Preprint Not A journal Science Review

(aka PNAjS Review)

Influenza A M2 Channel Oligomerization is Sensitive to its Chemical Environment

Julia A. Townsend, Henry M. Sanders, Amber D. Rolland, James S. Prell, Jun Wang, Michael T. Marty

https://www.biorxiv.org/content/10.1101/2021.05.07.443160v1

EDITORIAL COMMENTS

Reviewers agree that this is an excellent showcase of state of the art native MS as applied to membrane proteins. The detection of a small drug bound in the complex with the membrane is an impressive technical achievement. There is some concern that these experiments may teach us more about the limitations of native MS than about AM2 function specifically; even in face of that concern, this manuscript is valuable. The key technical considerations that merit further caveats/discussion in the manuscript are:

- contrasting how insertion into detergent/nanodisc vs. translation and incorporation into “real” membranes might affect the results

- given differences in native mass spec and biases about certain oligomers flying, etc better - is there any orthogonal metric to use to calibrate how each oligomer might be biased or to calibrate the reproducibility
    - See especially this comment by Reviewer #3: The authors offer two interpretations of their data in the discussion: 1) that it is very challenging to capture the pure tetramer 2) that the oligomeric states of AM2 are more complex than previously thought. The former is unlikely to have any physiological relevance while the latter could have important implications for development of novel therapeutics. A third interpretation could also be that the oligomeric profile observed is a byproduct of the native MS technique utilized. This manuscript would be much more impactful if this study included experiments to differentiate between these possibilities.

- the concentration dependence (of AM2 and of detergents) of the results

James Fraser (UCSF)

Note: I solicited some reviews and am acting as an “editor” and authenticator of their expertise to preserve their anonymity. Happy to facilitate any interactions between authors, reviewers, or any other interested party.

REVIEWER #1

In this study, Townsend and colleagues utilize native-state mass spectrometry to characterize the oligomeric state distribution of matrix protein 2 from influenza A (AM2) in response to varying environmental conditions and pharmacologic agents. AM2 is a well characterized viroporin, which are small transmembrane proteins which oligomerize into ion-conducting channels during viral infection. Viroporins are clinically validated drug targets, and investigating the structural and mechanistic properties of viroporins is important for understanding their roles in the viral replication cycle and could aid future drug discovery.

Most prior structural insights into AM2 have been obtained by X-ray crystallography or NMR. This manuscript adds to this structural investigation of AM2 by using native-state MS to investigate AM2 oligomeric states in the solution state and in nanodiscs, which could better reflect the physiologic membrane context. Their key findings are that 1) AM2 adopts a range of oligomeric states (monomers to hexamers) and 2) the distribution of these oligomers vary depending on environmental conditions (lipid composition, pH), small-molecule inhibitors, and mutations. The relative quantification of AM2 oligomer polydispersity is uniquely enabled by the authors' use of native-state MS. This contrasts with the predominantly tetrameric state that has been appreciated from prior structural studies of AM2. The authors' findings present a compelling case for investigators to employ careful experimental design and data interpretation when working on AM2/viroporins and other dynamic and oligomeric proteins. The implications of this polydispersity on AM2 function and viral replication remain unknown. Insights into the energetics and dynamics of interconversion of these oligomers, and application to other viroporin homologs are also areas for future investigation.

The manuscript is written clearly and the researcher's rationale and methods are described in detail. Specific comments are listed below:

1. How were the equilibration time and temperature of the samples for native-state MS analysis chosen? These two parameters (among others) can have significant effects on the population distribution of oligomers observed.

2. Page 5, first paragraph. "*The precise oligomeric state distribution varied substantially between replicate measurements, indicating variable and relatively nonspecific oligomerization*.".

    a. Could the authors provide some context/examples on this variation between replicates? For most figures, a representative spectra or an average with error bars (with no individual data points noted) are presented.

    b. Could the authors comment what implications the observed replicate variability would have on their interpretations of AM2 polydispersity?

    c. Could the authors explain why they conclude that the oligomerization is driven by relatively non-specific interactions? Prior structures of AM2, at least of the tetramer, show a symmetric oligomer with specific contacts being made at the interface between the monomers to form a conducting pore. Would the authors expect the interactions in the non-tetrameric states to be similar to or different from those observed in the tetramer?

