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Drug-Induced Conformational and Dynamical Changes of the S31N Mutant of the Influenza M2 Proton Channel Investigated by Solid-State NMR

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Abstract

The M2 protein of influenza A viruses forms a tetrameric proton channel that is targeted by the amantadine class of antiviral drugs. A S31N mutation in the transmembrane (TM) domain of the protein has caused widespread amantadine resistance in most of the currently circulating flu viruses. Recently, a new family of compounds based on amantadine- and aryl-substituted isoxazole were discovered to potently inhibit the S31N channel activity and reduce replication of S31N-harboring viruses. We now use solid-state NMR spectroscopy to investigate the effects of one of these isoxazole compounds, WJ352, on the conformation of the S31N TM segment and the dynamics of the proton-selective residue, His37. Chemical shift perturbations show that WJ352 changes the conformational equilibrium of multiple TM residues, with the maximal perturbation occurring at the crucial Asn31. ^{13}C - ^2H distance measurements and ^1H - ^1H NOE cross peaks indicate that the adamantane moiety of the drug is bound in the spacious pore between N31 and G34 while the phenyl tail resides near V27. Thus, the polar amine points to the channel exterior rather than to His37, in contrast to amantadine and rimantadine in the wild-type channel, suggesting that the drug is significantly stabilized by hydrophobic interactions between the adamantane and the TM peptide. ^{15}N and ^{13}C chemical shifts indicate that at low pH, His37 undergoes fast exchange among the τ tautomer, the π tautomer and the cationic state due to proton transfer with water. The exchange rate is higher than the wild-type channel, consistent with the larger single-channel conductance of the mutant. Drug binding at acidic pH largely suppresses this exchange, reverting the histidines to a similar charge distribution as that of the high-pH closed state.

Introduction

The M2 protein of influenza A viruses spans the viral envelope and forms a tetrameric channel that conducts protons across the membrane when the external environment is acidic. This proton channel activity is important for virus uncoating^{1,2} and in some viruses, also for

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Supporting Information

Additional NMR spectra, tables, and experimental methods are provided. This information is available free of charge via the internet at <http://pubs.acs.org>.

maintaining the high pH of the trans-Golgi network to prevent premature conformational changes of hemagglutinin³. A single histidine residue, His37, in the transmembrane (TM) domain of the protein is responsible for pH activation and proton selectivity of the channel⁴. Detailed information about how His37 conducts protons has been obtained from solid-state NMR studies of phospholipid-bound M2 TM peptides (M2TM)⁵. At acidic pH, the imidazolium rings undergo small-amplitude reorientation⁶ and exchange protons with water molecules at a rate of 10^5 s^{-1} ^{7,8}. The energy barrier of the ring motion, found to be at least 60 kJ/mol, is consistent with the proton-conduction energy barrier of ~100 kJ/mol measured in liposome assays⁹. ¹⁵N NMR chemical shifts allowed determination of the four pK_a's of the His37 tetrad^{8,10}. In virus-mimetic lipid bilayers, the four His37 residues protonate with pK_a's of 7.6, 6.8, 4.9 and 4.2. When compared with pH-dependent proton currents, these pK_a values indicate that the +3 channels conduct the majority of the proton current at the physiological pH of the endosome⁸. A 1.65-Å X-ray crystal structure showed that the four histidines form an extensive hydrogen-bonding network with a cluster of water molecules¹¹, suggesting proton delocalization. One helical turn away from His37, Trp41 is responsible for inward rectification of the channel¹². Cation- π interaction between His37 and Trp41 was observed in resonance Raman spectra¹³ and suggested by molecular dynamics (MD) simulations¹⁴. Recent solid-state NMR distance and dynamics data showed that at low pH Trp41 moved closer to His37 and underwent microsecond sidechain reorientations¹⁵. The resulting periodic cation- π interactions with the cationic His37 may restrict proton release from His37, thus explaining the low proton flux compared to the water-His proton exchange rate.

The M2 proton channel is inhibited by the amantadine class of antiviral drugs, which blocks the N-terminal region of the channel pore near residue Ser31¹⁶⁻¹⁹. Bound at this site, amantadine (Fig. 1a) dehydrates the aqueous pore²⁰, which prevents protonation of the His37 sidechains^{8,21}, in turn stopping conformational changes of the helix backbone and imidazole rings that are important for proton conduction⁸. However, in the last decade, several amantadine-resistant TM mutations have become widespread in the M2 proteins of circulating flu viruses²². The most prevalent drug-resistant mutant, S31N, dominates in H1N1, H5N1, and H3N2 strains isolated from humans, birds and swine²³⁻²⁵. The replacement of the hydroxyl group by the bulkier carboxamide at residue 31 not only caused loss of amantadine binding^{26,27}, but also gave rise to a moderately better proton channel, with ~20% higher single-channel conductance than the wild-type (WT) protein²⁸.

To inhibit the S31N-M2 channel, Wang *et al.* recently designed a family of isoxazole compounds²⁹. By functionalizing the heterocyclic isoxazole with amantadine and aryl substituents, they identified molecules that selectively inhibit S31N-M2 with a potency greater than that of amantadine against the WT channel. Solution NMR structures of the S31N M2(19-49) peptide in DPC micelles²⁹ bound with one such compound, N-([5-(thiophen-2-yl)-1,2-oxazol-3-yl] methyl)adamantan-1-amine (WJ332), showed both similarities and major differences to the structure of amantadine-complexed WT peptide¹⁸. This drug is a derivative of amantadine, in which the basic amine group is linked to a bi-heteroaryl group via a methylene group (Fig. 1b), which retains the basicity of the amine. In the solution NMR structure of the S31N-WJ332 complex, the adamantane group is located

in approximately the same region of the channel as amantadine in the WT, but it is flipped by 180° so that the pendant amino group projects upward towards the outside of the channel rather than downward towards His37, in contrast to the direction of amantadine and rimantadine in WT M2¹⁹. MD simulations based on the structure indicate that the N31 backbone carbonyl group helps solvate the ammonium group of WJ332 via water-mediated interactions, and the isoxazole group of the drug additionally interacts with S31N.

Given the reversed direction of the isoxazole drug in micelle-bound S31N-M2, the predicted small energy difference between the amine-up and amine-down orientations, and the possibility of detergent micelles affecting drug binding, we wish to verify the binding mode of the isoxazole drug in phospholipid-bilayer-bound S31N-M2 using solid-state NMR spectroscopy. To facilitate isotopic labeling of the drug, we chose WJ352, a potent analogue of WJ332 in which the terminal thiophene is replaced by a phenyl group (Fig. 1c). Solid-state NMR also allows us to answer important questions about the charge state of the tetramer recognized by the drug. Amantadine binds with greater affinity to the neutral form of the channel than to the low-pH forms³⁰. The neutral form may be stabilized by amantadine in part because the charged ammonium destabilizes the cationic form of His37 and in part because the neutral form of the channel has a conformation that is more complementary to the shape of the drug¹⁸. However, the ammonium group of the reverse oriented isoxazole is more distant from His37, so it was not clear whether the isoxazole would bind exclusively to the neutral state of the channel, or whether it might also associate with partially charged channels. Finally, studies in phospholipid bilayers allow us to examine the conformational and proton exchange dynamics of S31N-M2, since the mutant appeared more dynamic than the WT peptide in detergent micelles^{26,29}.

Using magic-angle-spinning (MAS) solid-state NMR spectroscopy, we have now measured the effects of WJ352 on the chemical shifts of key TM residues in S31N and on the proton exchange dynamics of His37. Chemical shift perturbation and drug-peptide distance measurements show that the adamantyl group is located between N31 and G34 while the phenyl tail points to the N-terminus. ¹⁵N and ¹³C chemical shifts of His37 show that S31N-M2 undergoes faster proton exchange with water than the WT. This exchange causes rapid interconversion among three states of His, which is observed for the first time. Drug binding suppressed most of the His37 exchange dynamics, providing direct evidence of the mechanism of inhibition of this mutant proton channel.

Materials and Methods

Isotopically labeled peptides and drug

Two peptide constructs were used in this study, one spanning residues 19-49 and the other spanning residues 22-46 of the S31N mutant of the Udorn strain of M2. The amino acid sequence of residues 19-49 is SNDSSPLVVAANIIIGILHLILWILDRLFFK. Three isotopically labeled peptides were synthesized using an optimized protocol as described previously¹⁹. After cleavage from the resin, the crude peptide was precipitated in cold ether and purified via HPLC (Vydac, C4 column). The first peptide contained ¹³C, ¹⁵N-labeled residues at G34, I35, H37 and L38 (GIHL₂₂₋₄₆, S31N). The second sample

contained ^{13}C , ^{15}N -labeled V27, A30, N31, I33 and G34 (VANIG₁₉₋₄₉, S_{31N}). The third sample contained ^{13}C , ^{15}N -labeled V28, G34, I35, H37 and L38 (VGIHL₁₉₋₄₉, S_{31N}).

5-phenyl-31(N-adamantyl-methylamine)-1,2-isoxazole (WJ352) was synthesized using a modified procedure from that described previously²⁹ to incorporate deuterium labels. The synthesis protocol is provided in the Supporting Information. Two compounds were synthesized, one with deuterated benzene (d_5 -WJ352) and the other with perdeuterated adamantane (d_{15} -WJ352) (Fig. S1). The purified drugs were verified by ^1H solution NMR and electrospray ionization mass spectrometry to have the correct chemical structures.

