Interaction between an Amantadine Analogue and the Transmembrane Portion of the Influenza A M2 Protein in Liposomes Probed by ^{1}H NMR Spectroscopy of the Ligand

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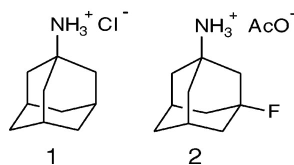
Received May 4, 2004

^{1}H NMR spectroscopy of a fluoroamantadine ligand was used to probe the pH dependence of binding to the transmembrane peptide fragment of the influenza A M2 proton channel (M2TM) incorporated into 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine liposomes. Above pH 7.5, when M2TM bound the ligand, fluoroamantadine resonances became too broad to be detected. Fluoroamantadine interacted weakly with the liposomes, indicating it may first bind to the bilayer and then block target channels after diffusion across the membrane surface.

In Europe in the 20th century pandemics of influenza A caused more fatalities than any other infectious disease. During less severe epidemics, ≥10% of the population can be infected, resulting in temporary debilitation, with significant economic consequences.[1a,b] The likelihood of future pandemics highlights the need to develop more effective therapies.

Amantadine (Am) **1** (1-aminoadamantane hydrochloride), a drug in the aminoadamantane series,[2] is li-



censed for use in the prophylaxis and therapy of influenza A virus infections. Am **1** inhibits the activity of the influenza A M2 protein, which is critical for virus replication. M2 is a small 97-residue integral membrane protein with an essential role in the acid-induced process of uncoating the viral RNA during infection. It comprises an extracellular N-terminal domain (21 residues), a transmembrane (TM) domain (25 residues), and an intracellular C-terminal domain (51 residues). M2 self-assembles as a homotetramer to form an ion channel. Each subunit of the tetramer contributes a single transmembrane α-helix (M2TM) that together constitute a proton selective pore that is activated by low pH environments, such as those found in endosomes.[3] The M2 channel allows protons to enter the interior of the viral particle, and this acidification dissociates the matrix protein M1 that coats the viral RNA genome.[1c]

It has been shown that the 25-residue M2TM peptide (the transmembrane portion of the wild-type M2 protein, corresponding to residues S22-L46 of the Singapore and Udorn strains: SSDPLVVAASIIGILHLILWILDRL) forms tetrameric amantadine-sensitive proton channels in planar lipid bilayers.[4,5] Insights from FT-IR,[6] solid-state NMR,[7] and molecular dynamics[8] have yielded a high-resolution structure of the monomer backbone of M2TM in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayers. Along with the measurement of a few intermolecular distance constraints,[7] these techniques have led to models of the M2TM tetrameric assembly as a left-handed parallel bundle of α-helices. These models account for cysteine mutagenesis data,[9] indicate that residues V6, A9, G13, H16, and W20 of M2TM line the pore, and are consistent with the imidazole side chain of H16 both acting as a proton shuttle and interacting with the indole side chain of W20 to occlude the pore. [7b]

Am **1** blocks the proton channel activity of M2. A current hypothesis is that this is achieved via interactions of the drug with the mouth of the M2 pore. Molecular modeling suggests that the luminal space between L26 and H37 is complementary in its shape, hydrophobicity, and polarity to Am **1**. Binding of the drug is expected to block proton channel activity by displacing water molecules that are essential for proton conduction.[9,10]

A variety of biophysical techniques have been used to study the monomer/tetramer equilibrium of M2TM and its interaction with Am **1** over a range of pH values in dodecylphosphocholine (DPC) micelles.[4a] In this paper we use the line shape of ligand resonances in ^{1}H NMR spectra to report on the interactions between amantadine analogue **2** and the M2TM peptide in DMPC liposomes, a more realistic lipid bilayer system.[4b] NMR spectroscopy has been widely used to study interactions between ligands and receptors by observing changes in the properties of either the macromolecule or the small molecule. Several different NMR parameters can be monitored for small molecules, including changes in chemical shift, relaxation rates, translational diffusion, and intermolecular or intramolecular magnetization transfer.[11] An advantage of monitoring the ligand is that

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10.1021/jm0496685 CCC: $27.50 © 2004 American Chemical Society
Published on Web 08/28/2004

there is no upper limit to the size of the receptor that can be investigated. This is an important consideration for studies of integral membrane protein systems by solution NMR techniques.

