The Chemical and Dynamical Influence of the Anti-Viral Drug Amantadine on the M₂ Proton Channel Transmembrane Domain

Jun Hu,† Riqiang Fu,‡ and Timothy A. Cross*†‡

*Department of Chemistry and Biochemistry, †Institute of Molecular Biophysics, Florida State University; and ‡National High Magnetic Field Laboratory, Tallahassee, Florida

ABSTRACT The M₂ proton channel plays a vital role in the life cycle of the influenza A virus. His37, the key residue in the M₂ transmembrane domain (M₂-TMD), plays a central role in the proton conductance mechanism. The anti-influenza drug, amantadine, inhibits the channel activity through binding to the pore of the M₂ channel. The nuclear spin relaxation data and polarization inversion spin exchange at the magic angle spectra show that both the polypeptide backbone and His37 side chain are more constrained in the presence of amantadine. Using 15N cross polarization magic-angle spinning NMR spectroscopy, the protonation of His37 of M₂-TMD in lipid bilayers was monitored in the absence and presence of amantadine as a function of pH. Binding amantadine lowers the His37 pKₐ values by approximately three orders of magnitude compared with the first pKₐ of histidine in amantadine-free M₂-TMD. Amantadine’s influence on the His37 chemical properties suggests a novel mechanism by which amantadine may inhibit proton conductance.

INTRODUCTION

In binding amantadine to the M₂ protein transmembrane domain of influenza A virus several significant effects on the structure, dynamics, and chemical properties of this proton channel are observed (1–5). Here, studies in planar lipid bilayers above the gel-to-liquid-crystalline phase transition and in liposomes below this phase transition temperature were performed using solid-state NMR. While the structural change in the polypeptide backbone as viewed by solid-state NMR has recently been reported (6), here the focus is on the change in dynamics and in the chemical properties of the histidine tetrad formed by His37 of this tetrameric protein. The M₂ protein from influenza A virus is a pH-modulated proton channel that is essential for viral replication (7,8). These viruses enter cells through receptor-mediated endocytosis and are encapsulated by endosomes having a low pH. The M₂ proton channels are activated by low pH and acidify the viral interior leading to the disruption of the interaction between the viral matrix proteins and ribonucleoproteins, a prerequisite process for the release of viral RNA into the cell. In addition to its function in the uncoating process, the M₂ channels also equilibrate the pH between the acidic trans-Golgi network and the cytoplasm. Amantadine (1-adamantanamine hydrochloride) is a specific anti-influenza A drug that inhibits viral replication, primarily by binding to the M₂ protein and thereby preventing proton conductance (9–11). Without the acidification of the viral interior, the uncoating process for the influenza A virus fails to occur (12,13). Moreover, the hemagglutinins in the trans-Golgi network are forced to adopt a low-pH conformation due to the blocked M₂ channels (14,15).

Although amantadine is effective as a preventative prophylaxis and as a treatment of the influenza A viral infections, its clinical use as an anti-influenza drug is limited due to its central nervous system side effects (16). Rimantadine (α-methyl-1-adamantamine-methanamine), an amantadine analog, has been considered a better anti-viral drug because of its enhanced activity and fewer side effects (17,18). However, in the past year, the prevalent Influenza A (H3N2) strain was the S31N variant that is amantadine-resistant (19). It is generally believed that the rigid amantadine molecule sterically blocks the M₂ transmembrane pore, thereby preventing proton conduction. However, this mechanism remains controversial as it is inconsistent with some of the electrophysiological observations for the M₂ channels (20) and may also be inconsistent with results obtained from amantadine-resistant mutants (4). While the mechanism of amantadine binding to the M₂ channel is unclear, many amantadine analogs have been synthesized to test their antiviral activities (21–23). The M₂ protein has 96 amino-acid residues with a single 19-residue transmembrane domain (24,25). The functional structure of the M₂ channel is minimally tetrameric (26–28). Electrophysiological studies demonstrate that M₂ protein selectively conducts protons through a variety of membrane systems, such as oocytes of Xenopus laevis, mammalian cells, and planar lipid bilayers (29–31). Significant suppression of proton conduction by amantadine or rimantadine in these systems is well documented.

