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^{19}F NMR detection of the complex between amantadine and the receptor portion of the influenza A M2 ion channel in DPC micelles

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Abstract—^{19}F NMR probes were used to follow interactions between ligands in the aminoadamantane series, amantadine (Am) 1 and 3-F-Am 2, and the 5-F-Trp20 transmembrane fragment of the influenza A M2 proton channel (F-M2TM 3) in dodecylphosphocholine micelles over the pH range 5–8. Above pH 7, when the peptide adopts a tetrameric state that is able to bind channel blocking ligands, ^{19}F-Trp signals from both the free and bound states of the M2TM tetramer are resolved. This differentiation of bound and unbound states of the M2TM receptor by ^{19}F NMR may provide a system for SAR studies.

Influenza presents a severe threat to public health. In Europe in the 20th century more casualties were inflicted by influenza than any other infectious disease.[1a] In 1997, outbreaks of the H5N1 influenza virus killed six of the 18 infected individuals. A highly pathogenic H7N7 avian influenza virus killed a veterinarian and infected poultry workers in 2003, while in the same year H9N2 viruses were isolated from individuals with mild influenza.[1b] The H5N1 avian influenza virus that emerged in southern China in 1996 is now endemic in domestic poultry in southeast Asia and has so far infected 121 humans, killing 62. In the summer of 2005, this H5N1 virus caused a lethal outbreak of influenza in wild birds in western China and subsequently spread to poultry in Europe.[1c] The M2 protein is one of the few drug targets that can be inhibited in order to stifle infections of the influenza A virus.[2]

M2 is a small 97-residue integral membrane protein which self-assembles as a homo-tetramer to form a proton-selective ion channel[3] that is activated by low pH environments, such as those found in endosomes.[4] The M2 channel allows protons to enter the interior of the virus and this acidification dissociates the matrix protein M1, releasing the viral RNA genome.[5] Similar small ion channels have been found in the other enveloped influenza viruses, e.g., the BM2 (influenza B) and CM2 (influenza C) proteins, and in the human immunodeficiency type-1 (HIV-1) virus (the Vpu protein).[6] It is important to study such viroporins and their interactions with ligands in order to realize their potential as drug targets.

It has been shown that the 25-residue M2TM peptide (the transmembrane domain of the wild-type M2 protein, corresponding to residues S22-L46 of the Singapore and Udorn strains: SSDPLVVAASIIGILHLIL WILDRL) forms tetrameric Am **1**-sensitive proton selective channels in planar lipid bilayers.[7] Early experimental insights from neutron diffraction and circular dichroism[8] and more recent FT-IR data[9] have revealed that in a lipid environment the M2TM tetrameric assembly adopts a left-handed parallel bundle of α-helices. The measurement of orientational and a few distance constraints by solid state NMR studies[10] indicates a helical tilt of 32–38° with respect to the lipid bilayer normal. These models, which account for cysteine mutagenesis data and inhibition with transition metal ions,[11] indicate that residues A9, G13, H16 and W20 of M2TM line the pore. Molecular dynamics simulations[12] have yielded detailed models of the M2TM tetramer structure, which were used along with mutational

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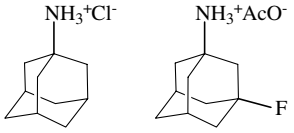
data[13a] and infrared spectroscopic measurements[13b] to propose a mechanism[13] for proton conductance.[3] According to the gating mechanism option,[13b] at low pH values the protonated His-37 imidazole tetrad of the M2TM tetramer is stabilized via cation-π interactions with Trp-41[13b] sidechains in the channel and a gate opens that can pass a proton but not other ions. At high pH, this π-complex is destabilized and the indole sidechains of Trp-41 move to occlude the pore. A recent model suggests that the M2TM channel conducts protons via three protonated His sidechains which line the pore of the tetramer and that at physiological pH the channel is closed by two imidazole–imidazolium dimers.[14] Conformational changes between the open and closed states have been followed using UV fluorescence, circular dichroism (CD) spectroscopy and by analytical ultra-centrifugation (AUC) in DPC micelles.[15]

Amantadine (Am) **1**, a drug in the aminoadamantane series,[16] blocks M2 proton channel activity[3] and inhibits virus entry and uncoating at micromolar concentrations.[5a,b] It has been proposed that this is achieved via interactions of the drug with the mouth of the M2 pore.[8b] Amantadine resistant mutations mostly target pore-lining residues, including V27,[17] A30 and G34.[5b,c] Molecular modelling[11,12,18] suggests that the luminal space between M2 residues L26 and H37 is complementary in its shape, hydrophobicity and polarity to Am **1**; binding of this drug is expected to block proton channel activity by displacing water molecules that are essential for proton conduction.



