Supplementary Information for

Influenza A M2 Channel Oligomerization is Sensitive to its Chemical Environment

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Supplemental Methods

Protein Expression and Purification

The full length AM2 with C-terminal polyhistidine tag and cysteines converted to serines was overexpressed in BL21(DE3) pLysS cells as previously described. AM2 was previously shown to retain its function without these cysteine residues. The sequence is shown in Figure 1A without the N-terminal methionine, which is cleaved during expression. Cells were resuspended in lysis buffer containing 150 mM NaCl, 50 mM Tris, 40 mM octyl-glucoside (OG), and protease inhibitor. After resuspension, cells were lysed using the LM20 Microfluidizer High Sheer Homogenizer. Lysed cells were then stirred at 4°C for 1–3 hours to allow for membrane solubilization. The lysate was clarified through centrifugation at 48,380×g for 20 minutes. Prior to protein purification, a HisTrap HP 5 mL column (GE Healthcare) was equilibrated with buffer A (150 mM NaCl, 50 mM Tris, 40 mM OG, 20% glycerol, and 20 mM imidazole). The sample was then filtered, loaded to the column, and washed with 10–15 column volumes of buffer A. To remove any nonspecific protein binding, the column was then washed with 5–10 column volumes of 10% buffer B (150 mM NaCl, 50 mM Tris, 4 mM OG, 20% glycerol, and 300 mM imidazole). AM2 was then eluted with 100% buffer B. It was then diluted with buffer A to a final monomer concentration of 580 µM, aliquoted, and flash frozen. The S31N mutant of AM2 was expressed and purified using the same protocol as AM2 wild type.

Membrane scaffold protein, MSP1D1(–) was expressed and purified as previously described. Briefly, MSP1D1 was expressed in E. coli and purified using immobilized metal affinity chromatography (IMAC). Following cleavage of the polyhistidine tag, MSP1D1(–) was purified by reverse IMAC.

Native Mass Spectrometry

Native mass spectrometry was performed as previously described using a Q-Exactive HF Orbitrap (Thermo Scientific, Bremen) mass spectrometer with Ultra-High Mass Range Modifications except where stated otherwise. Nano-electrospray ionization in positive ion mode was performed using borosilicate needles pulled using a P-1000 micropipette puller (Sutter Instruments).

Detergent-solubilized AM2 was analyzed with a range of 1,500–15,000 m/z at a resolution of 15,000. The trapping gas pressure was set to 5, and the spray voltage ranged from 1.1–1.5 kV. To aid in desolvation and detergent removal, 10–50 V of higher-energy collisional dissociation (HCD) energy and 10–50 V of source fragmentation were applied to each sample, as previously described. The precise collision voltages were adjusted slightly for each sample, and results are shown for the lowest value that gave a well-resolved spectrum. An open vial with 2–5 mL of acetonitrile was placed in the source of the mass spectrometer to allow for vapor charge reduction of all samples, which we found helped stabilize complexes during native MS. Mass spectrometry data was collected as single measurements for three sets of dilutions after the protein was buffer exchanged. Spectra are shown for a single representative replicate, and error bars show the standard deviation of the three replicates.

Nanodiscs were analyzed with a range of 2,000–25,000 m/z at a resolution of 15,000. The trapping gas pressure was set to 5 with a spray voltage of 1.1–1.3 kV. For nanodiscs with 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) or 1,2-dimyristoyl-sn-glycero-3-phosphorylglycerol (DMPG) lipids, 50–100 V of HCD collisional energy and 10–50 V of source voltage was applied to aid in the desolvation. To aid in the analysis of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) nanodiscs, a super charging reagent, propylene carbonate (Arcos Organics), was added prior to ionization at 5% propylene carbonate by volume. For DPPC nanodiscs, 100–200 V of HCD collisional activation was added to remove propylene carbonate. Representative spectra are shown from three replicate nanodisc assemblies.
Ion mobility-mass spectrometry (IM-MS) analysis was performed on a Synapt XS HRMS Q-ToF mass spectrometer (Waters Corporation, Manchester) using a nano-electrospray ionization source with borosilicate glass capillaries prepared as described above. MS conditions were applied to remove detergent adducts without disrupting structure prior to detection with instrument parameters as follows: capillary voltage, 1.5–1.8 kV; sampling cone, 150 V; trap collision energy, 100 V; transfer collision energy, 10 V; trap gas, 10 mL/min; helium cell gas, 120 mL/min; backing gas, 2.85 mbar. The parameters for IM were as follows: IM cell wave height, 40 V; IM cell wave velocity, 1000 m/s; transfer wave height, 4 V; transfer wave velocity, 69 m/s. Arrival time distributions (ATDs) were viewed using DriftScope 2.9 (Waters Corporation). CCS values were calculated as previously described using standards with published values. All reported CCS values were the result of triplicate experiments, and error bars are shown as the standard deviation of the CCS for different charge states.