The transmembrane domain of M₂ (M₂-TMD) also forms a proton selective channel in lipid bilayers that is sensitive to amantadine (32). Analytical ultracentrifugation experiments show that amantadine stabilizes the formation of the M₂-TMD tetramer (1). Structural information on M₂-TMD has been obtained by cysteine-mutagenesis (33), molecular dynamics (34), UV resonance Raman spectroscopy (2), Fourier transform infrared spectroscopy (35), solid-state NMR spectroscopy (36–38),

doi: 10.1529/biophysj.106.102103
and electron spin resonance (39). Uniquely, solid-state NMR has led to a high-resolution backbone structure of the monomer while in a tetramer configuration (PDB ID: 1MP6, (36)). The addition of a precise distance restraint between helices has resulted in a structure of the tetrameric transmembrane domain (PDB ID: 1NYJ; (38)). Interestingly other spectroscopic data suggests somewhat different tilt angles for the helices depending on the membrane mimetic environment (39). The M2-TMD structure built on the basis of solid-state NMR orientational and distance restraints indicates a channel pore lined by polar residues (Ser31, His37, and Trp31) that can accommodate an amantadine molecule (38). Nevertheless, spectroscopic evidence from fluorescence (1) and circular dichroism (3) indicates that some structural rearrangements takes place upon amantadine binding. The M2-TMD/amantadine model proposed by Gandhi et al. (40) reflects some insights for this structural alteration and suggests a potential hydrogen-bonding interaction between the nonprotonated His37 Nε1 and the amantadine amino group. Recently, the backbone structure of the amantadine-bound state has been determined (PDB ID: 2H95; (6)) showing a kinked helical structure, but the position of amantadine within the structure was not defined.

Here, by using 15N cross-polarization magic-angle spinning (CPMAS) NMR spectroscopy, we titrated the M2-TMD histidine in the presence and absence of amantadine as a function of pH. The effect of amantadine binding on the His37 side-chain motion was studied by comparing the cross polarization efficiency for the His37 15N 1H signal with and without amantadine. The conformational and dynamic changes in the M2-TMD backbone upon amantadine binding were also monitored by polarization inversion spin exchange at the magic angle (PISEMA) experiments (41,42).

MATERIALS AND METHODS

Peptide synthesis and sample preparation

15N 1 histidine and 15N 2 histidine were purchased from Cambridge Isotope Laboratories (Cambridge, MA) and chemically protected by trityl and 9-fluorenylmethoxycarbonyl (Fmoc) groups following literature procedures (43,44). The total yield of 9-fluorenylmethoxycarbonyl -His(trityl)-OH was 73–81%. The M2-TMD peptide, NH2-Ser22-Ser-Asp-Pro-Leu-Val-Val-Ala-Ala30-Ser-Ile-Ile-Gly-Ile-Leu-(15N 15N) 15N His37-Leu-Ile-Leu-(15N 15N) 15N His37-Trp-Ile-Leu-Asp-Arg-Leu 1COOH was chemically synthesized by solid-phase synthesis on an Applied Biosystems (Foster City, CA) model No. 430A synthesizer. The peptides were purified and characterized as described previously (37).

15N His37 labeled M2-TMD domain was incorporated into liposomes through the assistance of a detergent, 1-O-ctetyl-β-D-glucopyranoside (OG) (45). First 10 mg M2-TMD peptide, 40 mg 1,2-dimyristoyl-sn-3-phosphocholine (DMPC), 10 mg 1,2-dimyristoyl-sn-glycerol-3-[phospho-rac-(1-glycerol)] (DMPG), and 320 mg OG were codissolved in 20 ml 2,2,2-trifluoroethanol (TFE) and chloroform (v/v: 4/1). A OG/lipid ratio of 15:1 allows for the preparation of a highly homogeneous sample of vesicles after OG is dialyzed out later in this procedure (46). The organic solvent was evaporated from a rotary flask and then the mixture was placed under high vacuum for at least 6 h to remove residual organic solvent. Fifteen milliliters of 10 mM citrate-phosphate (CBP) buffer of a specific pH with 1 mM ethylenediaminetetraacetic acid was added to the dried mixture and a clear solution was prepared after the flash was shaken in a bath for ~20 min at 37°C (above the gel-to-liquid-crystalline phase transition temperature of DMPC (23–25°C) (47). This solution was then transferred into a dialysis bag with a 3 kDa cutoff and dialyzed at 4°C against the buffer, which was changed at least five times in five days to ensure virtually complete removal of the detergent. For an M2-TMD sample with 10 mM amantadine, 46.9 mg (250 μmol) amantadine hydrochloride (Thermo Fisher Scientific, Waltham, MA) in 5 ml CBP buffer was added to an M2-TMD loaded vesicle suspension (20 ml). The suspension was incubated at room temperature overnight and pelleted in 2.5 h by ultracentrifugation at 196,000 g. The pH value of the pellet was implied from a measurement of the supernatant. Finally, the M2-TMD in DMPC/DMPG liposomes with or without amantadine was packed into a 7 mm Bruker (Madison, WI) zirconia magic-angle rotor with a sealing cap ready for CPMAS NMR experiments.