Am 1 **F-Am 2**

The effects of the binding of Am **1** on the monomer/tetramer equilibrium of M2TM in dodecyl phosphocholine (DPC) micelles over the pH range 5–8 have recently been investigated by a variety of biophysical techniques.[15,19] Fluorescence spectroscopy, CD and AUC in DPC micelles showed that tetramerization of M2TM and binding of Am **1** are both favoured at pH 7.5–8. Compound Am **1** binds with its highest affinity to the neutral tetrameric closed state of the channel at elevated pH.[15a] Electrophysiological studies have also shown that Am **1** binds most tightly to the wild-type M2 protein at pH 8.[3a] Thus, properties of the full-length protein can be deduced from studies of the M2TM peptide.

There are several advantages when fluorine is used as a reporter group for the study of ligand–receptor interactions by NMR. The sensitivity of the ^{19}F nucleus is nearly as high as that of the proton, which keeps acquisition times short and results in strong NMR signals appearing against a background devoid of signals from endogenous nuclei. In addition ^{19}F is very sensitive to the local environment of a binding pocket, with free and bound species typically showing large changes in chemical shift.[21] Several ^{19}F NMR studies of drug binding to lipid membranes and ligand–receptor interactions have been reported, both in solution and in the solid state.[22]

In this paper, we describe a ^{19}F NMR study of the M2TM/aminoadamantane drug system in dodecylphosphocholine (DPC) micelles over the pH range 5–8. Detergent micelles of this sort can provide an environment that successfully mimics a lipid bilayer for segments of integral membrane proteins.[23] The isosteric replacement of the bridgehead and 5-indole ring hydrogen atoms of Am **1** and Trp20 of M2TM resulting in F-Am **2** and 5-F-Trp M2TM **3**, respectively, provided useful probe molecules for studying: (a) drug– or M2TM–lipid bilayer interactions (which are important for drugs that bind to membrane proteins since it can be assumed that the ligand must first interact with the lipophilic barrier in order to reach its receptor[24]); and (b) drug–M2TM receptor interactions. When the interaction of a membrane-associated peptide or protein is considered, tryptophan has been established to be a sensitive reporter of local dynamics.[25]

Results. In recent work we used the lineshapes of ligand resonances in ^{1}H NMR spectra to report on interactions between amantadine analogue **2** and the M2TM peptide in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) liposomes. Above pH 7.5, when M2TM bound the ligand, 3-fluoroamantadine **2** resonances became too broad to be detected.[26] When we attempted to acquire ^{1}H NMR spectra of F-M2TM **3** in DPC micelles over the pH range 5–8, no significant changes were observed in the absence or presence of ligand **2** (data not shown). We therefore turned our attention to ^{19}F spectroscopy, which is more sensitive to local environment changes.

^{19}F NMR spectra of F-Am **2** *in aqueous buffer*. Between pH 5 and pH 8 no significant changes in the spectra of F-Am **2** were observed (data not shown). This is consistent with F-Am **2** possessing a high pK_a and existing in the same protonated state throughout the pH range under consideration.[27]

^{19}F NMR spectra of F-Am **2** *with DPC micelles*. In the presence of DPC micelles at pH 5–8, the resonances of F-Am **2** were slightly broader (Fig. 1a–d) and T_1 relaxation times decreased from 1.5 s (panel a) to 1.2 s (panels b–d), suggesting that free drug molecules bind to and are released from the phospholipid assemblies at a rate that is fast compared to the changes in chemical shift between the bound and unbound states.[28] Increasing the sample pH from 5 to 8 made little difference to the ^{19}F chemical shift, which changed from −132.9 to −132.7 ppm (Fig. 1a–d). These observations are consistent with recent results from liquid-state NMR measurements of translational diffusion, which indicate that amantadine has a strong membrane partition potential but does not bind strongly to lipid molecules, tumbling freely and diffusing laterally in a model bilayer system.[29]

^{19}F NMR spectra of 5-F-Trp20 M2TM **3** *with DPC micelles*. After adding peptide **3** to DPC micelles, a

