Published in final edited form as:

J Am Chem Soc. 2010 August 18; 132(32): 10958–10960. doi:10.1021/ja101537p.

Magic Angle Spinning NMR Investigation of Influenza A M2₁₈₋₆₀: Support for an Allosteric Mechanism of Inhibition

Loren B. Andreas¹, Matthew T. Eddy¹, Rafal M. Pielak², James Chou², and Robert G. Griffin^{1,*}

¹ Francis Bitter Magnet Laboratory and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA, 02139

² Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, 02115

Abstract

The tetrameric M2 proton channel from influenza A virus conducts protons at low pH and is inhibited by aminoadamantyl drugs such as amantadine and rimantadine. We report magic angle spinning NMR spectra of POPC and DPhPC membrane embedded M2₁₈₋₆₀, both apo and in the presence of rimantadine. Similar linewidths in spectra of apo and bound M2 indicate that rimantadine does not have a significant impact on the dynamics or conformational heterogeneity of this construct. Substantial chemical shift changes for many residues in the transmembrane region support an allosteric mechanism of inhibition. A Rmt titration supports a binding stoichiometry of >1 Rmt molecule per channel while showing that nonspecific binding or changes in membrane composition are unlikely sources of the chemical shift changes. In addition, doubling of spectral lines in all of the observed samples provides evidence that the channel assembles with twofold symmetry.

The M2 protein from influenza A virus is a single pass membrane protein that assembles as a tetramer to form a H⁺ selective channel that functions at low pH and is critical in the viral lifecycle. A class of aminoadamantyl inhibitors has become ineffective against many influenza strains due to mutations in the N-terminal region of the channel¹, thus stimulating great interest in identification of the pharmacologically relevant binding site and the mechanism of inhibition and drug resistance. Discussion of an external, lipid-facing site and a pore-blocking site is ongoing^{2–8}. A solution NMR structure⁷ in DHPC micelles of M2₁₈₋₆₀ showed an external binding site at D44 via direct NOE measurement; however, the pharmacological relevance of this binding pocket was questioned due to possible detergent

rgg@mit.edu.

Supporting Information Available: Sample preparation and synthesis, description of assignments, additional spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>

effects, such as hydrophobic mismatch, which may impact structure, dynamics, and binding affinity⁴. Pioneering solid state NMR experiments by Cross, et al.⁹ using a shorter TM construct, M2₂₂₋₄₆, in lipid environments^{3,10} were followed by diffraction studies in detergent⁸. Some of these results and more recent NMR experiments³ suggest S31 as a binding site. Recently, an elegant ¹³C-²H REDOR experiment using ²H labeled drug and ¹³C labeled peptide on M2₂₂₋₄₆ in lipids detected inhibitor near S31, and at higher drug concentrations, near D44⁴. However, M2₂₂₋₄₆ exhibits reduced function and drastically reduced inhibition by drug when compared with M2₁₈₋₆₀⁶. Furthermore the similar construct, M2₂₁₋₆₁ has indistinguishable conduction compared to the full length protein⁵. It is presently unclear whether the discrepancy between the two observed binding sites arises due to detergent effects, the highly truncated construct, or other factors. We therefore initiated investigations of the fully functional construct, M2₁₈₋₆₀ in lipid bilayers with magic angle spinning (MAS) NMR. Our chemical shift data reveal global conformational changes upon drug binding that suggest an allosteric mechanism of inhibition, and peak doubling, indicating a twofold symmetric tetramer.

Figure 1 (**top**) shows a ¹⁵N-¹³C one-bond zf-TEDOR^{11,12} correlation spectrum of M2₁₈₋₆₀ in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers, demonstrating spectral assignments in both the absence (red) and presence (blue) of the inhibitor rimantadine (Rmt). Linewidths of ~1 ppm for both ¹⁵N and ¹³C are observed at 700 MHz for both drug-bound and apo samples, indicating conformational homogeneity. This narrow linewidth also indicates that the dynamics of this system are favorable for investigation by MAS NMR. The similar linewidths in the bound and apo states is in contrast to results with M2₂₂₋₄₆ where drug binding significantly narrowed the spectra³, and the improvement using M2₁₈₋₆₀ could be attributed to the larger construct, which remains tetrameric even in an SDS detergent environment⁶.