3. Were oligomers/aggregates larger than hexamers observed?

4. In Figure 4, the distribution with 0 uM AMT of WT AM2 solubilized in C8E4 appears quite different than in Figure S1 and in the Figure S9 QToF data. Could the authors comment on the reproducibility of these distributions?

5. Monomeric AM2 appears to be very low or non-existent in detergent, but is present in nanodiscs. Could the authors comment on how the detergent vs nanodisc environment could be responsible for the observed differences?

6. Did the authors investigate the dependence on the AM2 to nanodisc ratio on the oligomeric distribution of AM2?

7. The authors suggest that the S31N mutant is unable to bind amantadine because it is locked in a predominantly non-binding pentameric state (based on Figure 4 data). However, in nanodiscs, the S31N mutant forms monomers/dimers/trimers but no larger oligomers. Could the authors comment on this observed difference in their data, and how the authors' proposed mechanism of resistance relates to previous studies on the mechanism of the S31N mutant?

8. Page 9: "*Importantly, AM2 S31N nanodiscs did not show any mass defect shifts upon addition of amantadine, confirming specificity of drug binding*." Could the authors include this data, potentially in the supplementary file?

REVIEWER #2

The paper by the Marty group investigates by native MS of nanodiscs the oligomerization state and drug binding properties of the viral Matrix protein 2 from influenza A (AM2) at different chemical environments. Interestingly, AM2, which is thought to exist primarily as a tetramer, is shown in this study to be highly sensitive to the chemical environment and displays a distribution of assembly states, depending on pH and lipid composition. The findings that illuminate the polydispersity of Am2 provide new potential mechanisms of influenza physiology and pathology. The data is high quality and reproducible and the manuscript is well-written. I recommend addressing the points raised below.

1) According to the materials and methods section, the protein was analyzed at a concentration of 50 μM (of the monomer?), which is quite high. Understandably, if a tetramer is expected, then higher amounts of the monomer are needed. However, since the protein appears in a range of assembly states, non-specific oligomerization should be ruled out.

2) In the few cases in which dilution experiments were performed the extent of dilution is not indicated, i.e. what are the starting and end concentrations.

3) The data in Figs. 4, S1-S6 and S9 is processed, can the authors provide representative raw spectra, so the quality of data can be estimated.

4) The discussion section should be extended, with emphasis on the biological relevance of the results. Like what is the composition of the natural host membrane? How can polydispersity in assembly states benefit the influenza virus? and their similarity to the membranes tested. Does any of the tested conditions mimic the natural environment of the host membranes? Can any conclusions be drawn as to the endogenous assembly

state of AM2 in the host cells? In a structural and chemical point of view what is the mechanism in which pH or lipid content affect assembly?

5) AM2 is post-translationally modified. Can the author comment on this aspect and how do they think it affects the assembly state distribution?

6) In Figs 4, S1, S2 and S3 the concentration of Am2 is not indicated.

7) The mass defect analysis should be explained.

8) Raw data of the IM-MS results shown in Fig. S6 should be provided.

9) Theoretical and measured masses, including mass measurement errors should be added (also of drug binding). Perhaps in a table.

10) Figure 2, in panels E and F the y axis in the inset is distorted.

11) What does the cartoon in figure 5 demonstrate?

REVIEWER #3

In Townsend et al. the authors utilized native mass spectrometry to characterize the oligomerization state of the influenza A M2 channel in different environments and found that in contrast to what has been previously reported, AM2 exists in multiple oligomeric states depending on pH, lipid composition, and presence of drug. Of note, this study utilizes native MS to measure drug binding to a membrane protein in an intact lipid bilayer, which is technically challenging. Although this is a novel application of native mass spectrometry, additional experiments are needed to provide convincing data that would support the main conclusion, namely that the oligomeric state of AM2 is actually more polydisperse than previously reported. This manuscript would be greatly improved by addressing the following questions:

Major points:

1.The authors offer two interpretations of their data in the discussion: 1) that it is very challenging to capture the pure tetramer 2) that the oligomeric states of AM2 are more complex than previously thought. The former is unlikely to have any physiological relevance while the latter could have important implications for development of novel therapeutics. A third interpretation could also be that the oligomeric profile observed is a byproduct of the native MS technique utilized. This manuscript would be much more impactful if this study included experiments to differentiate between these possibilities.