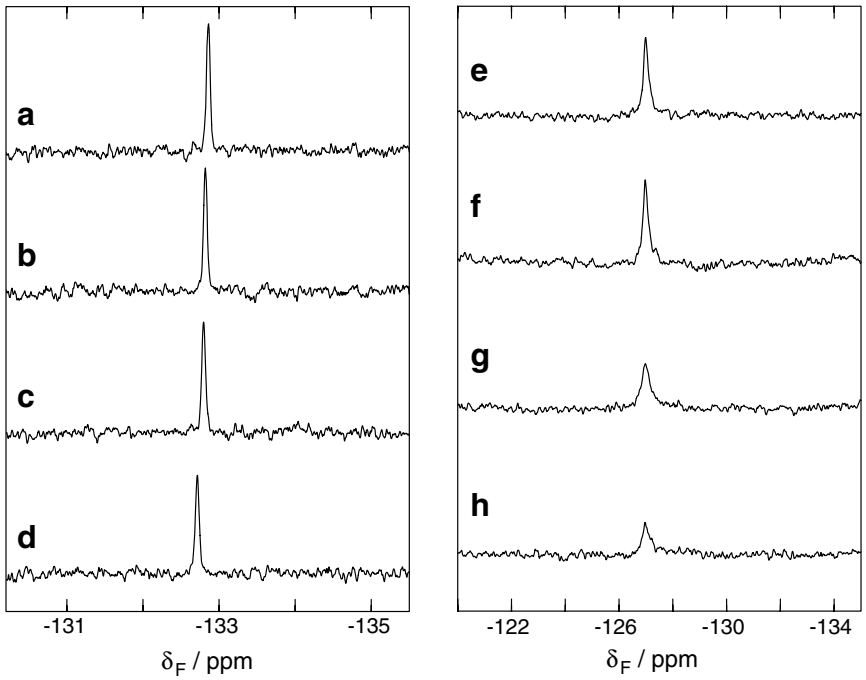


Figure 1. (a–d) ^{19}F NMR spectra of samples containing 1 mM F-Am **2** in 50 mM phosphate buffer containing 20 mM DPC and 100 mM NaCl at 298 K: (a) pH 5; (b) pH 6; (c) pH 7; (d) pH 8. (e–h): ^{19}F NMR spectra of samples containing 1 mM 5-F-Trp20 M2TM **3** monomer in 50 mM phosphate buffer containing 20 mM DPC and 100 mM NaCl at 298 K: (e) pH 5; (f) pH 6; (g) pH 7; (h) pH 8. All spectra averaged over 512 scans.

significant line broadening effect was observed when the pH was increased from 5–6 (Fig. 1e and f) to 7–8 (Fig. 1g and h). In DPC micelles the monomer–tetramer equilibrium of M2TM is reportedly reversible, with a tetrameric peptide assembly being favoured at high peptide/lipid molar ratios.[15] The DPC/M2TM molar ratio used in this study was 20, which is known to favour the formation of the tetrameric state of M2TM at pH 8.[15b]

The broadening effect observed in the ^{19}F spectrum of 5-F-Trp20 M2TM can therefore be explained in terms of the peptide tumbling more slowly when organized as a tetrameric assembly at pH 8, leading to a more effective dipolar interaction between the ^{19}F and ^{1}H spins, thereby reducing the ^{19}F transverse relaxation time (T_2) of the bound state form and increasing the linewidth of the NMR signal.

Throughout this pH range a small downfield resonance shift of 0.3 ppm was observed (from −127.8 ppm at pH 5 to −127.5 ppm at pH 8), but the ^{19}F T_1 relaxation time of 5-F-Trp20 remained constant at 0.6–0.7 s. Since longitudinal relaxation rates depend on the overall tumbling of the system containing the fluorinated aromatic ring, the same T_1 can be observed at the different correlation times associated with monomeric structure and a tetrameric assembly.[21]

^{19}F NMR spectra of F-Am **2** *or Am* **1** *with DPC micelles containing 5-F-Trp20 M2TM* **3**. When F-Am **2** was added to DPC micelles that contained the fluorinated peptide **3** at pH 5–6, two fluorine signals were detected at −132.9 and −127.8 ppm (Fig. 2a and b). These peaks correspond to the fluorine nuclei of F-Am **2** and 5-F-Trp20 M2TM **3**, respectively, and show no chemical shift or linewidth changes from the signals of binary mixtures with DPC (Figs. 1a and 2a). Both resonances

