| Mutant | Vrev $Na^+$ | SD | Vrev $NMDG^+$ | SD | Vrev $K^+$ | SD |
|---|---|---|---|---|---|---|
| A/M2 | 64.8 | 3.2 | 62.5 | 5.9 | 63.1 | 4.1 |
| V27A | 68.4 | 5.5 | 67.2 | 8.5 | 63.3 | 4.1 |
| V27D | 54.5 | 6.6 | 55.1 | 4.1 | 55.0 | 4.5 |
| V27W | 66.4 | 4.9 | 64.2 | 5.0 | 63.2 | 4.1 |
| V27G | 63.9 | 4.3 | 63.1 | 7.7 | 66.4 | 3.1 |
| V27T | 64.5 | 4.9 | 65.6 | 3.8 | 64.1 | 5.5 |
| V27K | 65.1 | 7.3 | 64.7 | 5.5 | 65.3 | 7.1 |
| V27S | 64.9 | 3.0 | 61.8 | 5.9 | 63.9 | 3.9 |
| V27R | 61.2 | 4.4 | 61.0 | 6.2 | 63.3 | 3.1 |
| V27F | NF | | NF | | NF | |
| A30D | 65.1 | 2.1 | 65.0 | 3.3 | 63.5 | 3.2 |
| A30T | 64.0 | 4.1 | 64.3 | 3.9 | 65.4 | 5.0 |
| A30G | 63 | 2.2 | 65 | 4.1 | 65 | 3.2 |
| A30F | NF | | NF | | NF | |
| A30K | -2.9 | 8.2 | -10.1 | 4.3 | 3.2 | 1.9 |
| S31N | 64.8 | 4.5 | 62.3 | 4.1 | 63.0 | 7.3 |
| S31A | 62.6 | 6.1 | 64.6 | 6.5 | 65.2 | 3.2 |
| S31G | 65.3 | 5.0 | 63.7 | 4.3 | 63.9 | 3.4 |
| S31T | 62.8 | 4.0 | 61.0 | 4.2 | 61.0 | 3.1 |
| S31D | 65.6 | 5.2 | 64.5 | 3.0 | 62.9 | 2.8 |
| S31K | 62.4 | 4.3 | 63.7 | 5.0 | 62.7 | 2.2 |
| S31F | NF | | NF | | NF | |
| G34A | 66.0 | 2.1 | 66.3 | 4.3 | 65.8 | 2.8 |
| G34E | 62.2 | 5.7 | 64.1 | 1.9 | 64.6 | 2.4 |
| G34T | 64.8 | 3.9 | 66.0 | 3.7 | 63.4 | 2.4 |
| G34K | 61.8 | 4.6 | 60.7 | 3.7 | 60.9 | 3.9 |
| G34F | NF | | NF | | NF | |
| G34V | NF | | NF | | NF | |
| G34L | NF | | NF | | NF | |
| H37A | -5.6 | 5.5 | 3.1 | 8.1 | -1.8 | 7.0 |
| H37S | 0.4 | 4.8 | 0.3 | 6.1 | 2.4 | 13.2 |
| H37G | 0.5 | 8.7 | 8.0 | 11.2 | 4.6 | 6.1 |
| H37K | -2.3 | 12 | -5.1 | 10. 0 | 0.1 | 7.9 |
| H37N | 6.9 | 3.0 | 1.9 | 3.1 | 3.7 | 7.3 |
| H37F | 4.3 | 5.3 | 2.5 | 9.4 | 5.9 | 11.0 |
| H37D | 11.0 | 6.6 | 6.4 | 3.0 | 8.2 | 5.8 |
| H37Q | 9.2 | 10.2 | 4.6 | 8.8 | 6.0 | 7.1 |
| W41A | 64.9 | 6.3 | 64.4 | 6.2 | 65.0 | 3.6 |
| W41G | 66.1 | 5.5 | 63.3 | 4.8 | 64.7 | 1.7 |
| W41Y | 65.3 | 3.7 | 62.3 | 2.9 | 64.1 | 6.1 |
| W41F | 63.8 | 5.1 | 66.4 | 2.2 | 64.0 | 3.2 |
| W41D | -2.1 | 1.8 | 1.6 | 2.4 | -1.2 | 4.2 |
| W41K | 4.9 | 7.9 | 0.3 | 7.0 | 6.1 | 7.2 |
| D44A | 65.2 | 7.1 | 65.4 | 2.9 | 63.8 | 4.8 |
| D44K | 62.7 | 4.1 | 63.0 | 2.1 | 63.2 | 1.5 |

