. AM2 shows the least oligomeric specificity in C8E4, so this set of conditions is perhaps most susceptible to shifts in the oligomeric state distribution caused by drug binding.

Another limitation is that only very small signals for bound drug were observed, despite the high concentrations added and clear shifts in the oligomeric state distribution induced by drug binding. The lack of signal from bound drug is likely due to gas-phase dissociation of the drug inside the mass spectrometer, where the activation required to remove the detergent micelle also likely removes the small (151 Da) bound drug. In any case, most AM2 structures have amantadine or an analogous AM2 inhibitor added, and our data suggest that the addition of
inhibitors may help shift AM2 from a polydisperse mixture to a monodisperse tetramer more suitable for structural biology.\textsuperscript{18,36,37}

**AM2 in Nanodiscs Shows Lipid Sensitivity and Drug Binding**

After screening AM2 in a range of detergent and pH conditions, we characterized its oligomerization in lipid bilayers by assembling AM2 into nanodiscs of different lipid types at a 4:1 ratio of AM2 per nanodisc. Using the shifts in the overall mass of the nanodisc measured by native MS, as well as mass defect analysis, we determined the stoichiometry of AM2 embedded within the intact nanodiscs (Table S1).\textsuperscript{38,39} We first incorporated AM2 into 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) nanodiscs, which showed AM2 stoichiometries from two through six (Figure 5A). We then incorporated AM2 into 1,2-dimyristoyl-sn-glycero-3-phosphorylglycerol (DMPG) nanodiscs, which showed less incorporation for the AM2 and only stoichiometries of one, two, or three within the nanodisc (Figure 5B). In both lipids, the incorporation of AM2 into the nanodisc showed a nonspecific distribution of oligomers. In contrast, when AM2 was incorporated into 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) nanodiscs, it incorporated with stoichiometries of only one and four, which shows that AM2 forms specific tetramers in DPPC bilayers under these conditions (Figure 5D). The increased oligomeric specificity in DPPC nanodiscs may be due to the increased thickness or saturation of the lipid bilayer.\textsuperscript{40,41}

We next added adamantane to the DPPC nanodiscs and measured drug binding by native MS. Without adamantane, there were two clear mass defect distributions for monomer and tetramer, respectively. Upon adding 40 µM adamantane, the mass defect of the monomer did not shift, confirming that monomeric AM2 did not bind the drug. However, there were clear shifts in the mass defect of nanodiscs with AM2 tetramers. The first shift corresponded to AM2 tetramers with one adamantane bound (Figure 5E and Table S2). Interestingly, there was also a second shift in the mass defect that corresponded to AM2 tetramer with four adamantane bound. At 80 µM adamantane, the relative intensity of the single-bound state diminished, and the four-bound state became more abundant. These data agree with existing structures that show AM2 can have one drug bound at lower concentration and four drugs bound at higher concentrations.\textsuperscript{37,42} Specifically, the allosteric binding site located at the helix interface has been previously shown by solution NMR.\textsuperscript{37} Surface plasmon resonance experiments further demonstrated the coexistence of pore binding and allosteric binding sites in AM2.\textsuperscript{42} Recent high-resolution X-ray crystal structures showed that adamantane binds specifically to the pore of the AM2 channel at a one drug per channel ratio at low drug concentrations.\textsuperscript{20} At high drug concentrations, rimantadine, an amantadine analog, also binds non-specifically to the AM2 helix interface at a four drug per channel ratio.\textsuperscript{42} DMPC nanodiscs also showed shifts characteristic of drug binding, but the more complex oligomeric state distribution prevented conclusive assignments.

To confirm specificity of drug binding, we incorporated drug-resistant AM2 S31N into DPPC nanodiscs (Figure 5C). AM2 S31N assembled into DPPC nanodiscs in stoichiometries of one, two, and three, suggesting that the mutant did not form specific complexes. Thus, the oligomerization of AM2 S31N appears to be different from the wild type in both detergent and
nanodiscs (Figures 4 and 5). Importantly, AM2 S31N nanodiscs did not show any mass defect shifts upon addition of amantadine, confirming specificity of drug binding.