Membrane sample preparation

The isotopically labeled peptides were reconstituted in two types of lipid membranes. The first membrane is 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and the second is a virus-mimetic lipid mixture (VM+) containing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), egg sphingomyelin (SM) and cholesterol (Chol) at molar ratios of 25.6% : 25.6% : 25.6% : 23%. DMPC lipid vesicles in desired buffer were prepared by eight to ten freeze-thaw cycles. The VM+ lipids were first mixed and dissolved in chloroform/methanol 1:1 (v : v). The bulk of the organic solvents were removed with nitrogen gas, then the lipids were dissolved in cyclohexane and lyophilized overnight. The resulting homogeneous dry powder was suspended in buffer, vortexed, and freeze-thawed eight times to create uniform vesicles. The M2 peptide was solubilized in the desired buffer using octyl- β -D-glucoside (OG) and mixed with the lipid vesicle solution. The peptide-lipid mixture was vortexed for 2 hours, then dialyzed at room temperature against 1 L of buffer for three days with 6 buffer changes. The dialyzed proteoliposomes were spun down at 55,000 rpm at 5°C for 4 hours to yield homogenous membrane pellets, which were then spun into 4 mm MAS rotors for solid-state NMR measurements. The peptide to lipid molar ratio was 1 : 15 for all the samples and the hydration level was 40-50% (by mass).

In total, nine membrane samples were used in this study. Three GIHL₂₂₋₄₆, S_{31N} samples were reconstituted into the DMPC membrane at pH 7.5 using a Tris buffer (10 mM Tris base, 1mM EDTA, 0.1mM NaN₃). The first sample contained no drug (apo), the second sample contained d_5 -WJ352 at a drug : tetramer molar ratio of 1 : 1, and the third sample contained d_5 -WJ352 at a drug : tetramer ratio of 8 : 1. Three VANIG₁₉₋₄₉, S_{31N} samples were reconstituted into DMPC bilayers at pH 6.5 using a phosphate buffer (10 mM NaH₂PO₄/Na₂HPO₄, 1 mM EDTA, 0.1 mM NaN₃). The first sample contained no drug, the second sample contained d_{15} -WJ352 at a drug : tetramer ratio of 1 : 1, and the third sample contained d_{15} -WJ352 at a drug : tetramer ratio of 10 : 1. Three VGIHL₁₉₋₄₉, S_{31N} samples were prepared in the VM+ membrane to investigate His37 structure and dynamics. Two of these samples were prepared at pH 5.4 using a citrate buffer (10 mM citric acid/sodium citrate, 1 mM EDTA, 0.1 mM NaN₃), one without drug and the other with protonated WJ352 at a drug : tetramer ratio of 10 : 1. The third sample was prepared at pH 7.5 using a phosphate buffer without drug.

For drug-bound samples, the desired aliquot of a stock ethanol solution containing the drug was dried down and redissolved in 20-30 μL of ethanol. This concentrated solution was

titrated into the membrane pellets from ultracentrifugation and mixed thoroughly to ensure homogenous distribution of the drug. The membranes were then dried to remove excess ethanol and rehydrated before being spun into the MAS rotors for solid-state NMR measurements.

Solid-state NMR experiments

All MAS NMR experiments were carried out on Bruker DSX-400 MHz (9.4 T) and AVANCE 600 MHz (14.1 T) spectrometers (Karlsruhe, Germany) using 4 mm triple resonance MAS probes tuned to $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ and $^1\text{H}/^{13}\text{C}/^2\text{H}$ frequencies. Typical radiofrequency (rf) field strengths were 50-63 kHz for ^{13}C , 42 kHz for ^{15}N , 50 kHz for ^2H and 62-83 kHz for ^1H . ^{13}C chemical shifts were referenced to the methylene signal of adamantane at 38.48 ppm on the tetramethylsilane scale and ^{15}N chemical shifts were referenced to the ^{15}N signal of N-acetylvaline at 122.0 ppm on the liquid ammonia scale. Sample temperature was controlled using a FTS Systems XR401 Air-Jet cooler, with an additional liquid nitrogen heat exchanger placed in series for temperatures below 240 K.

One-dimensional (1D) ^{13}C and ^{15}N MAS spectra were recorded using a cross-polarization (CP) sequence under 7 kHz MAS. Spectra were measured at 308 K to detect conformational dynamics and proton exchange, and between 190 and 243 K to determine the rigid-limit structure of the peptide and the protonation state and tautomeric structure of His37. 1D ^{13}C - ^{13}C double-quantum (DQ) filtered spectra were measured at 200 K under 7 kHz MAS using the SPC5 sequence³¹ to suppress the natural abundance ^{13}C signals of lipids. ^{15}N - ^{13}C dipolar filtered 1D ^{13}C spectra were measured at 7 kHz using a REDOR-based pulse sequence³². Two dimensional (2D) ^{13}C - ^{13}C ^1H -driven spin diffusion (PDS) experiments were conducted under 7 kHz MAS using a 30 ms mixing time between 230 and 308 K. 2D ^{15}N - ^{13}C correlation spectra were measured at 230 K and 308 K under 10 kHz MAS for the VGIHL₁₉₋₄₉, S_{31N} samples, and at 243 K under 7 kHz MAS for the VANIG₁₉₋₄₉, S_{31N} samples. A ^{13}C - ^{15}N REDOR period of 0.8 ms was used to obtain one-bond ^{15}N - ^{13}C cross peaks. Under 10 kHz MAS, the $\text{C}\gamma$ resonance of His37 was suppressed by rotational resonance with the directly bonded $\text{C}\beta$ ³³, thus allowing the distinction between the N δ 1 and N ϵ 2 signals in the 2D spectra.

^{13}C - ^2H REDOR experiments for determining peptide-drug spatial contact were conducted with and without ^{13}C - ^{13}C DQ filtering^{18,34,35}. The former was used to remove the lipid natural abundance signals in case of severe overlap with the peptide ^{13}C signals. Both types of REDOR experiments include a selective ^{13}C Gaussian pulse to suppress ^{13}C - ^{13}C J coupling³⁶. The DQ filtered REDOR experiments were carried out on the VANIG₁₉₋₄₉, S_{31N} sample under 5 kHz MAS, while the regular selective REDOR experiments were carried out on the GIHL₁₉₋₄₉, S_{31N} sample under 4.5 kHz MAS. In all REDOR experiments, a control (S_0) experiment without ^2H pulses and a dephasing (S) experiment with ^2H pulses on were conducted back to back. ^{13}C sites close to the ^2H spins have lower intensities in the S spectrum than the S_0 spectrum. The ratio S/S_0 thus reflects ^2H - ^{13}C distances: the shorter the distances, the lower the S/S_0 value. All REDOR spectra were measured at 243 K with 20,000-80,000 scans of signal averaging.

Solution NMR experiments

Peptide and drug were added to deuterated dodecylphosphocholine (DPC) (Cambridge Isotope Laboratory) powder from their ethanol stocks at the desired molar ratios. Ethanol was removed by nitrogen purging, followed by overnight lyophilization. A phosphate buffer (10% D₂O, 50 mM sodium phosphate, pH 6.8) was added to the dried mixture to make a 2 mM (monomer) S31N M2(19-49) sample with 20 mM WJ332 or WJ352 in 100 mM deuterated DPC.

Two 2D ¹³C-(¹H)-¹H NOESY spectra were measured on selectively labeled VANIG₁₉₋₄₉, S31N using a Bruker 900 MHz Avance II spectrometer equipped with a cryogenic probe. The experiments were conducted at 313 K with a mixing time of 150 ms, *t*_{1,max} of 7 ms and *t*_{2,max} of 71 ms, and 128 scans per *t*₁ slice. The ¹H carrier frequency was set to the water signal. The ¹H chemical shift was referenced to the residual water peak at 4.63 ppm relative to DSS while the ¹³C chemical shift was referenced indirectly via the gyromagnetic ratio. The spectra were processed and analyzed using the NMRPipe program³⁷. The first three time points of the indirect ¹³C dimension were extended by linear prediction. Time domain data were multiplied by sine square bell window functions shifted by 90° and zero-filled once before Fourier transformation.

Calculation of chemical shift perturbation

To examine drug-induced conformational change, we calculated the average chemical shift perturbation (CSP) of each residue between the apo and drug-bound states using the following empirical relation³⁸:

$$\Delta\delta_{av} = \left\{ \frac{1}{n} \left[\sum_{i=1}^{n-1} (\Delta\delta_{C,i})^2 + \left(\frac{\Delta\delta_N}{2.5} \right)^2 \right] \right\}^{1/2},$$

where *n* is the total number of chemical shifts measured in each residue and δ is the individual-site's chemical shift difference between the apo and drug-bound states. We used chemical shifts obtained from the DMPC-bound membrane samples, with the exception of V28, which was only labeled in the VGIHL₁₉₋₄₉, S31N sample bound to the VM+ membrane. For residues N31, I33, and G34, drug binding did not create new chemical shifts but changed the equilibrium between two sets of chemical shifts. We used the difference between these two sets of chemical shifts to represent drug perturbation of the conformation of these residues. For His37, a single set of backbone chemical shifts was observed for the apo peptide, and drug binding created a second set, so the average difference between these two sets of chemical shift was calculated. Only the N α , CO, C α and C β chemical shifts were used in the His37 calculation, because imidazole chemical shifts are sensitive to many chemical structure factors not directly related to conformational changes.