| | | | | | | |
|---|---|---|---|---|---|---|
| D44N | 64.3 | 4.3 | 66.7 | 8.1 | 61.9 | 2.9 |
| D44F | 64.6 | 1.1 | 63.1 | 2.0 | 62.4 | 4.4 |
| D44G | 63.4 | 4.1 | 63.0 | 2.2 | 64.7 | 6.4 |
| D44T | 67.5 | 6.3 | 66.2 | 3.3 | 65.6 | 5.9 |

Table S1: Summary of the reversal voltages (Vrev) of the A/M2 pore lining residues mutants. Vrev values for the A/M2 pore lining residues mutants were obtained from the current – voltage relationships (I/V) measured for wt and mutant channels expressed in *Xenopus* oocytes. The channels were activated by the application of acidic bathing solution (pH 5.5) and I/V relationships were measured using the voltage ramp from -60 to +80 mV in bathing solutions (pH 5.5 – activating solution, pH 8.5 – non-activating solution) containing $Na^+$, $NMDG^+$, or $K^+$ as a major monovalent cation. The Vrev values are the mean (±SD) measurements from 15-20 oocytes from 3-5 independent experiments. NF stands for "non functional".

| Mutant | Ion Selectivity | Amantadine Sensitivity (*remaining activity (%) after inhibition with 100 μM amantadine*) | Relative Specific Activity (%)* | pK1 | pK2 | r2/r1 |
|---|---|---|---|---|---|---|
| A/M2 | $H^+$ selective | 6.0±1.4 | 100±12 | 7.8±0.3 | 5.3±0.1 | 19.4 |
| V27A | $H^+$ selective | 97.5±4.2 | 148±11 | 6.6±0.5 | 5.1±1.1 | 3.8 |
| V27D | $H^+$ selective | 59.1±5.9 | 140±13 | 7.4±0.2 | 5.2±0.3 | 3.0 |
| V27W | $H^+$ selective | 13.1±1.9 | 35±2 | 7.7±0.4 | 5.3±0.1 | 9.2 |
| V27G | $H^+$ selective | 98.7±6.8 | 66±3 | 7.6±0.4 | 5.2±0.2 | 6.6 |
| V27T | $H^+$ selective | 26.5±4.3 | 67±7 | 7.6±0.3 | 5.2±0.1 | 6.3 |
| V27K | $H^+$ selective | 60.7±7.6 | 23±3 | ND | ND | ND |
| V27S | $H^+$ selective | 95.9±3.3 | 80±13 | 7.1±0.2 | 4.9±0.3 | 5.9 |
| V27R | $H^+$ selective | 96.1±4.2 | 22±2 | ND | ND | ND |
| V27F | NF | NF | NF | NF | NF | NF |
| A30D | $H^+$ selective | 102.6±6.5 | 276±36 | 7.7±0.7 | 5.7±0.1 | 12.4 |
| A30T | $H^+$ selective | 106.0±8.5 | 35±3 | 7.3±0.4 | 5.7±0.3 | 2.8 |
| A30G | $H^+$ selective | 82.3±2.2 | 77±9 | 8.2±1.8 | 5.4±0.1 | 37.6 |
| A30F | NF | NF | NF | NF | NF | NF |
| A30K | NIS | NIS | NIS | NIS | NIS | NIS |
| S31N | $H^+$ selective | 65.4±8.3 | 126±9 | 8.0±0.9 | 5.6±0.3 | 74.4 |
| S31A | $H^+$ selective | 17.0±5.2 | 42±4 | 6.8±0.5 | 5.5±0.3 | 4.4 |
| S31G | $H^+$ selective | 16.3±1.8 | 93±11 | 7.4±0.1 | ND | ND |
| S31T | $H^+$ selective | 74.0±6.8 | 25±1 | 7.5±0.2 | ND | ND |
| S31D | $H^+$ selective | 64.5±4.4 | 224±17 | 7.2±0.5 | 5.8±0.1 | 7.1 |
| S31K | $H^+$ selective | 86.1±20.5 | 36±3 | ND | ND | ND |
| S31F | NF | NF | NF | NF | NF | NF |
| G34A | $H^+$ selective | 72.7±8.4 | 40±5.7 | 7.7±2.7 | 6.0±0.1 | 14.6 |
| G34E | $H^+$ selective | 82.3±9.6 | 132±15 | 7.9±0.7 | 5.7±0.1 | 8.8 |
| G34T | $H^+$ selective | 42.0±1.9 | 118±15 | 7.5±4.0 | 5.7±0.2 | 38.7 |
| G34K | $H^+$ selective | 102.6±6.5 | 60±9 | ND | ND | ND |
| G34F | NF | NF | NF | NF | NF | NF |
| G34V | NF | NF | NF | NF | NF | NF |
| G34L | NF | NF | NF | NF | NF | NF |
| H37A | NIS | NIS | NIS | NIS | NIS | NIS |
| H37S | NIS | NIS | NIS | NIS | NIS | NIS |
| H37G | NIS | NIS | NIS | NIS | NIS | NIS |
| H37K | NIS | NIS | NIS | NIS | NIS | NIS |
| H37N | NIS | NIS | NIS | NIS | NIS | NIS |
| H37F | NIS | NIS | NIS | NIS | NIS | NIS |
| H37D | NIS | NIS | NIS | NIS | NIS | NIS |
| H37Q | NIS | NIS | NIS | NIS | NIS | NIS |
| W41A | $H^+$ selective | 42.5±9.8 | 89±11 | 7.3±0.1 | ND | ND |
| W41G | $H^+$ selective | 16.9±5.6 | 124±20 | 7.4±0.2 | ND | ND |
| W41Y | $H^+$ selective | 16.8±0.7 | 144±18 | 7.4±0.4 | 5.6±0.1 | 7.9 |
| W41F | $H^+$ selective | 67.3±5.4 | 172±29 | 7.4±0.1 | 5.2±0.1 | 2.8 |