Upon Rmt binding, we observe substantial (>1 ppm ¹³Cα/Cβ, >2 ppm ¹⁵N) chemical shift changes from residues 24 to 41, distributed across the entire range of unambiguously assigned residues and nearly spanning the transmembrane helix. Significant perturbations occur for pore lining residues 27, 34, 37, 41 and from residues 24, 25, 28, 29, 31, 32, 35, which are found in the helix-helix interface and lipid facing sites⁷. Only two assigned residues, 30 and 42, show no chemical shift perturbations >1 ppm in ¹³Cα/Cβ or >2 ppm in ¹⁵N. In Figure 2, these chemical shift perturbations are shown as a function of residue number and demonstrate significant changes on a length scale many times larger than the ~5 Å Rmt drug, indicating allostery. We note that the ~7 ppm shift change at S31 that was observed for M2₂₂₋₄₆ using Amt³ is also observed here for M2₁₈₋₆₀ using Rmt. In addition, we also observe a ~3.5 ppm shift in H37 Cα, which is comparable considering the ~2 fold increase in chemical shift variability of ¹⁵N compared to ¹³Cα. Thus, chemical shift data supports an allosteric effect, but does not locate the drug; it is therefore consistent with proposed sites of pharmacological relevance, S31 and D44.

An allosteric effect is also in agreement with previous measurements of aligned samples that detected a kink at G34 in amantadine (Amt) bound M2₂₂₋₄₆ and a modified conformation with drug binding¹³. A backbone structure of amantadine bound M2 was calculated in this previous study; however, broad apo spectra compromised a complete structural analysis of that state. From the present spectra, it is clear that the allosteric changes extend across the entire TM domain.

This conclusion relies on the significance of the chemical shift differences between the bound and apo states. Chemical shift changes can arise from several factors, which include changes in secondary structure but can also include variations in solvents, temperature, and pH. Comparisons of chemical shifts between solution and microcrystalline preparations of

the same model proteins reported strong agreement (~ 1 ppm and less for $^{13}\text{C}'$ and $^{13}\text{Ca} \sim 2$ ppm and less for ^{15}N) in the protein core, with somewhat larger differences observed for sites forming crystal contacts in the solid state preparations^{14–17}. Temperature and pH are constant for all of the data reported herein, and the possibility of nonspecific binding and membrane changes are addressed below and in Figures 3–4; we have therefore excluded these potential sources for chemical shift perturbations.

The solution NMR structure showed an external binding site with a specific interaction between the amine group of Rmt and D44 C γ . Therefore, we have also examined the Asp region of ^{13}C - ^{13}C proton driven spin diffusion¹⁸ (PDSD) spectra shown in Figure 1 (**bottom**) for perturbations. The G34 Ca-C' peak exhibits a well-resolved movement on drug binding and intense peaks in both states, suggesting that it is in a position in the peptide that is not influenced by dynamics. In contrast, Asp C β -C' and C β -C γ peaks show reduced intensity that is likely due to motion interfering with cross-polarization (CP) and decoupling^{19,20}. Addition of Rmt causes a 2-fold further decrease in these peak intensities and chemical shift changes of several ppm. A direct H-bond between the drug amine and an Asp C γ carboxyl, can explain these effects. However, these effects can also be explained by a large-scale reorganization of the channel resulting in altered conformation and dynamics in the vicinity of the Asp residues.

In Figure 3, zf-TEDOR spectra are shown at 0, 1, and 4 Rmt molecules per channel in order to investigate binding stoichiometry and rule out the possibility of nonspecific binding. Figure 3a shows an apo spectrum. Upon addition of one Rmt molecule per channel (Figure 3b), resonances of the Rmt-bound form appear and with approximately 25% of total intensity. At four Rmt molecules per channel (Figure 3c), resonances arising from Rmt-bound M2 are primarily observed, with apo resonances still detected at $<10\%$ total intensity. No gradual change in chemical shifts is observed; rather the resonances of the bound form appear in concert and increasing intensity with increasing Rmt. At 16 Rmt molecules per channel, the effect is saturated and only the bound form is observed (Figure 1, **blue**). If these chemical shift changes are due to specific binding, then resonance intensities suggest a binding stoichiometry of >1 molecule per channel. Notably, pore facing residues such as G34 and V27, which are unlikely to be affected by any nonspecific hydrophobic interactions or changes in lipid composition, clearly demonstrate the changes. Furthermore, although Rmt partitions strongly to membrane²¹, at one drug per channel it occupies only 2 mol percent of the *non-protein* membrane components. Neither protein nor M2 tetramer is in excess, yet all of the chemical shift perturbations are observed. Therefore if we assume that the pharmacological binding site has high affinity, then nonspecific binding and changes in membrane composition are excluded.