**Figure 5.** Native MS intensities as a function of normalized mass defect versus mass for (all except C) wild type and (C) S31N AM2 in nanodiscs with (A) DMPC, (B) DMPG, (C–F) DPPC lipids. (E) 40 µM and (F) 80 µM amantadine (AMT) were added, and shifts of the tetramer from the dashed reference line indicate 1 or 4 AMT bound. Illustrations to the upper left indicate observed stoichiometries, which are circled and annotated.

**Discussion**

Here, we used native MS to study the oligomerization of wild type and drug-resistant AM2 in different pH conditions, detergents, lipid bilayers, and with added drug. In all the detergent and pH combinations screened, AM2 had different patterns of oligomerization, which reveals two key conclusions. First, AM2 is not exclusively a tetramer. Second, AM2 is highly sensitive to its chemical environment, showing different oligomeric states in different pH and lipid/detergent conditions.
There are two potential interpretations of these surprising results. On one hand, it may be that the tetramer is the true physiological state of AM2. In this case, our results reveal that it is very challenging to capture the pure tetramer in detergent and even lipid bilayers. Even in cases where tetramer was the predominant form, it was not the only oligomer present. Native MS could thus guide structural biology to find the few select conditions that favor the true physiological oligomer. Our results could be a warning that AM2 and other viroporins may be very sensitive to their detergent or lipid environment. AM2 has a higher propensity to form tetramers in DPPC nanodiscs compared to detergent micelles, which might help guide future biophysical studies of viroporins such as AM2 and the SARS-CoV-2 E protein.

However, another interpretation of our results is that the oligomeric states of AM2 are more complex than previously thought. It is very challenging to measure the oligomeric state distribution for small membrane proteins like this, especially if they form polydisperse oligomers.\textsuperscript{43} Past studies may have underestimated the true polydispersity due limitations of the analysis techniques. Furthermore, most structural studies have been conducted in the presence of high drug concentrations, which may bias the drug towards a monodisperse tetramer, as we saw here (Figure 4). Native MS, despite the potential biases outlined above, provides a direct analysis of the oligomeric state distribution of AM2 that could reveal previously unseen oligomers. Past native MS studies have shown similar oligomeric pore-forming proteins, such as the mechanosensitive channel of large conductance (MscL),\textsuperscript{44} also form polydisperse oligomeric complexes that are sensitive to the local chemical environment.

These results present several new hypotheses for AM2 structure and function in a physiological context. First, AM2 is known to be activated by lower pH.\textsuperscript{45} Our results in LDAO detergent propose that this may be aided by shifts in oligomeric state distribution (Figure 3). Other detergents do not show as clear of a shift, but higher oligomers are preferred at lower pH in several different conditions. It may be that AM2 forms smaller oligomers at neutral pH, but acidic conditions in the endosome trigger formation of larger oligomeric pores that cause the influenza virus to fuse with the endosomal membrane and release the nucleic acid cargo for replication.\textsuperscript{46} Our results also suggest that changes in the lipid environment may affect the oligomerization of AM2 (Figure 5). DPPC nanodiscs showed specific tetramers whereas DMPC nanodiscs showed relatively nonspecific complexes. The thickness and fluidity of the lipid bilayer are known to influence AM2 activity, and these functional changes may be due, in part, to changes in the oligomeric state distribution.\textsuperscript{40,47}

Finally, our results propose a new potential mechanism of drug activity where the drug may pull the equilibrium of the system from polydisperse oligomers towards monodisperse tetramers. It may still block the channel directly or by inducing conformational changes, but it has the added effect of altering the oligomeric state distribution. Similar effects of AM2 stabilization by drug binding have also been observed in solution and solid-state NMR studies.\textsuperscript{18,48,49} Clearly, extensive future studies will be required to test all these hypotheses, but our results shed new light on AM2 oligomerization and prompt a fresh perspective on its mechanisms that may extend to other viroporins.
These experiments also mark a technical milestone in using native MS to measure drug binding to a membrane protein in an intact lipid bilayer. High-resolution native MS enabled detection of a 151 Da drug bound to a roughly 150 kDa intact nanodisc complex containing a polydisperse mixture of lipids and AM2. We were able to simultaneously determine the stoichiometry of the bound drug as well as which AM2 oligomer it was binding. Importantly, nanodiscs seemed to better preserve the drug bound complex inside the mass spectrometer than detergent micelles, which were unable to capture much of the bound drug. We suspect that the nanodisc better protects the protein-drug complex by preserving the membrane protein in its surrounding lipid bilayer.