Many important pharmacological agents are first solvated in the lipid bilayer prior to their interaction with their protein target.[12] Since the adamantane core of the aminoadamantane drugs is hydrophobic, we also undertook a ^{1}H NMR study of the interaction between aminoadamantane **2** and DMPC lipid bilayers. The isosteric replacement of a bridgehead hydrogen atom of Am **1** with fluorine, resulting in F-Am **2**,[13] provides a useful probe molecule for studying drug–M2TM and drug–lipid bilayer interactions by both ^{1}H and ^{19}F NMR.

The effects of the binding of Am **1** on the monomer/tetramer equilibrium of M2TM over pH 5–8 have recently been investigated by a variety of biophysical techniques. Fluorescence spectroscopy, circular dichroism (CD), and analytical ultracentrifugation (AUC) in DPC micelles have shown that tetramerization of M2TM and binding of Am **1** are both favored at pH 7.5–8. A pK_a of 6.8 was determined for the histidine side chain of M2TM in the monomeric state in DPC micelles by ^{1}H NMR.[4a] It was suggested that the M2TM channel conducts protons via one or two protonated His side chains that line the pore of the tetramer and that Am **1** binds to the neutral tetrameric closed state of the channel at elevated pH.[4a] Electrophysiological studies also showed that Am **1** possesses its highest binding affinity for the wild-type M2 protein at pH 8[14] and that M2TM forms proton-selective channels.[15] Thus, studies of the M2TM peptide have implications for the function of the full length protein.

In this work we describe a ^{1}H NMR study of the M2TM/aminoadamantane drug system in DMPC liposomes over the pH range 5–8. The signals of F-Am **2** were used as a probe to follow the binding of the drug to M2TM, in contrast to previous studies, which focused instead on the spectral characteristics of the peptide itself in DPC micelles.[4a] The DMPC liposomes used in the present study mimic native lipid bilayers more closely than detergent micelles,[4b] which have notable differences in curvature, lateral packing pressure, and surface charge density effects.

M2TM/Aminoadamantane Drug System in DMPC Liposomes

^{1}H NMR spectra of Am **1** and F-Am **2** in aqueous solution are shown in Supporting Information. Between pH 5 and pH 8 no significant changes in the spectra of Am **1** or F-Am **2** were observed. This is consistent with both molecules possessing a high pK_a[16] and existing in the same protonated state throughout the pH range under consideration.

Dynamic light scattering (DLS) and electron microscopy (EM) studies indicated that between pH 5 and 8 DMPC liposomes prepared by dialysis of mixed lipid/detergent micelles remained colloidal at room temperature for > 5 days and possessed diameters between 50 and 75 nm (not shown). In the presence of DMPC liposomes at pH 8, the ^{1}H NMR signals of F-Am **2** broadened considerably, but no changes in chemical shift were observed, as illustrated in Figure 1a,b. A

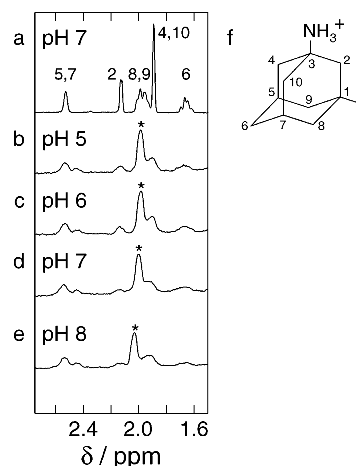


Figure 1. ^{1}H NMR spectra of samples containing (a) 0.6 mM F-Am **2** in phosphate buffer at 298 K, pH 8, (b–e) 0.6 mM F-Am **2** in phosphate buffer containing 30 mM DMPC at 298 K for pH range 5–8, and (f) F-Am **2**.

weak unassigned signal appeared at 2.45 ppm, close to the H-5,7 resonance of F-Am **2** in the absence of DMPC. The broadening of the signals of aminoadamantane **2** in the presence of DMPC vesicles suggests that free drug molecules bind to and are released from the large phospholipid assemblies at a rate that is fast compared to the changes in chemical shift between the bound and unbound states.[11] Although the unbound drug is likely to be in excess, the NMR spectrum is strongly affected by the rapid relaxation rate of the fraction that interacts with the liposomes. The line broadening effect could also be caused by the rate of exchange between the bound and free states being in the intermediate regime or by large changes in magnetic susceptibility at the bilayer interface. Increasing the acidity of the sample from pH 8 to pH 5 made little difference to the appearance of the ^{1}H spectrum, although the signal of unbound acetate at 2.0 ppm shifted slightly downfield toward pH 5 (Figure 1b–e). When Am **1** was added to DMPC vesicles at the same concentration, the signals from the drug were too broad to be observed (not shown), suggesting that Am **1** binds to the liposomes with higher affinity than F-Am **2**.