 Oriented samples of the 15N-(L26, L36, L38, L40, L43) and 15N 2 His labeled peptide in hydrated DMPC bilayers were prepared by first codissolving M2-TMD (20 mg) and DMPC (75 mg) in 10 ml TFE. TFE was removed by rotary evaporation and dried further under high vacuum. Fifteen milliliters of 2 mM CBP buffer (~37°C, pH 8.8) with 1 mM ethylenediaminetetraacetic acid was added to the dried mixture and shaken at 37°C. This lipid suspension was bath-sonicated for 10 min intermittently. The sonicated suspension was loaded into a 1 kDa cutoff dialysis bag. The dialysis bag was placed in 1 L of 2mM CBP buffer overnight to adjust the pH of the M2-TMD/DMPC liposomes. For the samples with amantadine, the outside buffer contained 10 mM amantadine. The liposomes were passed through a 2-μm filter and pelleted by ultracentrifugation at 196,000 g. As above, the pH of the supernatant was measured with an accuracy of ±0.05. The pellet was agitated at 37°C for 1 h until fluid. This viscous fluid was spread onto 50 glass slides (5.7 mm × 12.0 mm) (Marienfeld Glassware, Bad Margarethen, Germany) and dried in a constant humidity (70–75% relative humidity) chamber using an N2 atmosphere. The dehydrated slides were rehydrated with 1.5 μL, 2 mM CBP buffer per slide followed by stacking the slides into a glass tube. The sample was incubated at 43°C for 24 h in 96% humidity (saturated K2SO4) chamber. Finally, the glass tube was sealed at both ends with epoxy and two glass caps.

Solid-state NMR experiments

All CPMAS NMR experiments were conducted on a Bruker DMX-300 NMR spectrometer at 277 K using a spinning rate of 3 kHz. Two milliseconds cross-polarization contact time and 5 s recycle delay were used throughout the variable-pH NMR experiments. One-hundred Hertz exponential line broadening was applied to the free induction decay before Fourier transform. Static 13P NMR experiments on oriented samples at 303 K were also performed using the Bruker DMX-300 NMR spectrometer with an NHMFL 199H double resonance probe. The two-dimensional PISEMA experiments were performed at 303 K on a 400 MHz spectrometer using an NHMFL 13N 1H double resonance probe. Typically, a 6 s delay was applied before the 1H 90° pulse (52.1 kHz field). This RF field was also applied during the 1H-15N cross polarization (800 μs) and 1H continuous wave decoupling, while an RF field of 63.7 kHz was used during the Lee-Goldberg spin exchange at the magic angle. Proton carrier frequencies were carefully adjusted to minimize the offset effect (48,49). Thirty-two τ1 increments with 512 or 1024 transients were recorded for the two-dimensional PISEMA spectra. 15N chemical shift of a saturated 15NH4NO3 was defined as 0 ppm for all 15N spectra.