Results

Drug-induced chemical shift changes of S31N-M2

We first investigated the effects of the isoxazole drug on S31N-M2 structure by analyzing the chemical shifts of the TM residues. Figures 2 and 3 show representative 2D ^{13}C - ^{13}C and ^{15}N - ^{13}C correlation spectra of DMPC-bound S31N-M2 at pH 6.5. Among the five labeled N-terminal residues (V27, A30, N31, I33 and G34), the isoxazole drug either selected one of the conformations or changed the equilibrium between multiple conformations. For example, N31 exhibits two sets of $\text{C}\alpha$ and $\text{C}\beta$ chemical shifts whereas G34 exhibits two CO chemical shifts. Drug binding largely removed the signals of the more α -helical chemical shifts of N31, retaining the smaller $\text{C}\alpha$ and CO chemical shifts (Table S1). Similarly, the less α -helical (smaller) CO chemical shift of G34 is a minor component in the apo peptide but becomes equally populated as the more helical (larger) CO chemical shift in the drug-bound state (Fig. 2e). The less helical G34 chemical shift also exhibits cross peaks with I33 at long ^{13}C spin diffusion mixing times (Fig. 2c, d). For A30, conformational heterogeneity was less pronounced in the apo state, but drug binding also changed the $\text{C}\alpha$, $\text{C}\beta$ and CO chemical shifts to less helical values (Fig. 3).

Fig. 4 shows the aliphatic region of the 2D ^{13}C PDS spectra of VGIHL₁₉₋₄₉, S31N in the VM+ membrane. Three samples with different pH and drug-binding states are compared. The chemical shifts show complex dependences on pH, drug binding, and temperature. At low temperature, the low-pH apo peptide exhibited a single set of $\text{C}\alpha$ and $\text{C}\beta$ chemical shifts for I35, H37 and L38, but upon drug binding and pH increase, these residues showed two sets of $\text{C}\alpha$ and $\text{C}\beta$ chemical shifts, indicating two distinct backbone conformations. Drug binding at low pH gave rise to the same two chemical shifts as those of the high-pH apo peptide, indicating that drug binding creates the same conformational distribution as the closed state of the channel. The presence of two backbone conformations of H37 in the drug-bound state is qualitatively reproduced in the DMPC membrane (not shown). Thus, the conformation of the functionally essential His37 is largely dictated by pH and drug binding and is not sensitive to the membrane environment.

While I35 and L38 exhibit a single set of sidechain chemical shifts in the low-pH apo peptide at low temperature, at high temperature, both sidechains show two sets of chemical shifts (Fig. S2), which are similar to those of the high-pH and drug-bound samples. Taken together, these observations indicate that the energetic balance between the two sidechain conformations of these interfacial residues changes between the open state and the closed or inhibited state of the channel. The minor conformation is less stable in the open state and thus not populated at low temperature, but it is stabilized in the closed and inhibited states.

To compare the magnitudes of the chemical shift changes of the various residues by the drug, we calculated the CSP. Fig. 5 shows that N31 has the largest chemical shift perturbation, followed by H37 and G34. This trend is very similar to the effect of amantadine on the WT peptide, where S31 was the site of maximum chemical shift perturbation, followed by G34 and V28³⁹. H37 was not included in the WT chemical shift perturbation study.

Interestingly, while N31 exhibited the largest chemical shift perturbation, the Asn sidechain C γ chemical shift is relatively unaffected by the drug. This is seen in the 2D spectra in Fig. 2c, d and Fig. 3, and further verified in ^{15}N - ^{13}C filtered 1D ^{13}C spectra measured between 190 and 270 K (Fig. S3). Below 240 K, the C γ intensities are consistently 7-10% of the full spectral intensity, indicating full detection of this signal and ruling out the possibility of an invisible dynamic population. The absence of C γ chemical shift change indicates that the average N31 rotameric conformation is little affected by the drug. However, at 270 K, the C γ intensity in the drug-bound sample appears lower than in the apo sample (Fig. S3), suggesting the onset of rotameric dynamics, whose detail will require further experiments to elucidate.

WJ352 binding site in the S31N-M2 channel

The ability of WJ352 and its derivatives to inhibit the S31N-M2 channel²⁹ is somewhat surprising given the larger size of the isoxazoles compared to amantadine and rimantadine and the more constricted channel due to the replacement of Ser by Asn. To investigate how exactly the bulky isoxazole fits into the S31N channel, we selectively deuterated WJ352 either at the adamantane head (d_{15}) or at the phenyl tail (d_5). Using the versatile REDOR experiment, we measured the location and orientation of deuterated WJ352 bound to ^{13}C -labeled S31N-M2.

For d_5 -WJ352 complexed GIHL₂₂₋₄₆, S31N, we did not observe ^{13}C - ^2H REDOR intensity differences between the control (S_0) and dephased (S) spectra at drug : tetramer ratios of either 1 : 1 or 8 : 1. In particular, the well resolved G34 C α signal had no intensity change (Fig. 6a), indicating that the phenyl tail is far from this segment. Under excess drug, the lipid headgroup C γ showed moderate intensity reduction in the S spectrum, indicating that excess WJ352 partitions to the membrane-water interface, analogous to amantadine and rimantadine^{40,41}. In comparison, d_{15} -WJ352 complexed VANIG₁₉₋₄₉, S31N showed clearly reduced S intensities compared to the S_0 intensities for most labeled sites both at the stoichiometric drug concentration (Fig. 6b) and under excess drug (data not shown). At a REDOR mixing time of 9.6 ms, the I33/V27 C α peak exhibits an S/S_0 value of 0.81 ± 0.07 , the A30/N31 C α peak has an S/S_0 value of 0.88 ± 0.03 , while G34 C α has $S/S_0 = 0.86 \pm 0.05$. These REDOR spectra were measured under ^{13}C DQ filtering to suppress the lipid ^{13}C signals, thus the low sensitivity prohibits the use of longer mixing times to quantify the distances. Although the V27 and I33 C α peaks overlap, I33 likely contributes to the majority of the measured REDOR dephasing at 64 ppm because of the proximity of I33 to G34, whose C α signal also shows significant REDOR dephasing. Taken together, these spectra indicate that WJ352 binds to the channel lumen with the adamantane head spanning the segment from A30 to G34, while the phenyl tail is directed toward the N-terminus. Solution-state ^{13}C - ^1H NOESY spectra of VANIG₁₉₋₄₉, S31N in DPC micelles further confirmed this result: the adamantane H6, H7 and H8 protons show clear cross peaks with I33 sidechains while the phenyl H1-H4 protons exhibit cross peaks with V27 sidechains (Fig. 6c, d). Thus, the solution and solid-state NMR spectra indicate that in both lipid bilayers and DPC micelles, the isoxazole drug binds to the S31N channel pore with the amine pointing to the exterior (Fig. 6e).

Effects of isoxazole drug on His37 structure and dynamics

To investigate how S31N conducts protons and how drug binding interferes with this conduction, we measured the His37 ^{15}N and ^{13}C chemical shifts, since these are highly sensitive to the protonation state and tautomeric structure of the His sidechain⁴²⁻⁴⁵. Fig. 7 and Fig. 9 show 1D ^{15}N and ^{13}C spectra of VGIHL₁₉₋₄₉, S31N at high temperature (308 K) where dynamic processes are active and at low temperature where rigid-limit chemical shifts allow quantification of the various species of histidine present under a certain condition. The ^{15}N and ^{13}C chemical shifts were assigned based on the 2D spectra in Fig. 8 and Fig. S4 and by comparison with the WT data^{6,8}.

For the high-pH apo peptide, the ^{15}N spectra (Fig. 7c) show the N ϵ 2H peak of the τ tautomer (159 ppm) and the N δ 1H peak of the π tautomer (170 ppm), similar to WT M2TM⁶. The unprotonated ^{15}N peak at 251 ppm has high intensity, indicating that neutral histidines dominate at this pH. Since the multiple protonated nitrogens are not well resolved between neutral and cationic His, the presence of cationic His is better ascertained from the ^{13}C spectra (Fig. 9c). The C δ 2 chemical shift is an excellent indicator of the τ tautomer, π tautomer, and cationic His⁴⁶. At pH 7.5 all three species were detected: the τ tautomer (C δ 2 at 114 ppm) dominates, followed by the π tautomer (C δ 2 at 124 ppm), while a weak cationic C δ 2 peak at 117 ppm is also detected in the DQ filtered and ^{15}N - ^{13}C filtered ^{13}C spectra. Thus, cationic His indeed exists at a low percentage at this neutral pH, similar to the WT channel⁸.

In the low-pH apo peptide, the low-temperature ^{15}N spectrum shows a strong and broad peak at \sim 170 ppm (Fig. 7a). The large width of this peak distinguishes it from the π tautomer N δ 1H peak at high pH and identifies it as cationic N δ 1H, as verified by 2D correlation spectra (Fig. 8a, Fig. S4a). The unprotonated ^{15}N signal (251 ppm) is weak, further indicating that the percentage of neutral His is much lower than at pH 7.5. Increasing the temperature to 308 K dramatically changed the ^{15}N spectra: two dominant signals appear at intermediate ^{15}N chemical shifts of 213 and 185 ppm, indicating fast interconversion of various His species. Both peaks were also observed in the WT ^{15}N spectra, but with much large linewidths⁸. In addition to these two peaks, a significant intensity band between 160 and 180 ppm is also detected and constitutes about 30% of the total aromatic intensity.