Sensitivity to membrane composition was further investigated by collecting TEDOR and PDSD spectra in another lipid, 1,2-diphytanoyl-*sn* glycerol-3-phosphocholine (DPhPC). Spectra recorded in DPhPc are remarkably similar to spectra of POPC embedded M2, with maximum chemical shift differences of 0.3 ppm and 0.7 ppm for ^{13}C and ^{15}N , respectively. PDSD Spectra recorded in these two lipids are overlaid in Figure 4. The fact that this change in membrane composition causes small changes in the spectra provides further evidence that drug induced chemical shift changes are caused by a specific drug interaction and not caused by altering the membrane composition. Clearly the state of this construct of M2 in lipids is stable to the change in membrane composition between DPhPC and POPC.

Two distinct sets of peaks are observed for many residues in both apo and drug bound M2₁₈₋₆₀, with approximately equal intensity, providing evidence that the tetramer is twofold symmetric. These are most obvious in the P25 cross peaks in Figure 1 (**top**), but are also apparent in more crowded regions of the spectra. Multiple peak sets could indicate the

presence of multiple conformations or arise due to incomplete drug binding. However, peak doubling appears with equal intensity between the two sets of peaks, and is found in *both* the apo and drug bound states, suggesting that the tetrameric assembly has twofold symmetry, which may arise due to packing of bulky W41 and H37 side chains. This is in agreement with previous work, which showed that the doubly protonated state of M2 contains two imidazole-imidazolium dimers of H37 and is therefore twofold symmetric at this position²². It is also qualitatively consistent with the diffraction structure at neutral pH, which shows conformational heterogeneity in the C-terminal region⁸. Other structural studies have assumed fourfold symmetry. For example, a single set of resonances was observed for this construct in DHPC micelles⁷ and may be the result of fast interconversion between two states at higher temperatures. Also, peak doubling may be present and within the linewidth observed in previous MAS NMR studies.

Dimerization of two tetramer channels could also lead to two sets of resonances, with different chemical shifts at the interface. However, some of the largest separations in doubled peaks appear at residues inside the channel such as H37 and W41. We therefore find that the most likely explanation for the peak doubling is a twofold symmetric channel.

In summary, large drug induced chemical shift changes observed across the entire TM region support a large-scale reorganization of the channel by an allosteric mechanism. In addition, doubling of peaks is likely due to a twofold symmetric tetramer and drug titration data are consistent with a binding stoichiometry of >1 Rmt molecule per channel. Determination of the inhibitor binding site based on maximal chemical shift perturbation alone is not possible given the magnitude and distribution of chemical shift changes. Therefore, a direct dipolar coupling measurement⁴ between the drug and the M2 is needed to determine the binding location(s) and thereby elucidate the mechanism of inhibition in a construct, such as M2₁₈₋₆₀, that retains full function in conductance assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Marcelo Berardi, Alexander Barnes and Eric Miller for helpful discussion. This work was supported by the National Institute of Health (EB001960 and EB002026). L.B. Andreas was supported by a National Science Foundation Graduate Research Fellowship.

References

1. Bright RA, Shay DK, Shu B, Cox NJ, Klimov AI. JAMA. 2006; 295:891–4. [PubMed: 16456087]
2. Pielak RM, Chou JJ. Protein & Cell. 2010; 1:246–258. [PubMed: 21203971]
3. Cady SD, Luo W, Hu F, Hong M. Biochemistry. 2009; 48:7356–64. [PubMed: 19601584]
4. Cady SD, Schmidt-Rohr K, Wang J, Soto CS, DeGrado WF, Hong M. Nature. 2010; 463:689–692. [PubMed: 20130653]
5. Ma C, Polishchuk AL, Ohigashi Y, Stouffer AL, Schon A, Magavern E, Jing X, Lear JD, Freire E, Lamb RA, DeGrado WF, Pinto LH. Proc Natl Acad Sci U S A. 2009; 106:12283–8. [PubMed: 19590009]
6. Pielak RM, Schnell JR, Chou JJ. Proc Natl Acad Sci U S A. 2009; 106:7379–84. [PubMed: 19383794]
7. Schnell JR, Chou JJ. Nature. 2008; 451:591–5. [PubMed: 18235503]
8. Stouffer AL, Acharya R, Salom D, Levine AS, Di Costanzo L, Soto CS, Tereshko V, Nanda V, Stayrook S, DeGrado WF. Nature. 2008; 451:596–9. [PubMed: 18235504]
9. Kovacs FA, Cross TA. Biophys J. 1997; 73:2511–7. [PubMed: 9370444]