The 1H spin-lattice relaxation time in the rotating frame (T1p) was measured using a standard pulse sequence described previously (50,51). In short, after a 90° pulse on the 1H channel, the 1H magnetization was spin-locked for a period of time τSL before making CP contact with the 15N spins. With a fixed CP contact time (2 ms), the polarized 15N signals detected with 1H decoupling monitored the 1H magnetization in the spin-lock field as a function of τSL, thus allowing one to measure T1p indirectly. For variable-contact
RESULTS AND DISCUSSION

Amantadine binding effect on M2-TMD resonances

Chemical structures and the chemical shift resonances of histidine side chains in M2-TMD depend upon the local environment. In addition, neutral histidines may also undergo tautomerization. Fig. 1 illustrates the resonance assignment of histidines for the different chemical states in the M2-TMD channel (52). Generally, $^{15}\text{N}^{2\text{d}}^{1\text{H}}$ of positively charged histidines contributes to signals in the range of 153–156 ppm (53), while the neutral histidine side chains give rise to chemical shifts between 144 and 147 ppm. Neutral $^{15}\text{N}^{6\text{d}}^{1\text{H}}$ loses a proton due to tautomerization, resulting in $^{15}\text{N}^{6\text{d}}$ signals resonating at 230 ppm. His$^{+}$-His hydrogen-bonding shifts $^{15}\text{N}^{2\text{d}}^{1\text{H}}$ to a lower field, while the nonprotonated nitrogen shifts to a higher field (60). For M2-TMD in the absence of amantadine, the protonated nitrogen was observed between 162 and 167 ppm (52).

Fig. 2 A displays the CPMAS NMR spectra of $^{15}\text{N}^{2\text{d}}$ labeled His$^{37}$ M2-TMD with and without amantadine at pH 8.8. The $^{15}\text{N}$ chemical shifts of 144 ppm indicate that histidines are neutral in both cases at this pH. The striking difference in these spectra is the improvement in linewidth upon binding amantadine, suggesting that the amplitude of low frequency motions has been dramatically reduced or that conformational heterogeneity has been greatly reduced upon amantadine binding. Shown in Fig. 2 B is PISEMA data from the $^{15}\text{N}$-Leucine labeled M2-TMD prepared in an analogous fashion to the $^{15}\text{N}$ His$^{37}$ samples. Once again, a dramatic improvement in linewidth is observed upon binding amantadine. Consequently, if there is a reduction in low frequency motion it pertains to both the histidine side chain and the backbone throughout the TM helix. Furthermore, since the
PISEMA resonances directly and sensitively reflect the orientation of these backbone sites with respect to the laboratory frame, the well-resolved resonances in the M$_2$-TMD/amantadine spectrum define a unique structure with little orientational mosaic spread. Either local (peptide plane) or global (helix) disorder would broaden the resonances in the PISEMA spectrum. Such a situation is observed when amantadine is absent, but not in its presence. In addition, the PISEMA spectrum of $^{15}$N$^{2}$-His$^{37}$ M$_2$-TMD in the presence of amantadine at pH 9 shows a well-defined splitting (Fig. 2) consistent with a unique conformation having little disorder (54). As indicated from thermal stability studies of M$_2$-TMD mutants (5), the dynamics or conformational heterogeneity in the absence of amantadine when the channel is functional appears to require structural flexibility to be sensitive to the chemical stimulus (e.g., H$^+$.)

**Slow dynamics of the His$^{37}$ side chain**

In the CPMAS experiments, it is not possible to quantitatively compare the intensities of the protonated and nonprotonated $^{15}$N resonance intensities directly. For the case where $T^H_{1p}$ is approximately equal to the CP buildup time $T_{NH}$, Fu and co-workers developed a Lee-Goldburg frequency-modulated CP approach to improve the $T^H_{1p}$, and CP buildup rate (50). Under long CP contact conditions, a relatively accurate estimate of the ratio between nonprotonated and protonated histidine resonances is achieved by integrating the signals in the Lee-Goldburg frequency-modulated CP spectrum. For membrane protein samples in hydrated lipid bilayers, $T_{NH}$ is often similar to or even longer than $T^H_{1p}$. In this case, it is inappropriate to apply the above methods to define the relative amount of each component in the CPMAS NMR spectrum and we have resorted here to analyzing the cross polarization kinetics followed by the development of a correction factor to calculate the relative number of protonated and nonprotonated sites. In addition to the measurements of $T^H_{1p}$ and $T_{NH}$, the intensities of the spinning side bands for the nonprotonated state must also be included (50).