Drug binding to the low-pH channel largely reverted the ^{15}N spectra to that of the high-pH apo state (Fig. 7b): at low temperature, the broad cationic ^{15}N signals between 170 and 180 ppm sharpened to become the signature of the π tautomer N δ 1H peak, while the 251-ppm peak greatly increased its intensity. The spectrum resembles the high-pH spectrum at low temperature. Increasing the temperature gave rise to residual exchange intensities at 213 ppm and 185 ppm, but the spectrum remained dominated (\sim 70%) by the sharp signals at the frequencies of neutral His. Thus, isoxazole binding at low pH converted most of the cationic His's to the neutral, non-conducting conformation.

To elucidate the origin of the 213-ppm and 185-ppm peaks, we measured 2D ^{15}N - ^{13}C correlation spectra (Fig. 8), supplemented with 2D ^{13}C - ^{13}C correlation spectra (Fig. S4). The 185-ppm ^{15}N peak is correlated to two ^{13}C signals (134 ppm and 117 ppm) while the 213-ppm peak is correlated only to a 134-ppm peak (Fig. 8a). These 2D ^{15}N - ^{13}C correlation

spectra were measured under 10 kHz MAS, near the rotational-resonance condition³³ between C β and C γ of His37, which broadens the C γ signal. As a result, N δ 1 has only one correlation peak (to C ϵ 1) while N ϵ 2 has two correlation signals (to C ϵ 1 and C δ 2). Thus, the 213-ppm signal must be assigned to N δ 1 while the 185-ppm peak must be assigned to N ϵ 2. Similarly, at high pH (Fig. 8c), the 1701ppm ¹⁵N peak can be assigned to N δ 1 while the 1601ppm peak is confirmed to result from N ϵ 2. The 2D ¹⁵N-¹³C correlation spectrum of the drug-bound sample (Fig. 8b) confirms the 1D observation that the sample is a mixture of a small fraction of unbound channels with exchange peaks and bound channels in which His adopts the high-pH apo structure.

The low-temperature 1D ¹³C spectra (Fig. 9) allow us to quantify the concentrations of the three His species in each sample based on the distinct C δ 2 chemical shifts⁴⁶. At pH 7.5, the concentration ratios are 65% : 25% : 10% (\pm 5%) for τ : π : cationic His, indicating the predominance of neutral His. For the apo pH 5.4 sample, the ratios are 36% : 12% : 52% (\pm 5%), indicating that half of the His are cationic. The drug-bound sample showed concentration ratios of 60% : 28% : 12% (\pm 10%) for τ : π : cationic His, quantitatively indicating that isoxazole converted most His to the neutral form at this low pH.

Discussion

Isoxazole binding site and the effects of drug on S31N-M2 conformation

The observed ¹³C and ¹⁵N chemical shifts indicate that the isoxazole drug perturbed the conformation of S31N in a similar fashion as amantadine to wild-type M2TM. Most importantly, the drug significantly changed the C α and C β chemical shifts of residue 31, thus achieving the design purpose of targeting this amantadine-resistant mutation site. Both amantadine and isoxazole perturbed the chemical shifts of G34, whose conformation is known to be sensitive to the environment^{39,47,48}. However, a major difference exists in the way that the two drugs are oriented in the pore. While amantadine and rimantadine are oriented with the polar amine pointing down to H37¹⁹, the biaryl isoxazoles are flipped by 180° and point the polar amine up to the exterior, as evidenced by ¹³C-²H REDOR data and NOE cross peaks (Fig. 6). This opposite orientation suggests that both amantadine and isoxazole drugs are significantly stabilized by hydrophobic interactions of the adamantane with the peptide, mainly at the segment between N31 and G34 (Fig. 6e)¹⁸. In addition to the hydrophobic interaction, MD simulations show the presence of multiple hydrogen bonds between the amine and the Asn sidechains and between the isoxazole nitrogen and the Asn sidechains, which should further stabilize the amine-up orientation²⁹.

Isoxazole binding shifted the conformational equilibrium of various residues, selecting one of two conformations at N31, I33 and G34 while creating two conformations at H37. The nature of the two conformations in apo S31N-M2 is not yet fully understood. A longer construct of the protein, S31N M2(18-60), was also reported to show peak doubling, which was found for most residues²⁷. The current data on the TM construct suggest that one possible cause of the two conformations is tautomerism of His37, since the two His37 C β chemical shifts are individually correlated to the τ and π resonances (Fig. S4). This hypothesis is supported by the observation that the two C α -C β peaks of Leu38 have similar intensity ratios as the His37 C α -C β peak intensity ratios in the high-pH sample (Fig. 4b, c).

Interestingly, while the drug selected one of the two backbone conformations of N31, the carboxamide C γ chemical shift is largely unaffected by the drug at low temperature (Fig. S3), indicating that the average rotameric conformation of N31 is unperturbed by the drug. On the other hand, the N31 sidechain chemical shifts of DMPC-bound S31N M2(19-49) measured here differ significantly from the N31 chemical shifts of S31N M2(18-60) bound to the diphytanoylphosphocholine membrane²⁷: the C γ and N δ chemical shifts are about 173 ppm (DSS scale) and 106 ppm in the current study (Table 1) but 175-178 ppm and 113-115 ppm, respectively, in the previous study. These differences indicate that the rotameric structures of N31 may be sensitive to the lipid membrane and/or the peptide construct length.

An increasing number of membrane proteins have been found to adopt multiple conformations under different conditions and undergo slow exchange among these conformations⁴⁹. For example, the structures of phospholamban⁴⁰, the G-protein coupled receptor β_2 -adrenoreceptor³⁵, wild-type influenza M2 transmembrane peptide^{47,50}, and viral fusion peptides⁵¹⁻⁵³ are all significantly influenced by the membrane composition, and other factors such as temperature⁵⁴ and pH⁵⁵ can also modulate membrane protein structure. Such conformational plasticity is usually associated with function, such as ion channel opening, closing and inactivation^{41,56,57}, enzyme catalysis⁵⁸ and protein-protein interactions⁴⁰. The fact that drug binding to S31N-M2TM did not simply cause a conformational change but selected a subset of conformations that already pre-existed in the apo peptide for many residues⁵⁹ suggests that the energy landscape of the M2 transmembrane domain, even in the lipid bilayer instead of detergent micelles⁶⁰, is rough, and this roughness may be relevant for the multiple functions of M2, including proton conduction², membrane scission⁶¹, virus assembly⁶², in addition to drug binding⁵.

Proton-exchange dynamics of His37 in S31N-M2

The conformation and dynamics of His37 in S31N reveal information about the proton conduction mechanism of the M2 channels. Similar to the WT channel, S31N shows His37 exchange dynamics at low pH, as manifested by the 213-ppm and 185-ppm ¹⁵N chemical shifts and the associated ¹³C exchange peaks⁸. Also similar to the WT channel, these exchange peaks are highly sensitive to temperature (Fig. 7a), indicating significant energy barriers, which support the assignment of these peaks to His-water proton transfer^{5,7} rather than strong His-His hydrogen bonding¹⁴, which would be essentially barrier-less⁶³. The rate of this His-water proton transfer is discernibly faster for S31N than for WT M2, as evidenced by the narrower linewidths of the ¹⁵N exchange peaks. This difference is qualitatively consistent with the higher proton conductance of S31N than the WT channel²⁸. On the other hand, the conductance difference is only about 20%, while the His-water proton transfer rate difference appears much larger. We recently calculated an exchange rate of $\sim 10^5$ s⁻¹ for WT M2⁸ based on its exchange linewidths. This rate is already more than two orders of magnitude larger than the proton flux per second⁹. We attribute the smaller proton flux partly to the interaction of His37 with the gating residue, Trp41¹². Recent solid-state NMR data of WT M2TM indicate that at low pH, Trp41 moves closer to His37 by ~ 2 Å and undergoes larger-amplitude sidechain reorientation¹⁵. The resulting periodic cation – π interactions with His37 should reduce the number of protons released from His37 to

water. Similar Trp41-His37 interactions should be present in S31N as well, which would reduce the number of protons transported into the virion.

Further insight into the mechanism of His-mediated proton transfer is obtained by determining the nature and populations of the molecular species that give rise to the ^{15}N exchange peaks. The similar linewidths of the two ^{15}N exchange peaks (Fig. 7a, 8a) suggest that they originate from the same exchange process. Since protonated nitrogens have ^{15}N chemical shifts below ~ 185 ppm, the 213-ppm ^{15}N exchange peak is only possible when unprotonated nitrogens constitute roughly 50% of the interconverting His species⁸. Since both τ and π tautomers contain unprotonated nitrogens, all three His species can in principle participate in exchange. Using the rigid-limit ^{15}N chemical shifts (Table 1), we solve for three simultaneous linear equations,

$$\begin{array}{r} 213 \text{ ppm} = 251 \text{ ppm} \cdot f_{\tau} + 170 \text{ ppm} \cdot f_{\pi} + 170 \text{ ppm} \cdot f_{+} \\ 185 \text{ ppm} = 159 \text{ ppm} \cdot f_{\tau} + 251 \text{ ppm} \cdot f_{\pi} + 175 \text{ ppm} \cdot f_{+} \\ 1 = f_{\tau} + f_{\pi} + f_{+} \end{array}$$

which gives molar fractions of $f_{\tau} = 53\%$, $f_{\pi} = 24\%$ and $f_{+} = 23\%$ for τ , π and cationic His.