| | | | | | | |
|---|---|---|---|---|---|---|
| W41D | NIS | NIS | NIS | NIS | NIS | NIS |
| W41K | $H^+$ selective | NIS | NIS | NIS | NIS | NIS |
| D44A | $H^+$ selective | 13.3±5.3 | 244±32 | 7.9±3.7 | 5.8±0.1 | 32.9 |
| D44K | $H^+$ selective | 12.1±4.8 | 297±56 | 8.6±18.3 | 5.8±0.2 | 161.1 |
| D44N | $H^+$ selective | 7.0±0.3 | 151±13 | 8.1±2.6 | 5.8±0.2 | 8.5 |
| D44F | $H^+$ selective | 11.7±2.5 | 171±42 | 8.1±1.3 | 5.7±0.2 | 12.6 |
| D44G | $H^+$ selective | 7.2±0.7 | 275±35 | 8.1±1.2 | 5.6±0.2 | 10.6 |
| D44T | $H^+$ selective | 20.3±2.7 | 390±66 | 7.7±0.4 | 5.7±0.2 | 4.1 |

Table S2: Summary of the A/M2 mutant channel properties. The table summarizes the data obtained from the measurements of the channel ion selectivity, amantadine sensitivity, relative specific activity and pH-dependent proton conductance. *Relative specific activity is presented as a (%) activity relative to the activity of the wt A/M2 channel. NF stands for "non functional". NIS stands for "non ion selective". ND stands for "not determined". The pK1, pK2 and r2/r1 values (±SD) obtained from the fits presented on the Figures 3 and 4 using Eq. 2A (see Results).