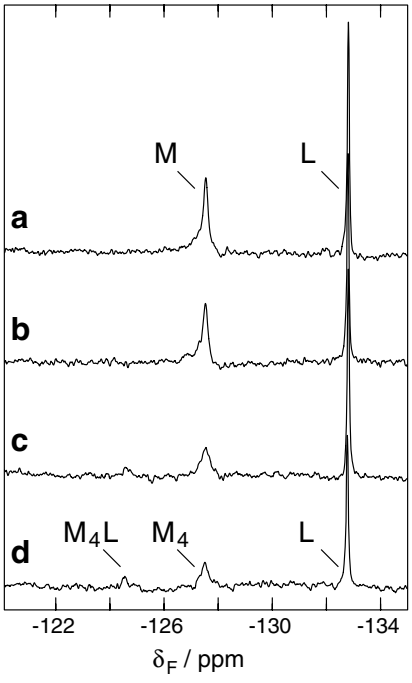


Figure 2. ^{19}F NMR spectra of samples containing 1 mM F-Am **2** in 50 mM phosphate buffer containing 20 mM DPC, 100 mM NaCl and 1 mM 5-F-Trp20 M2TM **3** monomer at 298 K: (a) pH 5; (b) pH 6; (c) pH 7; (d) pH 8; *Annotations used*: M_4L, bound F-M2TM **3** tetramer peaks; M_4, free F-M2TM **3** tetramer peaks; L, F-Am **2** peaks. All spectra are averaged over 512 scans.

shifted slightly upfield by ~0.3 ppm when the pH was raised to 8 (Fig. 2d). However, at pH 7, a broad signal of lower intensity appeared at −124.6 ppm (Fig. 2c), ~3 ppm downfield from the resonance of the F-M2TM **3** tetramer; the definition of this feature was slightly improved at pH 8 (Fig. 2d or 3c). The ^{19}F spectrum of F-M2TM **3** in DPC micelles contains a similar signal in the presence of Am **1**, which contains no fluorine (Fig. 3a). We therefore surmised that the new signal represents the bound state of the F-M2TM **3** tetramer rather than a change in the environment of F-Am **2**. Supporting this conclusion, the intensity of the free F-M2TM signal decreased and that of the bound state increased when the concentration of F-Am **2** was increased by 10-fold (Fig. 3b).

The DPC/M2TM molar ratio used in this study (20) is likely to favour the formation of the tetrameric state of M2TM at pH 8.[15] This pH value is also optimal for the binding of Am **1** to both full-length M2 and M2TM.[15,3a] The appearance of the fluorine signal at −124.6 ppm at pH 8 is therefore consistent with a state formed by the drug binding tightly to an intact tetrameric F-M2TM **3** channel. ^{19}F relaxation measurements for the ligand were consistent with this suggestion, as the T_1 value decreased significantly from 1.2 s in DPC alone to 0.6 s in DPC micelles containing peptide **3** at pH 8.

Discussion. Previous experiments have shown that the tetramerization of M2TM which occurs in DPC micelles at pH ⩾ 7.5 can be monitored using a small but significant increase in ellipticity at 223 nm in the CD spectrum. This change has been interpreted in terms of the Trp41 indole ring experiencing an increasingly hydrophobic and/or rigid environment. The ellipticity change is more pronounced upon binding Am **1**, suggesting that the peptide experiences a subtle conformational change. It has been proposed that on going from acidic to neutral conditions or from the monomeric to the tetrameric state of M2TM, a reorientation of the indole ring occurs; this movement of the Trp sidechain is possibly governed by a favourable cation–π interaction between His16 and Trp20.[13,30]

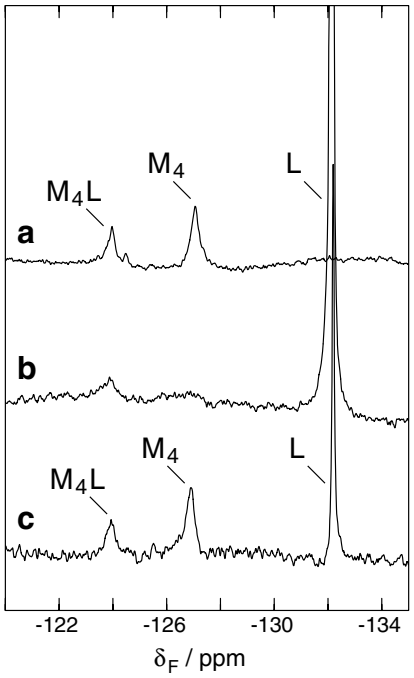


Figure 3. ^{19}F NMR spectra at 298 K of samples containing 20 mM DPC, 100 mM NaCl and 1 mM 5-F-Trp20 M2TM **3** monomer in 50 mM phosphate buffer pH 8 containing: (a) 1 mM Am **1**; (b) 10 mM F-Am **2**; (c) 1 mM F-Am **2**; *Annotations used*: M_4L, bound F-M2TM **3** tetramer peaks; M_4, free F-M2TM **3** tetramer peaks; L, F-Am **2** peaks. All spectra are averaged over 2048 scans.