This three-site exchange model is confirmed by the high-temperature ^{13}C spectrum of the apo peptide (Fig. 9a), where the main C $\delta 2$ signal at 118 ppm can be distinguished from the cationic C $\delta 2$ peak (117 ppm) as well as the neutral C $\delta 2$ peaks (124 ppm for π and 114 ppm for τ) at low temperature. This 118-ppm peak is consistent with the weighted average of the three rigid-limit C $\delta 2$ chemical shifts, but inconsistent with any two-site exchange models such as τ - cationic or τ - π exchange. For example, a τ - cationic exchange model that agrees with the 213-ppm ^{15}N peak would require a 1 : 1 ratio, which would lead to an average C $\delta 2$ chemical shift of less than 117 ppm, in contradiction with the measured C $\delta 2$ chemical shift. The three-site exchange with the above ratios predicts an average C γ chemical shift of 133 ppm, which is also detected in the high-temperature spectrum. C $\epsilon 1$ is insensitive to exchange because all three states of His have the same rigid-limit C $\epsilon 1$ chemical shift of 134 ppm. Taken together, the high-temperature ^{13}C spectra and the clear identification of the 185-ppm ^{15}N peak as an exchange peak revise our previous understanding of the interconverting His species during His-water proton transfer, by also including the π tautomer in the exchange.

The τ : π : cationic ratio of 53% : 24% : 23% obtained from the exchange-averaged ^{15}N chemical shifts is noteworthy, because the cationic fraction is much lower than expected, and lower than the low-temperature ^{13}C spectra derived cationic His concentration of $\sim 52\%$ (Fig. 9a). This apparent discrepancy is resolved by the fact that the observed exchange peaks mainly results from tetramers with low charge states of +1 and +2, where only one or two His residues are cationic. Higher-charged +3 and +4 tetramers, which are also populated at pH 5.4 based on the measured pK_a 's of the WT channel⁸, do not contribute to these specific ^{15}N exchange peaks because they will give smaller average ^{15}N chemical shifts in the 160-190 ppm range, which cannot be easily distinguished from non-exchange protonated nitrogen signals. Indeed, the 160-180 ppm band in the low-pH high-temperature ^{15}N spectrum (Fig. 7a) constitute $\sim 30\%$ of the total aromatic intensity, and is consistent with the

presence of exchange signals from higher charged tetramers. In other words, exchange within low-charged tetramers is made more detectable by a high population of unprotonated nitrogens, which shifts the average ^{15}N frequencies downfield, away from the protonated ^{15}N signals. If we combine the 30% cationic His intensity from the 160-180 band with the cationic His fraction from the exchange peaks, which is ~23% of the remaining 70% aromatic intensity ($\sim 70\% \times 23\% = 16\%$), then we indeed obtain a higher cationic fraction of close to 50%, in good agreement with the low-temperature ^{13}C intensities.

In addition to charge-conserved interconversion of the four histidines within a tetramer, the charge state of a tetramer can also change due to net His-water proton transfer, which may be considered as inter-tetramer exchange. More definitive information about the second process will require further experiments. What is clear from the current data is that high pH suppresses both intra-tetramer fast exchange and whatever inter-tetramer exchange may be present, even though a small fraction (10%) of cationic His remains.

The fast $\tau - \pi$ - cationic His interconversion at low pH refines our model of the His37-mediated proton transfer in M2 channels (Fig. 10)⁶. In this model, in addition to proton acquisition by a neutral His to convert to the cationic state and proton release by the cation to a neutral His, τ and π tautomers can exchange rapidly by coupled release of a proton on one nitrogen (e.g. N δ 1) and acquisition of a proton on the other nitrogen (e.g. N ϵ 2). Such prototropic tautomerism of heterocycles has been studied extensively^{64,65}, and is known to involve much lower energy barriers when it is mediated by solvent molecules instead of proceeding purely by an intramolecular mechanism. Thus, His37 interaction with water molecules on both sides of the ring is most likely required for this tautomerism to occur. Indeed, exchange-averaged ^1H chemical shifts associated with the His37 nitrogens were recently measured for WT M2 and were shown to be close to the water ^1H chemical shift at high temperature⁷, indicating that the proton-transfer partner of all three His species is water. Ring motion remains important in the model, to orient the imidazolium with water molecules to achieve proton transfer.

Although the model in Fig. 10 depicts only the dynamic transformations of a single His residue, in reality the process must reflect the ensemble average of all His's in each tetramer, since protons relayed from one His can be transferred via water to a neighboring His. This model is applicable to both S31N and WT channels, based on the fundamental similarity of the observed exchange frequencies.

Effects of the isoxazole drug on His37-water proton exchange

Binding of the isoxazole drug, although only as deep as G34, largely suppressed the His-water proton exchange, and changed the His37 chemical shifts to a form similar to the high-pH closed state. These are fully consistent with the observed C α and C β chemical shifts, which also indicate a reversion of the peptide conformation to that of the closed state. Thus, isoxazole binding dehydrates the S31N channel, and interrupts the water wire that leads to His37⁵, which inhibits the proton shuttling dynamics at His37.

Although we did not directly measure the pK $_a$'s of the His37 tetrad in S31N-M2, the similar pH-dependence of the ^{15}N spectra between the S31N and WT peptides⁸ suggests similar

thermodynamic parameters for protonation and deprotonation. This implies that the pH 5.4 apo S31N sample in the VM+ membrane should contain significant amounts of +2 and +3 tetramers. We showed recently that the +3 channel has the highest specific conductance for the WT peptide, and in this state the imidazole undergoes ring reorientation and proton exchange^{6,8}. Any effective drug must therefore inhibit the +3 charged channels. In general, the drug affinity to the channel is pH dependent: for example, titration of WJ352 to DPC-bound S31N showed an optimal pH of 6.8 for drug binding²⁹, which is close to the third pK_a of S31N-M2 in DPC micelles. But the effective pK_a 's are sensitive to the membrane environment: a more dynamic membrane causes higher pK_a 's.^{8,10} In the chain-saturated virus-mimetic membrane the WT M2 has a third pK_a of 4.9. The more dynamic, unsaturated, VM+ membrane⁶⁶ used here is expected to increase the His pK_a 's moderately, so the third pK_a should shift to between pH 5 and 6. Thus, S31N in the VM+ membranes at pH 5.4 may have a very similar charge-state profile as S31N in DPC micelles at pH 6.8: both should be dominated by +2 and +3 channels, and both are inhibited by the isoxazole drugs based on the NMR spectra. Therefore, both micelle and bilayer data suggest that the isoxazole drugs significantly inhibit activated channel at acidic pH. This may result from the reduced sensitivity of the amine-up drug to the charge state of His37 and from the bulky size of the drug, whose larger contact area with the peptide makes it less sensitive to small shape differences between the low-pH and high-pH channel pores.

Conclusion

In conclusion, we found that the isoxazole drug WJ352 perturbed the conformational equilibrium of S31N-M2, shifting the TM segment to a less ideal helical conformation. N31 exhibits the largest chemical shift perturbation, similar to S31 of the WT protein in complex with amantadine. In the low-pH S31N channel, the four His37 residues undergo proton transfer with water more rapidly than the WT channel, causing fast interconversion among τ , π , and cationic His. This exchange is more easily detected for the low-charge tetramers because of the high populations of neutral His, whose ¹⁵N signals cause the exchange peaks to be well resolved from unexchanged ¹⁵N signals. Drug binding at low pH suppressed most of the exchange process, reverting the His37 residues to a similar charge distribution as that of the high-pH closed state. The conformation of other TM residues also reverts to the closed state structure. Therefore, the isoxazole drug, while bound more than one helical turn away from His37, potently arrests the atomic processes that shuttle protons in this mutant channel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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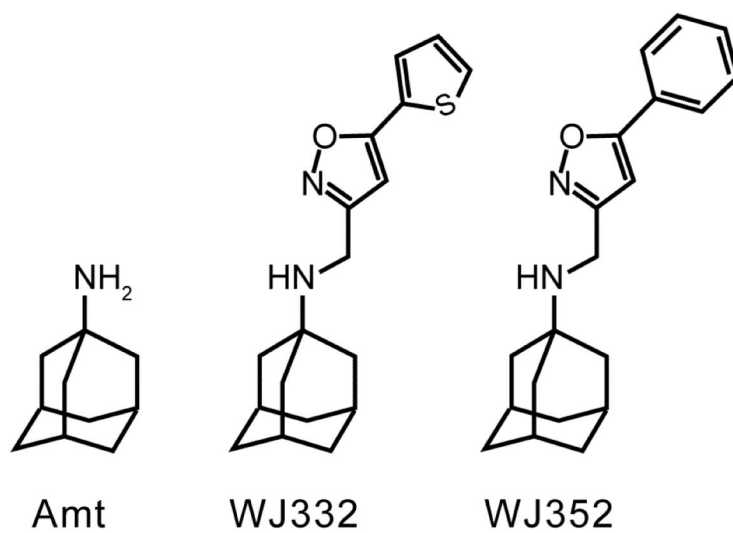


Figure 1.

Chemical structures of adamantyl-based drugs against influenza M2 proton channels: amantadine (Amt) against the WT protein, and WJ332 and WJ352 against the S31N mutant.