The mobility of the His$^{37}$ side chain can be assessed by measuring the relaxation parameters controlling the $^{15}$N CP process. Here, we make the reasonable assumption that $T_{NH}$ is much shorter than the $^{15}$N spin-lattice relaxation time in the rotating frame ($T^N_{1R}$ (55)). Then the signal intensity of a given species, $M(t_{CP})$, can be characterized by the equation (56)

$$M(t_{CP}) = M_0 \left(1 - \frac{T_{NH}}{T_{1p}}\right)^{-1} \left[\exp\left(-\frac{t_{CP}}{T_{1p}}\right) - \exp\left(-\frac{t_{CP}}{T_{NH}}\right)\right],$$

(1)

where $t_{CP}$ is the CP contact time and $M_0$ is a constant that is proportional to the number of spins. Using a standard CP experiment (51), $T^H_{1p}$, $M_0$, and $T_{NH}$ in the absence and presence of amantadine are listed in Table 1.

The inverse of the CP buildup time of the dilute spin $^{15}$N signal cross-polarized from the abundant spin $^1$H, 1/T$_{NH}$, more often called the CP rate constant, is predominantly controlled by the NH dipolar coupling. Roughly, the CP rate is proportional to the square of the dipolar coupling between $^1$H and $^{15}$N (57,58). This explains why the signal buildup for the protonated $^{15}$N$^{61}$ (147 ppm) resonance is approximately two orders-of-magnitude faster than for the nonprotonated resonance (230 ppm). Consequently, the observed intensities for protonated and nonprotonated sites at a single cross-polarization time cannot be compared directly without taking the cross-polarization kinetics into account.

Molecular motions can average and hence reduce dipolar interactions and therefore $T_{NH}$ can provide a qualitative description of the motional difference in the presence and absence of amantadine. Here we assume that the magic angle spinning rate does not affect the $T_{NH}$ values (59) and that the Hartmann-Hahn condition was well matched. In the presence of amantadine, $T_{NH}$ is approximately half that in its absence. In other words, the $^{15}$N$-^1$H dipolar interaction in the absence of amantadine is 22% smaller due to the motional averaging in the His$^{37}$ side chain. For amantadine-bound M$_2$-TMD, both the backbone (Fig. 2 B) and side-chain (Fig. 3) PISEMA data suggest less conformational heterogeneity and/or reduced motional amplitude. Coupled with the $T_{NH}$ results, it is clear that the amantadine-bound state is more rigid and conformationally more homogeneous. In addition, the PISEMA data for amantadine-bound M$_2$-TMD has been used to characterize an altered backbone structure involving a kinked α-helical structure (6).

![FIGURE 3 $^{15}$N PISEMA spectra of $^{15}$N$^{2}$ His$^{37}$ labeled M$_2$-TMD in aligned DMPC planar bilayers with amantadine bound at pH 9.0. The PISEMA spectra were acquired on a 400 MHz spectrometer at 298 K.](image-url)
His\textsuperscript{37} tautomerization

On the basis of the values of \( M_0 \) for the 147 and 230 ppm resonances, the nonprotonated \( d_1 \) tautomer was found to be at least three times more favorable than the protonated \( d_1 \) tautomer (Table 1). Amantadine does not appear to change the population of the two tautomers. In solution, free histidine favors the nonprotonated \( d_1 \) tautomer with a 4:1 ratio (60).

It is intriguing that the neutral histidines in the M2-TMD channel behave similarly as they do in the bulk solution. This ratio suggests that one out of four histidines is in the \( d_1 \)-protonated tautomeric state and hence is unlikely to be adjacent to another \( d_1 \)-protonated His\textsuperscript{37} residue.