2. The author's note that "There are several dozen X-ray or NMR structures of the AM2 TM domain in a variety of membrane mimetics, all depicting monodisperse homotetramers" yet most of their conditions do not replicate this finding. Could the authors please comment in more detail on how their conditions differ from the previously reported structural studies which indicate AM2 is present as a homotetramer? The authors mention that most studies used high concentrations of drug - are there other explanations as to why they observed high variability and complex instability where others did not? Do all the previous studies use drug to stabilize the complex? In cases where they did not use drug, what was different?

3. The fact that the replicate measurements showed significant variation suggests that these results may be due to technical complications rather than truly reflecting distinct complex formation. Did the authors consider using a

positive control - perhaps something else known to form a tetrameric complex of similar molecular weight for comparison? This would help build confidence that utilizing native MS for this application can provide reliable data.

4. In figure 2 and S1, please provide intensity values associated with each condition. Larger complexes are harder to ionize and more likely to inadvertently dissociate in the gas phase. It is impossible to understand how well AM2 ionized in each of these conditions when it is presented as a percent of total. Have the authors considered creating covalently bonded versions of dimer, trimer, and tetramer AM2 to use as standards to accurately quantify the amount of each complex in each condition?

5. In figure S2, as protein concentration increases, a shift towards higher molecular weight complexes is observed. Is it possible this is due to protein aggregation and unlikely to be observed in physiological conditions?

6. The "orthogonal measurements confirm oligomeric sensitivity" section is confusing. What do the authors mean by oligomeric sensitivity? It is also unclear how the SEC data supports the authors' claims about the oligomeric state of AM2.

7. Please explain the statement "very small signals for bound drug were observed". Does this refer to the signal from AM2 or from the drug itself or for drug bound to AM2?

Minor:

1. Could the authors please comment on why the select conditions were chosen for figure 2? Supplemental figure 1 is more informative and is worth including in the main figures. Similar question for the other figures where parital datasets are shown in the main text.

2. Please clarify the concentration of AM2 used in Figures 1, 2, 3, 4 and S1 and S3.

3. Clarify which detergent was used in figure S9.

REVIEWER #4

The authors of this manuscript explore the effects of detergents, drugs, pH, and lipids on the oligomerization state of a well-studied viroporin from the influenza A virus, the M2 channel. Using native mass spectrometry as their main approach, the authors show that pH and the chemical nature of the membrane or membrane mimetic influence the observed polydispersity of M2. While native mass spectrometry captures a distribution of oligomeric states that was not seen in previous analytical studies, the question, ultimately, is whether this polydispersity is physiologically relevant or whether it highlights the need for rigorous testing and vetting of membrane mimetics for structural and functional studies.

In the initial detergent study, the authors investigate how various detergents affect oligomerization of the channel at different pH. They show that certain detergents favor different oligomeric states over others and capture an array of states in the detergents tested. They then show that the binding of drug to the WT shifts the observed population distribution to favor the tetramer. They repeat these experiments with the S31N mutant, which forms pentameric assemblies in the given conditions.

To see the effects of lipid bilayers on the oligomerization state of M2, they assembled M2-incorporated nanodiscs. They show that choice of lipid composition of the nanodiscs is crucial to the observed distribution of states with DPPC being the lipid that favors the homotetramer. Moreover, they show that they are able to detect mass defect shifts from drug binding, corroborating earlier work in the field. The authors repeat the nanodisc studies with the S31N mutant. From their lipid studies with and without drug, they again rationalize

that the drug-resistance of the mutant to amantadine and rimantidine may arise from the formation of small oligomers that preclude binding.

The big question is whether these newly observed states are physiologically relevant or whether they're an artifact of the physicochemical nature of the local environment. Overall, the authors clearly show that the assembly of M2 is sensitive to its chemical environment, and from their data, seem to suggest that the observed polydispersity reflects the true distribution of states in the physiological context. The data showing the polydispersity is very convincing and serve as a reminder that the choice in membrane mimetics plays a critical role in determining which oligomeric state, whether functional or otherwise, is favored. However, if the point is that these non-tetrameric states have some biological or channel function, then the authors bear the burden of proof.