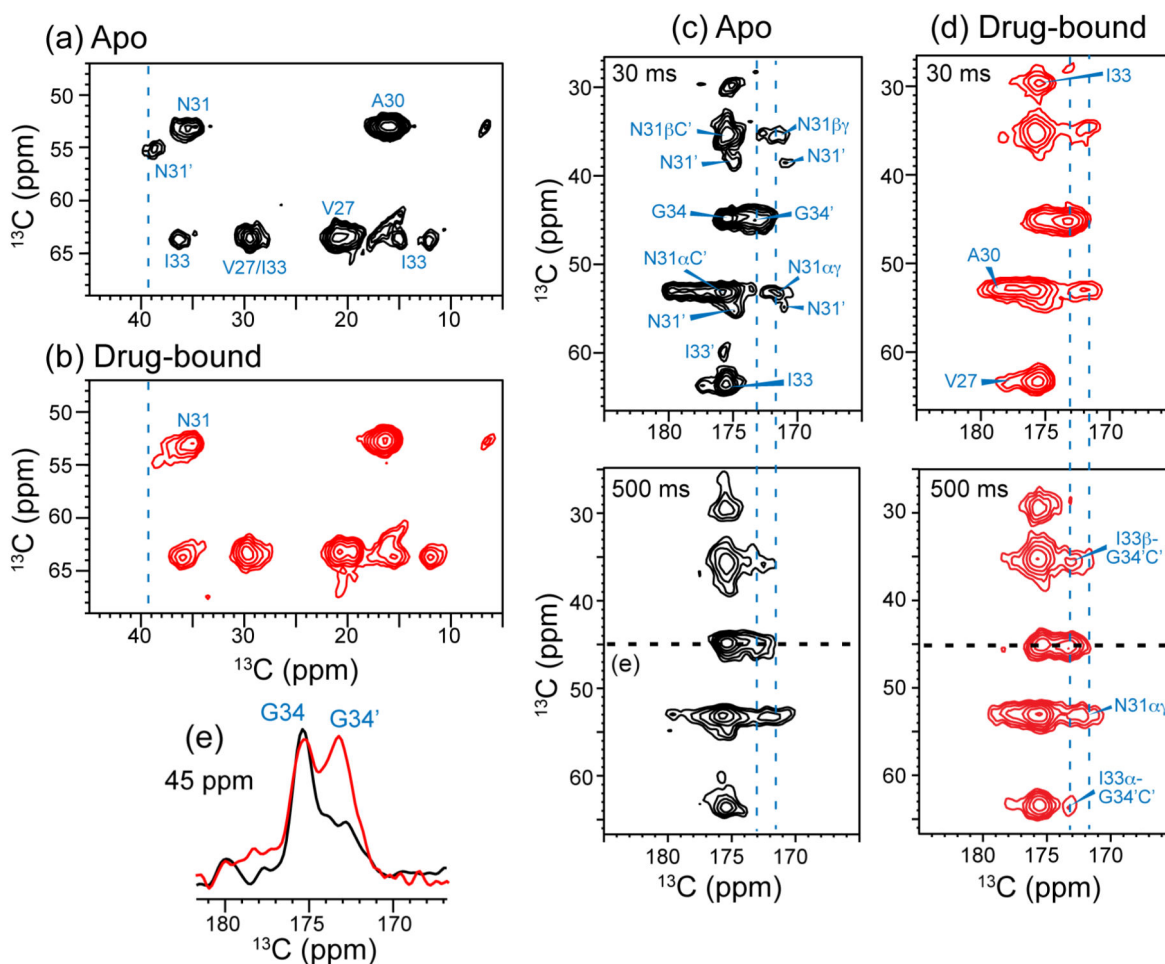


Figure 2.

$2\text{D } ^{13}\text{C}$ - ^{13}C correlation spectra of VANIG₁₉₋₄₉, S₃₁N in DMPC bilayers without and with WJ352 at pH 6.5. (a, b) Aliphatic region of the 30 ms spectra of the apo peptide (a) and drug-bound peptide (b). Drug binding changed the conformational equilibrium of the N31 Ca-C β peaks. (c, d) Carbonyl region of the 30 ms (top) and 500 ms (bottom) spectra of the (c) apo and (d) drug-bound peptide. N31, I33 and G34 exhibit two sets of chemical shifts. (e) 45-ppm cross section of the apo (black) and drug-bound (red) peptide, showing the G34 Ca-CO cross peak. All spectra were measured at 243 K under 7 kHz MAS.

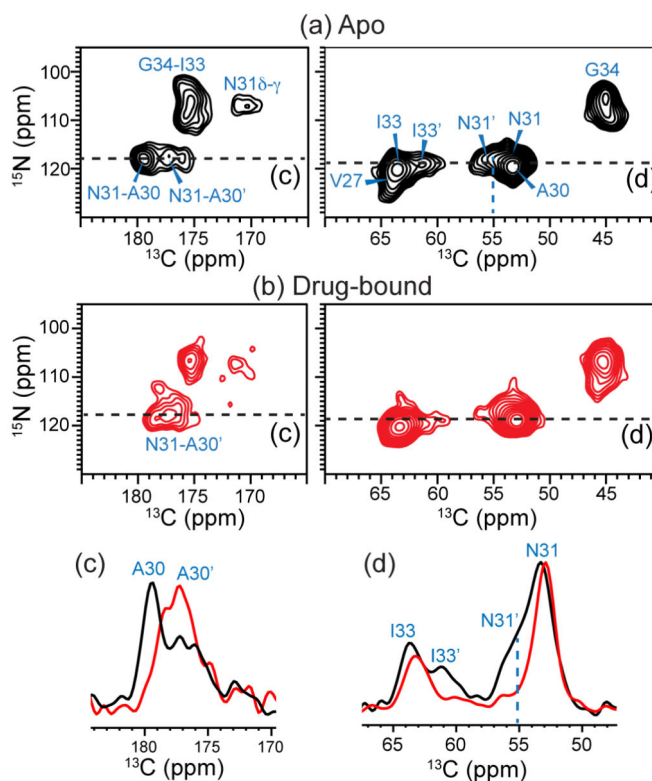


Figure 3.

2D ^{15}N - ^{13}C correlation spectra of VANIG₁₉₋₄₉, S_{31N} in DMPC bilayers at pH 6.5. (a) Without drug (black). (b) With drug (red) at the 10:1 drug : tetramer ratio. (c) A30 CO cross section at the ^{15}N chemical shift of 117.6 ppm. Drug binding changed the intensity distribution of two A30 CO peaks. (d) I33 and N31 $\text{C}\alpha$ cross section at 119.1 ppm ^{15}N chemical shift. Drug binding selected one of the two peaks of I33 and N31. The spectra were measured at 243 K under 7 kHz MAS.

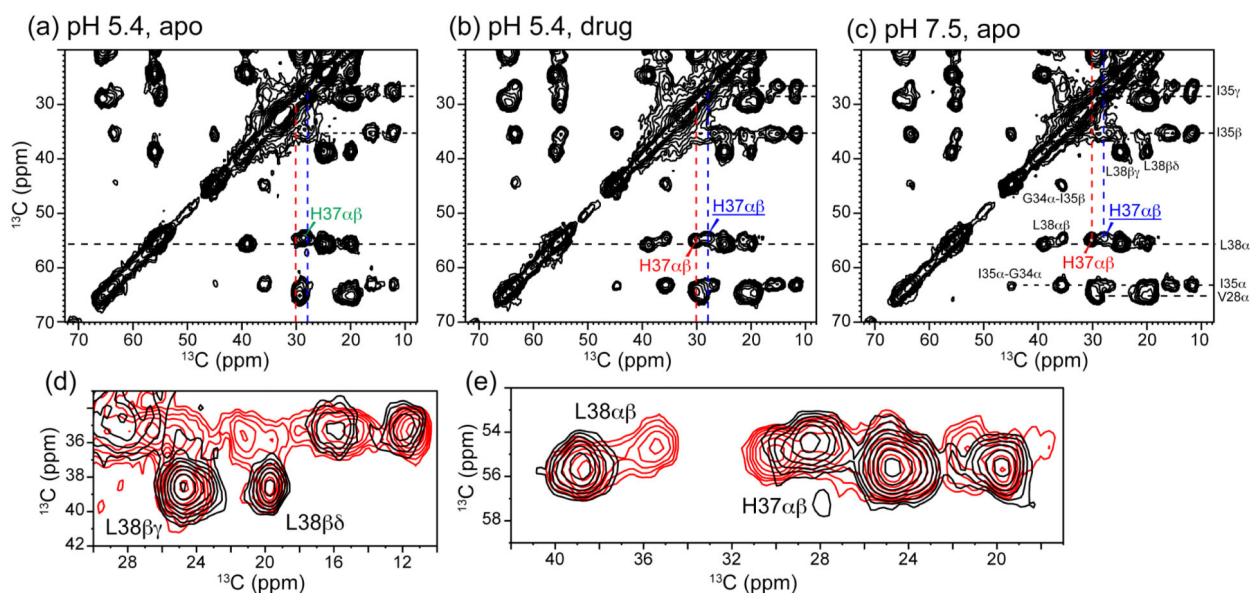


Figure 4.