Even without charged residues, the presence of both tautomeric forms breaks the fourfold symmetry of the structure. The observation of distinct nonprotonated (230 ppm) and protonated (147 ppm) sites clearly demonstrates that the His\textsuperscript{37} residues undergo side-chain tautomerization at a rate slower than 2.5 kHz based on the separation of these two resonances in Hz for a sample in a 7 Tesla magnet (30 Hz/ppm \( \times \) 83 ppm). At the same time, one might expect structural differences based on the tautomer heterogeneity that would be observed in the PISEMA spectra. We can expect a protonated \( ^{15}\text{N}^2 \)-imidazole to be located in each of the three positions in the tetrad with a nonprotonated residue in the other site. Each of these imidazoles has a different local environment based on the protonation of the imidazoles on either side and each configuration could result in a different orientation for the \( ^{15}\text{N}^2 \)-imidazole. The observation of a unique \( ^{15}\text{N}^1 \)-H dipolar coupling and \( ^{15}\text{N} \) chemical shift for both the \( ^{15}\text{N}^2 \) sites (Fig. 3) and the polypeptide backbone (Fig. 2 B) suggest that the structural difference causes \(<2 \text{ kHz} \) change in the dipolar splitting; otherwise, multiple splittings would be observed. Such a small change in dipolar splitting translates to very small changes in either the backbone amide planes or the imidazole ring orientations.

Low proton affinity of His\textsuperscript{37} with amantadine

Fig. 4 shows the pH titration for the \( d_1 \) site in the presence and absence of amantadine. Resonances at 230 and 147 ppm arise from the nonprotonated and protonated (neutral) \( ^{15}\text{N}^8 \) sites, respectively (54). In addition to the neutral tautomeric states, two additional resonances are observed. The resonances at 156 ppm represent the biprotonated charged state and the broad resonance at 167 ppm (only in the absence of amantadine) reflects the formation of the His-HisH\textsuperscript{+} dimer (52,61). In the absence of amantadine the observed resonance intensities (corrected for cross-polarization kinetics and spinning side-band intensities) have been interpreted to yield a set of \( pK_a \) values for this histidine tetrad: 8.2, 8.2, 6.3, and <5.0 (Fig. 5). As discussed previously (52) this titration results in a couple of unique conclusions. The first two histidines to titrate appear to titrate with cooperative proton binding and at very high pH compared to the normal \( pK_a \) of histidine. Secondly, the binding of these protons appear to form His-HisH\textsuperscript{+} dimers that distribute the charge through the formation of low-barrier hydrogen bonds.

![Figure 4](image_url)

**FIGURE 4** CPMAS NMR spectra of His\textsuperscript{37} (\( ^{15}\text{N}^8 \)) M\textsubscript{2}-TMD with and without amantadine at different pH values. Spectra were obtained on a Bruker DMX-300 NMR spectrometer at 277 K with a spinning rate of 3 kHz. Signals at \(<100 \text{ ppm} \) are from the natural abundance amides from the M\textsubscript{2}-TMD backbone. Spinning side bands are marked with asterisks.

<table>
<thead>
<tr>
<th>Peak position (ppm)</th>
<th>( T_{1p}^\text{am} ) (ms)*</th>
<th>( T_{\text{NH}} ) (ms)*</th>
<th>( M_0 )</th>
<th>Relative amount*</th>
</tr>
</thead>
<tbody>
<tr>
<td>230 (( \equiv \text{N} ))</td>
<td>4.2 ( \pm ) 0.9</td>
<td>5.2 ( \pm ) 1.6</td>
<td>6900 ( \pm ) 590</td>
<td>11400 ( \pm ) 420</td>
</tr>
<tr>
<td>147 (( &gt;\text{N-H} ))</td>
<td>4.8 ( \pm ) 1.6</td>
<td>5.1 ( \pm ) 0.9</td>
<td>65 ( \pm ) 30</td>
<td>170 ( \pm ) 30</td>
</tr>
</tbody>
</table>

*The errors are obtained from the curve fitting to Eqs. 1 and 2.

†The relative amount is calculated from the ratio of \( M_0 \) between different species.