Major Comments:

- Why are the lipid nanodisc experiments only done at pH 7.4 and not other pH? In the detergent study, we clearly see a change in the oligomerization state brought on by a change in pH, and the authors speculate that the change in pH in the endosome could change the oligomerization state to higher order oligomers, so why is there no pH-dependent study of M2 in nanodiscs?

- There have been several studies that look at the effects of a completely different set of detergents on the conformational landscape of the channel using solution NMR (Thomaston et al. *JACS* 2019) or different lipids using solid state NMR (Mandala et al. *JMB*, 2017): how does this study compare to these results? If the authors do the detergent study with solution state NMR, would they see evidence for polydispersity? Similarly, if the authors do these same native MS experiments using the detergents and/or lipids discussed in these two manuscripts, would they see polydispersity or do these conditions favor the exclusive formation of the homotetramer? The choices for lipids/detergents are orthogonal to what has been published in the literature, so a couple of experiments with the same sample conditions (i.e. lipid/detergent and pH) would be insightful as to whether the previous conditions just happen to favor the homotetramer.

- In the amantadine-binding study of the WT and S31N in detergent micelles, the authors noted no major changes to the oligomeric state distribution for the mutant and conclude that the absence of a shift is indicative of lack of drug binding. They also suggest that the known drug resistance of the S31N variant arises because this mutant is locked into a novel pentameric state that is impervious to drug-binding. While this is an interesting hypothesis, their MS data does not prove that the drug is not binding. Moreover, they note that even in their WT samples, which show clear shifts, there is a lack of signal from the bound drug in their MS results, so how can the authors make the claim that S31N is not binding the drug? A similar comment can be made about the S31N nanodisc study, although the experimental evidence for drug-binding in the WT lends more support to this conclusion than the one made in the detergent study.

Minor Comments:

- Can the authors rule out effects from the varying peptide:detergent ratios? Each of these samples was run at 2x CMC (seemingly standard in the native MS field) with a constant monomer concentration of 50 uM, which works out to very different peptide:detergent ratios. At the same peptide:detergent ratios, how do the distributions compare to each other?

- Since the higher order oligomers (i.e. hexamers) in LDAO seem stable, could they potentially crosslink these samples to get a low-resolution structure of the hexamer?

- Is there polydispersity evident in other detergents for S31N?

- Previous studies (Ref #35 in this manuscript, for example) which look at the oligomerization of M2 using analytical ultracentrifugation used dodecylphosphocholine (DPC) micelles as the membrane mimetic. Using this particular detergent, the authors of the JMB publication showed that the monomer-tetramer equilibrium was cooperative in the presence and absence of the drug amantadine. Is there a reason why DPC was not used in this study? It would be interesting to see what distribution of states this technique captures in the detergent primarily used for the classical analytical ultracentrifugation experiments.

- Can the authors comment on why the drug-binding studies were only done in C8E4 detergent? How does the drug affect the distributions of the oligomers in other detergents? Would the larger hexamer observed in LDAO also bind the drug?

- The authors comment that the thickness and fluidity of the membrane is known to modulate M2 activity and suggest that these changes are due to a shift in the observed population of states in their discussion. Functional studies (i.e. liposomal proton flux assays) in the various lipids tested would be helpful to drive this point home. I would like to see how the activity of M2 changes in these lipids and how it relates to the distribuition of states observed in the native MS.

- The authors commented on the bilayer thickness/saturation of DPPC as a potential reason for the tetrameric specificity of M2 in these conditions. Similar speculation into the chemical or physical properties of the detergents that give rise to the observed oligomeric distributions would be welcome.

- Figures

    - Figure 2: Since the main take-home message from the figure is the deconvolved mass spectra, which clearly illustrate the polydispersity of the sample, it may help to flip the inset and the mass spectra or move the mass spectra to the supplemental. To someone who isn't in the field of native MS, the representative mass spectra are distracting and detract from conclusions illustrated in the deconvolved spectra.

    - Figure 3: A similar comment to the remarks made in Figure 2 can be made for this figure as well.

    - Figure 5: Is there a reason for the exclusion of S31N data? Since the drug-binding can be clearly seen in the corresponding WT samples, it would be better to swap out one of the WT-AMT figures (since they both are very similar) for one that shows the S31N with the drug even if no clear mass defect shift is seen. The two concentrations of AMT binding to WT is probably meant to show