In the present study, a ^{19}F-labelled tryptophan analogue was used to investigate the monomer/tetramer equilibrium of M2TM in DPC micelles and the effects of drug binding. Such an approach presents a risk of inducing structural or electronic perturbations, but ^{19}F-labelled analogues are generally compatible with native structures, causing only small changes.[25] According to the NMR spectra presented in Figure 1e–h, under optimal conditions for M2TM tetramer formation (pH 8 and a DPC/M2TM molar ratio of 20) the only changes in the F-M2TM peak were a significant broadening and a small downfield resonance shift (0.3 ppm). In parallel with the minor changes observed in CD spectra,[15a] differences in the ^{19}F spectrum were small but could nevertheless be used to follow the tetramerization of M2TM. We interpret our experiments as indicating that the tetrameric state of F-M2TM **3** appears at pH 7, that is ~0.5 units below the pH at which tetramer formation had previously been identified by CD for M2TM.[15a]

The ^{19}F NMR spectra of F-Am **2** and Am **1** with 5-F-Trp20 M2TM **3** in DPC micelles obtained at pH 5–6 (Fig. 2a and b) show two peaks at −132.9 and −127.8 ppm, corresponding to F-Am **2** and F-M2TM **3**, respectively. These signals are similar to those in samples containing only F-Am **2**/DPC or peptide **3**/DPC (Fig. 1a and e), indicating that there is no special interaction between the drug and F-M2TM **3** at low pH. This result was not unexpected, since M2TM exists mainly in a monomeric state in DPC micelles below pH 7.5.[15a] However, the ability of the extracellular domain of the full-length M2 protein to form intersubunit cysteine bridges indicates that the intact channel remains tetrameric under all conditions in native membranes. Thus, the monomeric state observed for M2TM in DPC micelles is an artefact of the non-native membrane-mimetic environment and the peptide model of the channel.

It has been proposed that at pH ⩾7.5 in DPC micelles Am **1** inserts into the pore of the M2TM channel, where it is stabilized through favourable hydrophobic and polar interactions.[8b,11,19] At pH 8, 5-F-Trp20 M2TM **3** gave rise to a second fluorine signal at −124.6 ppm in the presence of both Am **1** and F-Am **2** (Figs. 2c and d; 3a–c). Since M2TM exists mainly as a neutral tetramer above pH 7.5 in DPC micelles, this behaviour 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; on addition of the ligand the environment of 5-F-Trp-20 in the tetramer is altered,

resulting in the new peak. Figure 2c indicates that this signal began to manifest itself at pH 7. Consistent with the previous observation on the spectra of the F-M2TM/DPC vesicle system (Fig. 1e–h), this confirms that a tetrameric state of the F-M2TM **3** peptide is at least partially formed at pH 7.

The observation of two signals from F-M2TM suggests that chemical exchange between the free and bound tetrameric states is slow compared with the chemical shift difference between the two sites. This agrees with conductivity experiments which showed that Am **1** is a relatively strong inhibitor, exhibiting an almost irreversible block with a binding constant of 3×10^6 M^{-1} and a very low reverse reaction constant (3×10^{-4} s^{-1}).[3a] Our results represent the first time that resonances from a complex between amantadine analogues and an M2TM receptor have been resolved by NMR. A significant fraction of the F-M2TM **3** tetramer appears to remain unbound at pH 8 (Fig. 3a). Taking into account that the molar ratio of Am **1** or F-Am **2** to F-M2TM **3** was 1 and that, according to the conductivity experiments, Am **1** binds to M2TM in a first order reaction,[3a] this suggests that a significant number of ligand molecules diffuse into DPC micelles.

Synopsis. Our investigation used ^{19}F labels and simple 1D NMR spectroscopy to study the interactions between Am **1** or F-Am **2** and F-M2TM **3** incorporated into DPC micelles. Consistent with previous studies on unfluorinated M2TM, F-M2TM **3** is not able to bind the aminoadamantane ligand at pH 5–6, due to the formation of a non-native monomeric state. Above pH 7, F-M2TM **3** adopts a neutral tetrameric form that binds the drug with high affinity. Under these conditions, two separate NMR signals assigned to the free and bound forms of the F-M2TM **3** tetramer were detected.

These results could possibly be used for SAR by NMR[22c,28] studies of the aminoadamantane series and will guide new investigations into the formation of the M2TM–drug complex. Since M2TM is also a minimal model for the study of other viral ion channels and of proton channel proteins in general,[5] such experiments would provide information about how ion channel/blocker interactions can be described and possibly contribute to the design of successful channel blockers.

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