‡The amantadine-free data were adopted from Hu (54).
In the presence of amantadine, there are two striking changes in the pH titration. First, the nonprotonated δ1 resonance, indicative of the uncharged monomeric state, is present even at pH 5, while in the absence of amantadine this resonance disappeared between pH 7.0 and 8.0. Consequently, the first pKₐ values for this histidine tetrad have shifted to much lower pH values in the presence of amantadine. Secondly, there is little or no evidence for a resonance at 167 ppm, suggesting that the His-HisH⁺ dimer does not form in the presence of amantadine. As shown in Fig. 5, the pKₐ values appear to be in the vicinity of 5.4. The protonation at this pH does not appear to be associated with the dissociation of amantadine from M2-TMD, based on the narrow linewidths observed even at very low pH in the presence of amantadine. Neither is it very reasonable to propose uniform pKₐ values for each of the four His37 residues, unless the tetrameric structure is not stable below this pH. The tetramerization studies for M2-TMD suggest that the tetramer is not very stable below pH 6 in micelles (1); however, it is anticipated to be much more stable in lipid bilayers, as suggested by the conductance studies of M2-TMD (32,52). In addition, it has been shown that binding amantadine to M2-TMD (1) stabilizes the tetrameric complex. Consequently, we do not anticipate that the tetramer is dissociating at this pH. However, it is likely that there is a structural change that is permitting multiple protonations of the His37 residues at this low pH. Unfortunately, it is difficult to obtain structural data at this pH due to conformational heterogeneity (54).

The lack of His-HisH⁺ dimer suggests that water is in an appropriate orientation to access the imidazole nitrogens and to compete for these hydrogen-bonding sites, thereby preventing the formation of low-barrier His-HisH⁺ hydrogen bonds. This appears to be in conflict with the Trp31 fluorescence emission results reported by Salom et al. (1), in which they suggest that, upon binding amantadine, the environment for Trp31 becomes more hydrophobic. However, other explanations for the failure to form His-HisH⁺ dimers are also possible; for instance, the cooperativity in proton binding in the absence of amantadine suggests that a structural change is induced by the formation of the first His-HisH⁺ dimer and that the resultant tense state is relieved when a second proton is bound, forming a second and symmetric His-HisH⁺ dimer. The observed titration in the presence of amantadine may then be consistent with the formation of individual biprotonated histidines in the M2-TMD.

The influence of amantadine on the chemical properties of this histidine tetrad observed here suggests the possibility of a novel mechanism for how amantadine may inhibit proton conductance. It has long been assumed that amantadine sterically blocks the pore formed by the M₂ protein four-helix bundle. However, Arkin and co-workers (4), in studying amantadine-resistant mutants by surface plasmon resonance, has observed that a subset of these mutants still bind amantadine and yet proton conductance is not blocked. While it is possible to make an argument on steric grounds to explain this result, it is also possible to speculate that the influence of amantadine on the chemical properties of the histidine tetrad could be modified in these mutants and hence form a distinct functional mechanism.

In conclusion, the structure of the transmembrane domain of M₂ at high pH and in the absence of amantadine is more dynamic and structurally heterogeneous compared with the structure when amantadine is bound. It is likely that the dynamics and structural plasticity are essential for the functional complexity of channel opening, gating, and proton conduction, both for the isolated transmembrane domain and for the full-length protein. There is growing evidence in the literature that there is a large number of membrane proteins for which the transmembrane domain and water-soluble domain, at least in part, function separately. For instance, the CorA Mg²⁺ transporter structure was recently solved by crystallizing the water-soluble domain and then using that structure to solve the full-length protein structure (62). Many properties of the M₂ transmembrane domain and the full-length protein appear to be, at least, qualitatively the same, such as tetramerization, proton conductance, and amantadine binding. Here, amantadine appears to interfere with the unique chemistry of the histidine tetrad in the transmembrane domain; we would anticipate the same in the full-length protein. Not only are the pKₐ values drastically altered, but the formation of low-barrier hydrogen bonds appears to be impossible in the presence of amantadine—suggesting that the mechanism by which amantadine prevents proton conductance may not be steric hindrance, but instead a mechanism that interferes with the histidine facilitation.
The authors are grateful to the staff of National High Magnetic Field Laboratory NMR facility (A. Blue) and the staff of Bioanalytical Synthesis and Service Laboratory (H. Hendricks and U. Goli) for their expertise, assistance, and maintenance of the instruments essential for this work.

This work was supported by the National Science Foundation (grant No. MCB02-35774) and the National Institutes of Health (grant No. AI23007). The experiments were largely performed at the National High Magnetic Field Laboratory, supported by Cooperative Agreement No. DMR-0084173 and the State of Florida.

REFERENCES