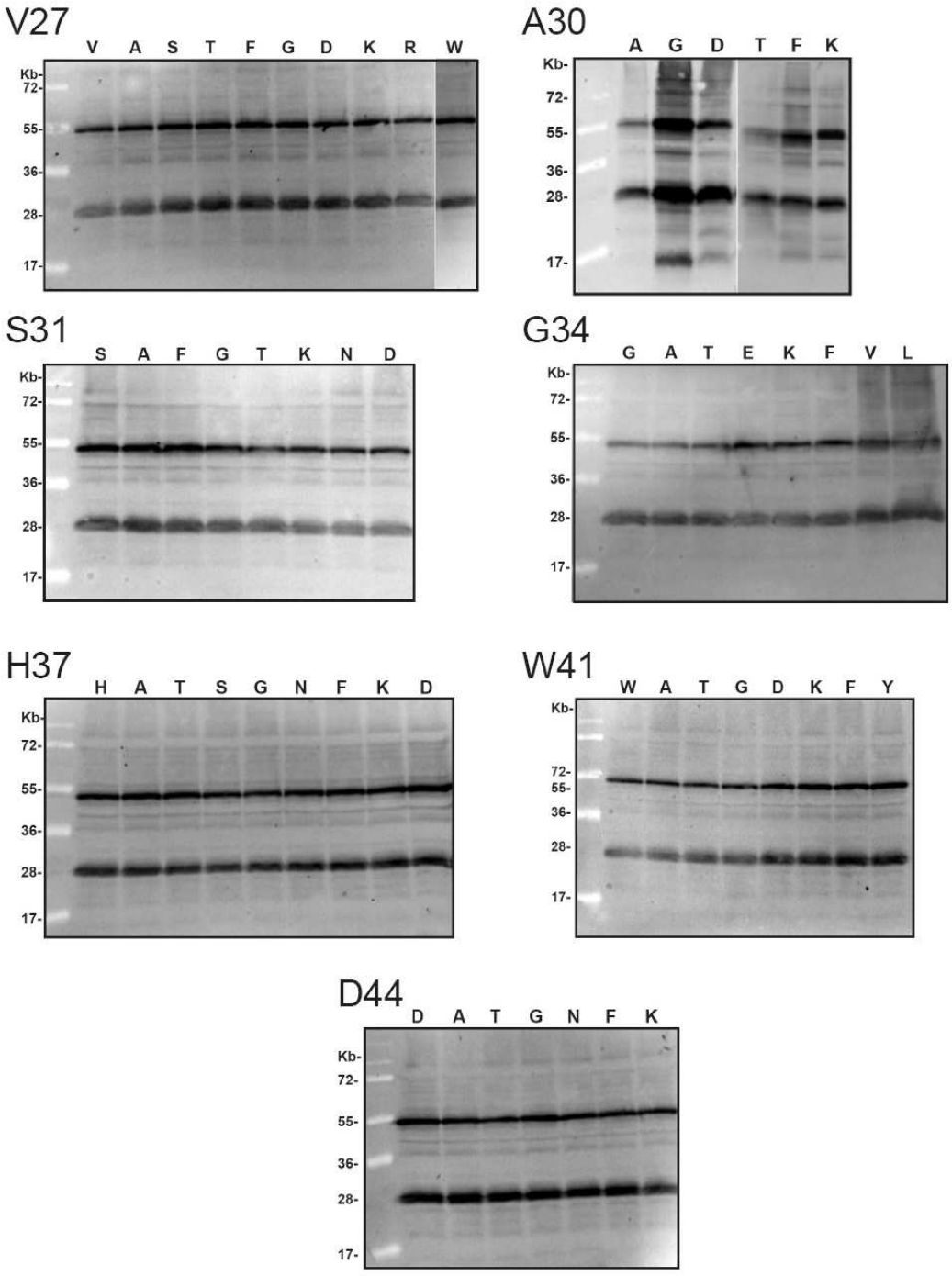


Figure S1: Protein expression of wt and mutant A/M2 proteins. *Xenopus* oocytes were injected with cRNA of the appropriate mutants. Following current measurements the oocytes were homogenized, examined on a 12% SDS-PAGE under non-reducing conditions, immunoblotted and reacted with anti-A/M2 monoclonal antybody. Wt and

mutant A/M2 proteins form complexes which correspond to spontaneously formed dimers and tetramers