Aliphatic region of the 2D ^{13}C - ^{13}C correlation spectra of VM+ membrane bound VGIHL₁₉₋₄₉, S31N. The spectra were measured at 243 K under 7 kHz MAS. (a) Apo peptide at pH 5.4. (b) Drug-bound peptide at pH 5.4. (c) Apo peptide at pH 7.5. The signals of τ , π , and cationic His37 are annotated in red, blue underline, and green, respectively. (d) Amplified L38 and I35 sidechain region of the apo (black) and drug-bound (red) spectra at low pH. (e) Amplified H37 and L38 $\text{C}\alpha$ - $\text{C}\beta$ region of the apo and drug-bound spectra at low pH. The low-pH drug-bound spectrum resembles the high-pH spectrum, indicating that WJ352 shifts the backbone conformation of S31N to that of the closed state.

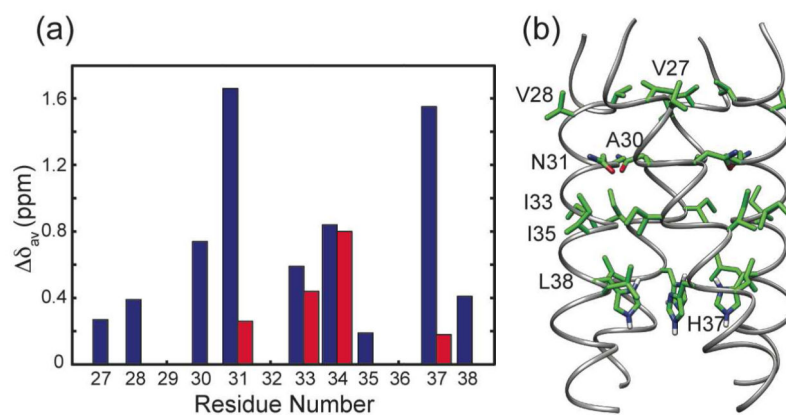


Figure 5.

(a) Residue-specific average chemical shift change in S31N M2TM induced by the drug WJ352. Residues with a single set of chemical shifts in both the apo and drug-bound states have a single blue bar for the average CSP, while residues with two sets of chemical shifts in the apo state but only one set in the drug-bound state have two CSP values. (b) Solution NMR structure of S31N M2TM (PDB: 2LY0), showing the positions of most residues whose CSPs were measured.

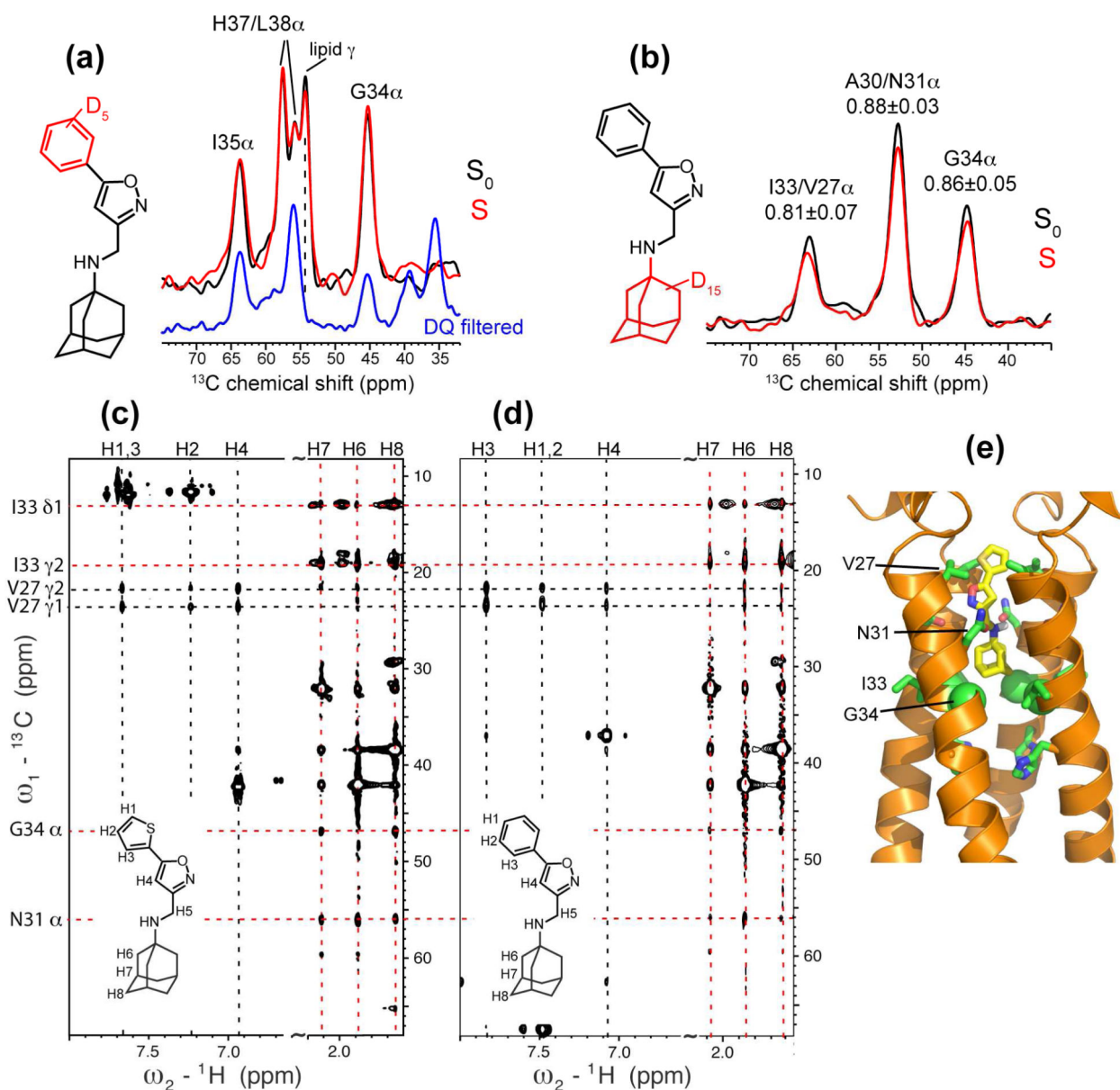


Figure 6.

Binding site and orientation of WJ352 in S31N bound to DMPC bilayers (a, b) and DPC micelles (c, d). (a) ^{13}C - ^2H REDOR S_0 (black) and S (red) spectra of d_5 -WJ352 complexed GIHL₂₂₋₄₆, S_{31N} in DMPC bilayers. The mixing time was 14.2 ms and the drug : tetramer ratio was 8 : 1. The deuterated phenyl group did not dephase any peptide ^{13}C signals, especially G34. The REDOR spectra were measured without DQ filtering, thus showed a lipid headgroup C γ peak at 54 ppm. This is verified by the absence of the peak in a ^{13}C DQ filtered spectrum (blue). (b) ^{13}C - ^2H DQ filtered REDOR spectra of d_{15} -WJ352 bound VANIG₁₉₋₄₉, S_{31N} in DMPC bilayers. The mixing time was 9.6 ms and the drug : tetramer ratio was 1 : 1. The deuterated adamantane dephased multiple ^{13}C signals. (c, d) 2D ^{13}C -(^1H)- ^1H NOESY (150 ms) spectra of VANIG₁₉₋₄₉, S_{31N} in DPC micelles with bound WJ332 (c) or with bound WJ352 (d). Drug – peptide cross peaks are observed and consistent with the amine-up orientation. (e) Solution NMR structure of S_{31N}-M2 with bound WJ332 (PDB: 2LYO)²⁹.