Between pH 5 and 8 the presence of M2TM enhanced the stability of DMPC liposomes, which remained colloidal for several weeks at room temperature.[17] The dimensions of these vesicles (diameter of ~70 nm by DLS and EM) are similar to the natural membranes in which M2 is known to function, the virion surface, and the trans Golgi network.[1] When F-Am **2** was added to DMPC/M2TM proteoliposomes at pH 8, the line broadening of ligand signals in the ^{1}H NMR spectrum was more dramatic (Figure 2a). Resonances from F-Am **2** could no longer be detected, with only signals from residual molecules of DMPC in solution and unbound acetate remaining. This result indicates that F-Am **2** interacts more strongly with lipid vesicles in the presence of the M2TM peptide than with DMPC liposomes alone, reducing the off rate so that the contribution from the transverse relaxation rate of the bound state dominates. After acidification of the sample to pH 5, resonances from F-Am **2** reappeared (Figure 2b), with line widths similar to those found in the presence of DMPC vesicles alone (Figure 1b). At low pH in the

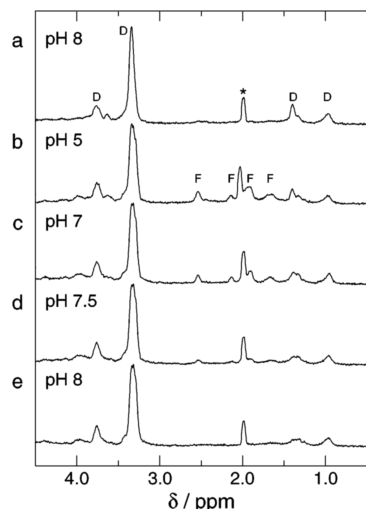


Figure 2. ^{1}H NMR spectra of samples containing 0.6 mM F-Am **2** in phosphate buffer with 30 mM DMPC and 0.6 mM M2TM monomer at 298 K: (a) pH 8; (b) pH 5; (c) pH 7; (d) pH 7.5; (e) pH 8. Annotations used are the following: D, DMPC peaks; F, F-Am **2** peaks; *, free acetate.

presence of M2TM, the drug appeared to lose the increased affinity for the bilayer environment that it had displayed at pH 8. Increasing the pH to 7 had little further effect (Figure 2c), but after a further increment to pH 7.5 the resonances of F-Am **2** showed signs of additional broadening (Figure 2d). When the sample was returned to pH 8, all resonances from F-Am **2** disappeared once again (Figure 2e).

The DMPC/M2TM molar ratio used in this study (25) favors the formation of the tetrameric state of M2TM at pH 8.[4b] This pH value is optimal for the binding of Am **1** to both full length M2 and M2TM.[4,14] The disappearance of the signals of F-Am **2** at pH 8 in the presence of the peptide is therefore consistent with the drug binding tightly to intact M2TM channels that are embedded in the bilayers of slowly tumbling lipid vesicles.

The ^{1}H NMR spectra obtained at pH 5−7 (Figure 2b,c) were similar to the spectra of samples containing only F-Am **2** and DMPC (Figure 1b−d), indicating that under these conditions there is no special interaction between the drug and the M2TM channel. This result was not unexpected because in DPC micelles M2TM exists mainly in a monomeric state that does not bind Am **1** between pH 5 and pH 7.[4a] The oligomeric state of M2TM in DMPC vesicles has not yet been established, although tetramers have recently been observed in studies of a slightly longer construct, $M2TM_{19-46}$.[4b] The formation of intermolecular cysteine bridges in the extracellular domain of the full length M2 protein indicates that the channel is tetrameric under all conditions in native membranes. The monomeric state observed for M2TM in DPC micelles at low pH is therefore probably an artifact of this non-native membrane-mimetic environment. The dissociation constant of the tetrameric state of $M2TM_{19-46}$ in DMPC liposomes has been estimated to be smaller than that in DPC micelles by a factor of $>10^2$.[4b] Hence, although M2TM probably remains tetrameric between pH 5 and 7 in DMPC liposomes, it is likely that the conformation of the tetramer is different from that at pH 8 because of the opening of the proton channel. This could explain why F-Am **2** is no longer able to bind M2TM under acidic conditions; the altered structure of the tetramer has lower affinity for the drug so that any residual interactions cannot be distinguished from those between the drug and DMPC.