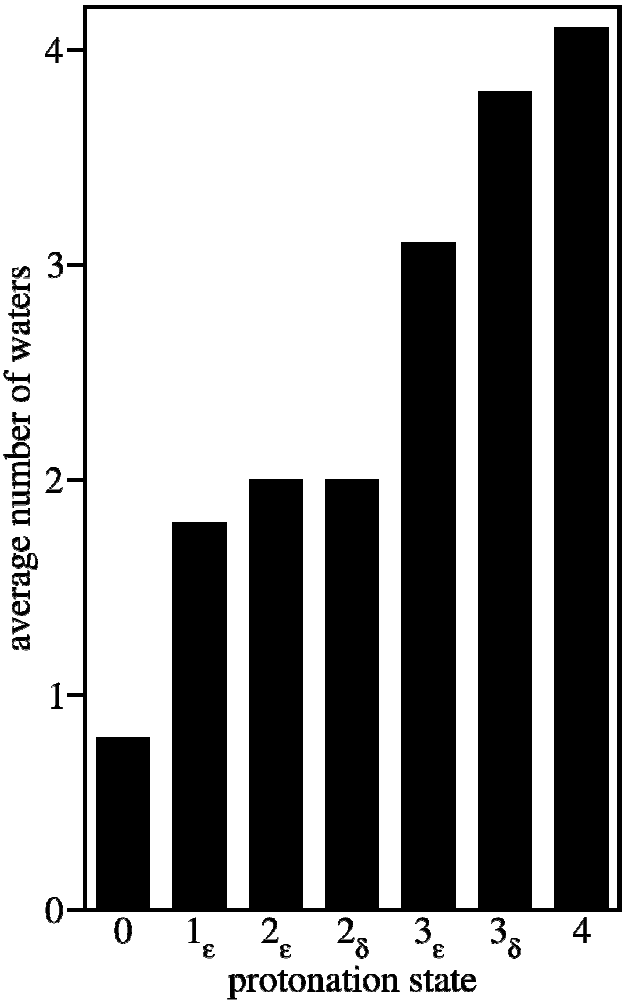


Figure S2: Number of water molecules localized in the pocket between the His37 and the Trp41 residues (2 in the X-ray structure) for different protonation states. Average numbers are obtained from 15 ns-long classical MD simulations.

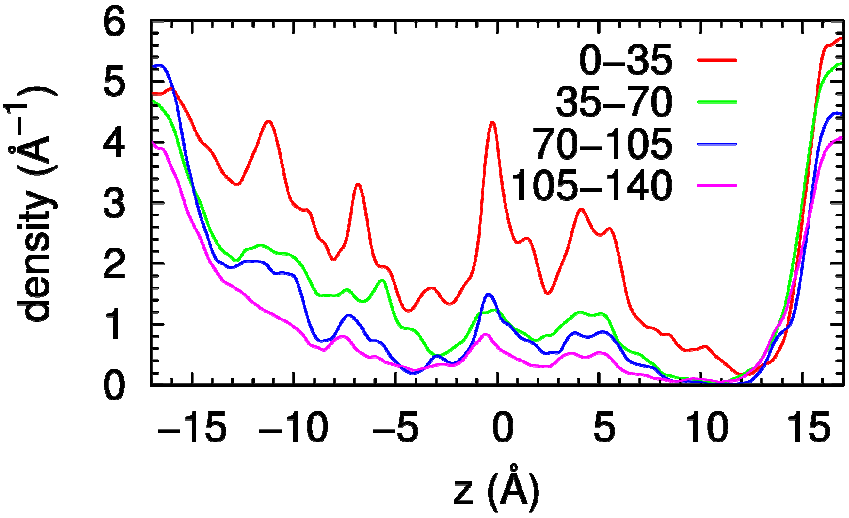


Figure S3: The density of water oxygen in the pore as a function of transmembrane displacement ($z$) is shown for four subsequent time intervals (0 to 35 ns, 35 to 70 ns, 70 to 105 ns, and 105 to 140 ns) of the S31F MD simulation. In particular, the density between the F31 residues (~9 Å to ~12 Å) converges to nearly zero.