**Native MS Data Analysis**

The native mass spectra for AM2 solubilized in detergent were deconvolved using UniDec as previously described. The settings for the deconvolution of AM2 in all conditions included a mass range of 1–110 kDa, a charge range of 1–50, and a FWHM of 1 m/z. A curved background subtraction of 100, as well as point smooth width of 1 and a beta value of 50 were also applied for all data. The native mass spectra for the AM2 nanodiscs were analyzed using UniDec as previously described. The mass range was extended to an upper limit of 250 kDa. For nanodiscs made of DMPG and DMPC lipids, the charge range was set 1–25. For nanodiscs made of DPPC lipids, the charge range was set 1–16. The mass of the lipid was used with mass smoothing set to -1.

To determine the stoichiometry of AM2 in nanodisc samples, we used mass defect analysis. Mass defect analysis divides the mass of the sample by a reference mass (the mass of the lipid), and the remainder of the division is then plotted. The plotted remainder is then normalized between 0 and 1. The addition of each AM2 incorporated into the nanodisc shifted the overall mass of the complex by about 10 kDa, which helped in the assignment of the stoichiometry of the protein-nanodisc complexes.

**Size Exclusion Chromatography**

Analytical size exclusion chromatography (SEC) was performed using a Superdex 200 Increase 10/300 (GE Healthcare) equilibrated with 1 column volume of each solution, and 100 µL of concentrated AM2 (580 µM) was injected in duplicate.

**AM2 Model Structures and Predicted Collisional Cross Sections**

CCS values expected for native, globular proteins were determined using the empirical relationships between mass and CCS for a variety of proteins. Model structures of AM2 oligomers were generated in PyMol using PDBs 2N70 and 4N8C as templates for the transmembrane and intracellular domains. Any discrepancies in sequence were changed, and missing extracellular domain residues were added manually in PyMol to generate a model AM2 monomer. This monomeric structure was then subjected to a brief (1 ns) relaxation in water using GROMACS, and the resulting simulated structure was used to construct all model oligomeric complexes with PDB 2KIH as a template for subunit arrangement. In vacuo molecular dynamics simulations of each model AM2 structure were then performed using the GROMOS96 43a2 force field in GROMACS, as previously described. Briefly, the
center of the experimental charge state distribution was chosen for each AM2 oligomer, and a low-energy configuration of positive charges was determined using the charge placement algorithm in Collidoscope. This configuration was used to assign charges during topology file generation, and then each model AM2 structure was allowed a brief energy minimization step, followed by a 5 ns \textit{in vacuo} MD production run at 300 K with a modified Berendsen thermostat. CCSs for simulated structures were computed using nitrogen buffer gas and the Trajectory Method in Collidoscope after identifying a low-energy charge configuration for the compacted structures.
**Supplemental Figures**

**Fig. S1**: The average relative peak areas measured by native MS of different oligomeric states of AM2 at pH 5 (A, D, G, J, M, P), pH 7 (B, E, H, K, N), and pH 9 (C, F, I, L, O, Q) while solubilized in C8E4 (A–C), LDAO (D–F), DDM (G–I), OG (J–L), DPC (M–O), and LMNG (P, Q). AM2 was not stable in pH 7 with LMNG and no mass was detected under these conditions. Error bars indicate the standard deviation of measurements from triplicate samples. Representative native mass spectra of select conditions are shown in Figure 2.