10. Wang J, Kim S, Kovacs F, Cross TA. *Protein Sci.* 2001; 10:2241–50. [PubMed: 11604531]
11. Jaroniec CP, Filip C, Griffin RG. *J Am Chem Soc.* 2002; 124:10728–42. [PubMed: 12207528]
12. Hing AW, Vega S, Schaefer J. *J Magn Reson.* 1992; 96:205–209.
13. Hu J, Asbury T, Achuthan S, Li C, Bertram R, Quine JR, Fu R, Cross TA. *Biophys J.* 2007; 92:4335–43. [PubMed: 17384070]
14. Franks WT, Zhou DH, Wylie BJ, Money BG, Graesser DT, Frericks HL, Sahota G, Rienstra CM. *J Am Chem Soc.* 2005; 127:12291–305. [PubMed: 16131207]
15. Igumenova TI, Wand AJ, McDermott AE. *J Am Chem Soc.* 2004; 126:5323–31. [PubMed: 15099118]
16. Seidel K, Eitzkorn M, Heise H, Becker S, Baldus M. *Chembiochem.* 2005; 6:1638–47. [PubMed: 16094694]
17. van Rossum BJ, Castellani F, Pauli J, Rehbein K, Hollander J, de Groot HJ, Oschkinat H. *J Biomol NMR.* 2003; 25:217–23. [PubMed: 12652133]
18. Szeverenyi NM, Sullivan D, Maciel GE. *J Magn Reson.* 1982; 47:462–475.
19. Long JR, Sun BQ, Griffin RG. *J Am Chem Soc.* 1994; 116:11950–11956.
20. Maus DG, Copie V, Sun BQ, Griffiths JM, Griffin RG, Luo S, Schrock RR, Liu AH, Seidel SW, Davis WM, Grohmann A. *J Am Chem Soc.* 1996; 118:5665–5671.
21. Wang J, Schnell JR, Chou JJ. *Biochem Biophys Res Commun.* 2004; 324:212–7. [PubMed: 15465004]
22. Hu J, Fu R, Nishimura K, Zhang L, Zhou HX, Busath DD, Vijayvergiya V, Cross TA. *Proc Natl Acad Sci U S A.* 2006; 103:6865–70. [PubMed: 16632600]

Figure 1. (top) ^{15}N - ^{13}C zf-TEDOR spectra ($\tau_{\text{mix}} = 1.3$ ms) showing assignments of ^{13}C , ^{15}N [^{12}C , ^{14}N -ILFYJ M218-60 in the drug bound (blue) and unbound (red) states. Unless otherwise indicated, cross peaks arise from 1-bond N- α magnetization transfer. (bottom) ^{13}C - ^{13}C PDS spectra ($\tau_{\text{mix}} = 50$ ms) showing Asp and Gly cross-peaks of these samples. Sizable chemical shift changes are observed in the N and or α sites for residues 25, 27, 28, 31, 34, 35, 37, and 41. Many peaks are doubled (see supplemental Figure S1 for expansion), notably P25 and A29, supporting the existence of a twofold symmetric tetramer. Spectra were recorded $\sim 0^\circ\text{C}$, just above the phase transition of pure POPC lipids. Labels such as D44 are shown in italics to indicate less certainty in assignments (see Supplemental Information).

Figure 2.

Chemical shift perturbations ($\Delta\delta = \delta_{\text{bound}} - \delta_{\text{apo}}$) are distributed across the channel and support an allosteric effect upon drug binding. (left) Chemical shift perturbations as a function of residue number. (right) A comparison of the Rmt drug size with the transmembrane tetramer assembly from the solution structure. Blue residues indicate a shift of >2 ppm in N and/or >1 ppm in Cα/Cβ. One of the four helices has been removed for clarity.

Figure 3.

TEDOR spectra acquired at 0, 1, and 4 Rmt molecules per channel in a, b, and c, result in cross peaks due to M2 bound to Rmt and present at ~0%, 25% and >90%, respectively. The apo spectrum is simultaneously observed at 100%, 75% and <10% of total site-specific signal intensity. Unless otherwise indicated, cross peaks arise from 1-bond N-C α magnetization transfer. Resonances that clearly show the titration are displayed in red (unbound form) and blue (Rmt bound resonances). Dashed lines at G34 and other resonances serve as a guide. Signal to noise is ~10 for strong signals. M2 samples used in the titration were embedded in DPhPC lipids and show nearly identical spectra as those recorded in POPC lipids (see Figure 4).

Figure 4. ^{13}C - ^{13}C PDSD spectra ($\tau_{\text{mix}} = 15$ ms) of POPC embedded (red) and DPhPC embedded (black) M2 show nearly identical spectra with maximum chemical shift differences of 0.3 ppm. With 15 ms mixing, mostly 1-bond correlations are observed.