In conclusion, we discovered that AM2 is more polydisperse than previously thought and is heavily influenced by both the pH and the surrounding membrane environment. In some conditions, AM2 assembles into specific complexes, but others create a dynamic mixture of oligomers. Overall, the application of new analytical approaches revealed unexpected biophysical insights into the polydispersity and pharmacology of AM2 that may have implications for the structures and functions of other viroporins.

Materials and Methods

Preparation of AM2 in Different Detergents and pH

Full-length AM2 was expressed and purified as previously described, and details are provided in the Supporting Information. A series of ammonium acetate solutions were first adjusted to pH 4, 5, 6, 7, 8, and 9 with acetic acid or ammonium hydroxide. All detergents were purchased from Anatrace. Each detergent solution was created by adding twice the critical micelle concentration (CMC) of the detergent to the ammonium acetate solution at each pH. AM2 was exchanged into each of these detergent solutions using Bio-Spin 6 columns (Bio-Rad) and diluted to a final concentration of 50 µM prior to analysis in the relevant solution, except where different concentrations are noted. For drug binding experiments, amantadine (Sigma Aldrich) was diluted to 1.5, 0.75, 0.375, and 0.188 mM in water. 0.5 µL of amantadine was added to 4.5 µL of AM2, for a final drug concentration of 150, 75, 37.5, and 18.8 µM. Mixtures were incubated with amantadine for 5–10 minutes prior to analysis.

Nanodisc Assembly and Sample Preparation

AM2 nanodiscs were assembled using a 4:1 AM2 to nanodisc ratio. For nanodiscs containing DMPC and DMPG lipids, the lipids (Avanti Polar Lipids) solubilized in cholate (Sigma Aldrich) were added at an 80:1 ratio of lipid to membrane scaffold protein (MSP). Details on MSP expression and purification are provided in the Supporting Information. For nanodiscs containing DPPC lipids, the lipids were added at a 90:1 ratio of lipid to MSP. All nanodiscs were assembled overnight by adding Amberlite XAD-2 hydrophobic beads (Sigma Aldrich) at the phase transition temperature of the lipid. To isolate nanodiscs containing AM2 from empty nanodiscs, nanodiscs were purified using a HisTrap HP 1 mL column (GE Healthcare). The column was equilibrated with buffer containing 40 mM Tris, 0.3 M NaCl, and 20 mM imidazole at pH 7.4. AM2 nanodiscs were then eluted from the column with buffer containing 40 mM Tris, 0.3 M NaCl, and 400 mM
imidazole at pH 7.4. Nanodiscs were then concentrated and purified on a Superose 6 10/300 GL (GE Healthcare) equilibrated with 0.2 M ammonium acetate. For all nanodisc drug binding experiments, 1 µL of 400 or 800 µM drug were added to 9 µL of nanodiscs for final drug concentrations of 40 and 80 µM. These samples were allowed to incubate for ten minutes at room temperature prior to analysis.

**Native Mass Spectrometry**

Native MS was performed using a Q-Exactive HF Orbitrap (Thermo Scientific, Bremen) mass spectrometer with ultra-high mass range modifications except where noted as a Synapt XS Q-ToF mass spectrometer (Waters Corporation, Manchester). The native mass spectra were deconvolved and quantified using UniDec, and macromolecular mass defect analysis was used to quantify the stoichiometries of AM2 and amantadine in nanodiscs. Full details are provided in the Supporting Information.

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**Data Availability**

Native mass spectra, ion mobility data files, SEC chromatograms, and modeled structures have been deposited in Figshare (DOI: 10.6084/m9.figshare.14558079.v1).

**References**