**Fig. S2**: The average relative peak areas measured by native MS of different oligomeric states of AM2 solubilized in C8E4 at AM2 monomer concentrations of 13, 25, 50, 100, and 200 µM at pH 5 (A–E) and pH 9 (F–J).
Fig. S3: The average relative peak areas measured by native MS of different oligomeric states of AM2 at pH 4 (A, G, M), pH 5 (B, H, N), pH 6 (C, I), pH 7 (D, J, O), pH 8 (E, K), and pH 9 (F, L, P) while solubilized in C8E4 (A–F), LDAO (G–L), and DDM (M–P). AM2 was not stable in DDM at pH 6 and 8, so no data is shown. Representative native mass spectra of AM2 in LDAO are shown in Figure 3.

Fig. S4: The average relative peak areas measured by native MS of different oligomeric states of AM2 solubilized in LDAO at an AM2 monomer concentrations of 13, 25, 50, 100, and 200 µM at pH 5 (A–E) and pH 9 (F–J).
**Fig. S5:** The average relative peak areas measured by native MS of different oligomeric states of AM2 solubilized in DDM at an AM2 monomer concentrations of 13, 25, 50, 100, and 200 µM at pH 5 (A-E) and pH 9 (F-J).

**Fig. S6:** Experimental collisional cross sections (CCS) of AM2 oligomers in C8E4 at pH 9 (*purple*) compared to CCSs expected for native globular proteins (*green*) and to CCSs calculated for model structures (*blue*). A linear fit to the experimental data is annotated and shows that each monomer added to the oligomeric complex contributed around 638 Å² in CCS.
**Fig. S7:** Native mass spectrum measured with the Synapt XS Q-ToF mass spectrometer of AM2 solubilized in LDAO detergent at pH 5 with the deconvolved mass spectrum in the inset. Similar to results from an Orbitrap mass spectrometer (Figure 2D), a mostly monodisperse hexamer of AM2 is observed. The CCS value for the hexamer is 4497 ± 66 Å².

**Fig. S8:** The relative absorbances at 280 nm of AM2 in LDAO pH 5, C8E4 pH 5, OG pH 5, C8E4 pH 9, and C8E4 pH 9 with 300 µM amantadine during size exclusion chromatography. For comparison, standards were analyzed on the same column: thyroglobulin (eluted at 9.2 mL), catalase (9.6 mL), alcohol dehydrogenase (12.96 mL), carbonic anhydrase (16.39 mL), and ribonuclease A (17.59 mL). Duplicate injections are shown for each.
Fig. S9: The average relative peak areas of different oligomeric states of AM2 with increasing concentrations of amantadine added measured with the Synapt XS Q-ToF mass spectrometer. Increasing concentrations of drug drive formation of more monodisperse tetramer complexes.
Supplemental Tables

**Table S1:** Mass defect values for nanodiscs with different stoichiometries of AM2 that contain 2 × 22044 Da MSP belts with DMPC, DMPG, or DPPC lipids.

<table>
<thead>
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<th>AM2 Stoichiometry</th>
<th>DMPC</th>
<th>DMPG</th>
<th>DPPC</th>
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<tr>
<td>0</td>
<td>0.03</td>
<td>0.09</td>
<td>0.09</td>
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<tr>
<td>1</td>
<td>0.44</td>
<td>0.81</td>
<td>0.16</td>
</tr>
<tr>
<td>2</td>
<td>0.86</td>
<td>0.51</td>
<td>0.25</td>
</tr>
<tr>
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<td>0.22</td>
<td>0.34</td>
</tr>
<tr>
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<td>0.92</td>
<td>0.43</td>
</tr>
<tr>
<td>5</td>
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<td>0.51</td>
</tr>
<tr>
<td>6</td>
<td>0.54</td>
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**Table S2:** Mass defect shifts for binding different stoichiometries of amantadine in nanodiscs made of DPPC lipids. Contributions from AM2 and MSP are not included, so measured mass defect values will correspond to the values from Table S1 plus the shift indicated here.

<table>
<thead>
<tr>
<th>Amantadine Stoichiometry</th>
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<th>2</th>
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<th>4</th>
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<td>Mass Defect Shift</td>
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SI References