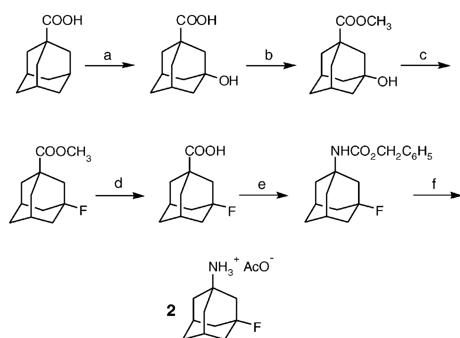
At pH 7.5 the resonances of F-Am **2** were significantly broadened in the presence of M2TM and DMPC liposomes; by pH 8 they were no longer detectable (Figure 2d,e). Since M2TM exists mainly as a neutral tetramer above pH 7.5 in DPC micelles, this behavior can be interpreted in terms of the recovery of a peptide tetramer conformation that is capable of binding to the drug in a reversible manner. In agreement with this view, at pH $\geq$ 7.5 in DMPC liposomes Am **1** has been shown to insert into the pore of the M2TM channel, where it is stabilized through favorable hydrophobic and polar interactions.[10] It is worth noting that simple ^{1}H NMR spectroscopy was successful at following the binding of F-Am **2** to M2TM only because the interaction between this drug and DMPC was relatively weak. The disappearance of resonances due to Am **1** in the presence of DMPC leaves no way of discriminating further interactions with peptides incorporated into the bilayer. Although the chemical shift degeneracy of Am **1** gives it the potential to act as a high-sensitivity probe of interactions with the closed state of the M2TM channel, our results highlight the utility of the fluorinated derivative. Our ongoing work with this system aims to exploit the high sensitivity and low background signal of ^{19}F NMR by introducing fluorine labels into the M2TM peptide as well as the F-Am **2** ligand.

Conclusions

The work presented in this paper is an extension of previously reported studies of the pH dependence of the monomer/tetramer equilibrium of and the binding of Am **1** to the M2TM peptide in DPC micelles. Our investigation used simple ^{1}H NMR spectroscopy to focus on a fluorinated aminoadamantane analogue and its interactions with M2TM incorporated into DMPC liposomes. Both approaches demonstrate that M2TM is not able to bind the aminoadamantane ligand between pH 5 and pH 7, either because of the formation of a non-native monomeric state or a change in the conformation of the tetrameric M2TM assembly. Above pH 7.5, M2TM adopts a neutral tetrameric form that binds the drug with high affinity. Under these conditions NMR signals from the drug cannot be detected. A broadening of drug resonances in the presence of DMPC suggests that the drug interacts with the lipid bilayers first and then possibly diffuses across the membrane until it encounters a tetrameric M2TM assembly in the closed state, whereupon it binds into the channel pore. [12]

Since we focused on inspection of the aminoadamantane ligand rather than its peptide M2TM receptor, we anticipate that this study will have implications for future work on the medicinal chemistry of this series of drugs. In principle, similar ligand focused experiments could be extended to investigate the interaction of aminoadamantane drugs with full length receptors on intact virus particles.[18] Future ^{19}F NMR experiments using F-Am **2** will aim to elucidate further aspects of how aminoadamantane drugs exert their anti-influenza A activity. Since M2TM is also a minimal model for the study of proton channel proteins, such results would

Scheme 1[a]



[a] Reagents and conditions: (a) $KMnO_4$, KOH, 3 h, 60 °C (yield 70%); (b) $TBAHSO_4$, $NaHCO_3$, CH_3I, acetone, 48 h, room temp (99%); (c) DAST, CH_2Cl_2, 3 h, −80 °C → 25 °C (48%); (d) NaOH, MeOH, THF, H_2O, 12 h, room temp (85%); (e) DPPA, TEA, 45 min, reflux, then BnOH, benzene, 72 h, reflux (quantitative); (f) H_2, 10% Pd/C, AcOH, 50 psi, 6 h, room temp (84%).

open new avenues for discovering how ion channel−blocker interactions can be described.

Experimental Section

The synthesis of F-Am **2** is illustrated in Scheme 1. Details of the preparation of liposomes and proteoliposomes and the ^{1}H NMR spectroscopy are reported in Supporting Information.

Acknowledgment. A.K. gratefully acknowledges the Royal Society for the award of a travel grant to perform NMR experiments in Cambridge.

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM0496685