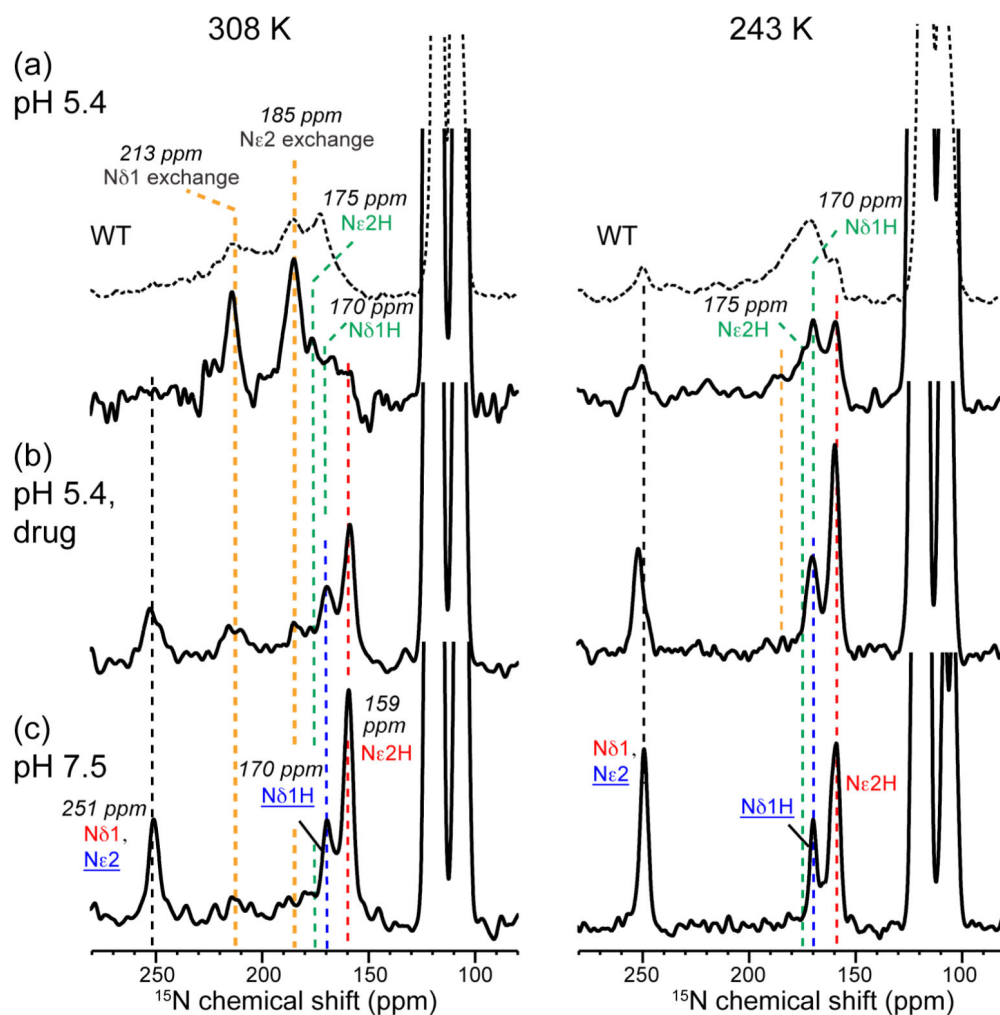


Figure 7.

1D ^{15}N CP-MAS spectra of His37 in VM+ membrane bound VGIHL₁₉₋₄₉, S_{31N} at 308 K (left) and 243 K (right). The spectra were scaled to show roughly equal integrated intensities for the imidazole nitrogens. (a) Apo peptide at pH 5.4. (b) Drug-bound peptide at pH 5.4. (c) Apo peptide at pH 7.5. Assignments shown here and in subsequent figures are annotated in red for the τ tautomer, blue underline for the π tautomer, and green for cationic His. For comparison, the pH 5.2 spectra of WT His37 are shown (dotted line) above the S_{31N} spectra in (a). At low pH and high temperature, S_{31N}-M2 exhibited sharp ^{15}N exchange peaks. WJ352 binding largely suppressed these peaks.

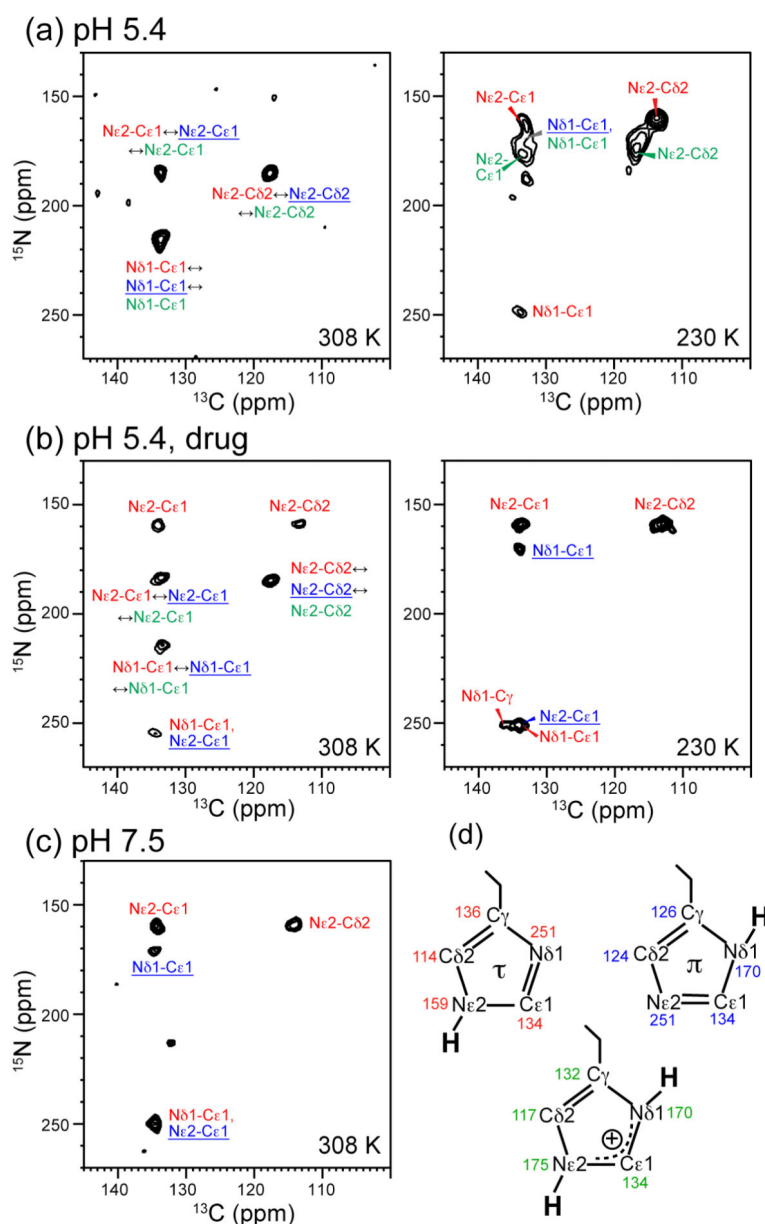


Figure 8.

2D ^{15}N - ^{13}C correlation spectra of VM+ bound VGIHL₁₉₋₄₉, S31N at 308 K and 230 K under 10 kHz MAS. (a) Apo peptide at pH 5.4. (b) Drug-bound peptide at pH 5.4. (c) Apo peptide at pH 7.5. The C γ signal was not detected due to rotational resonance with C β , thus allowing the distinction between N δ 1 and N ϵ 2 signals. (d) Imidazole chemical shifts for the τ tautomer, π tautomer, and cationic His.

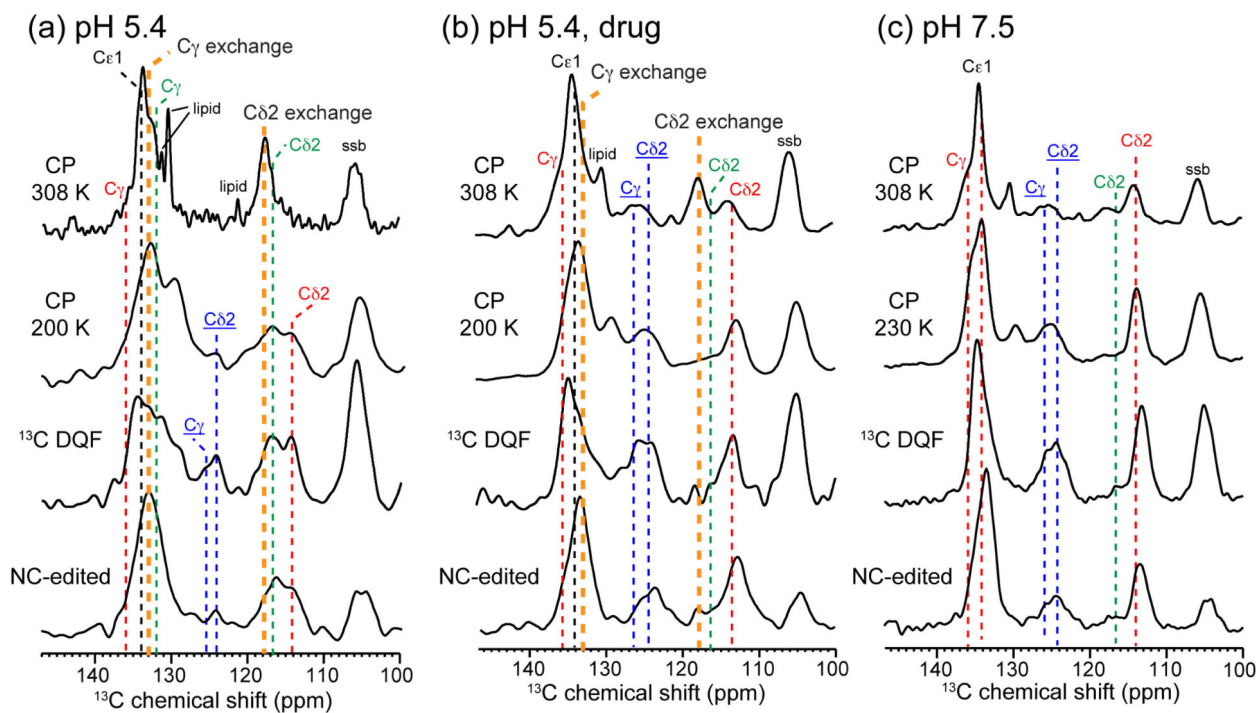


Figure 9.

Aromatic region of the 1D ^{13}C MAS spectra of His37 in VM+ bound VGIHL₁₉₋₄₉, S_{31N}. From top to bottom are the 308 K ^{13}C CP-MAS spectra, the low-temperature CP-MAS (200 or 230 K) spectra, the low-temperature ^{13}C - ^{13}C DQ filtered (DQF) spectra, and the low-temperature ^{15}N - ^{13}C filtered spectra. (a) Apo peptide at pH 5.4. (b) Drug-bound peptide at pH 5.4. (c) Apo peptide at pH 7.5. Spinning sidebands are denoted as ssb. All spectra were measured under 7 kHz MAS.

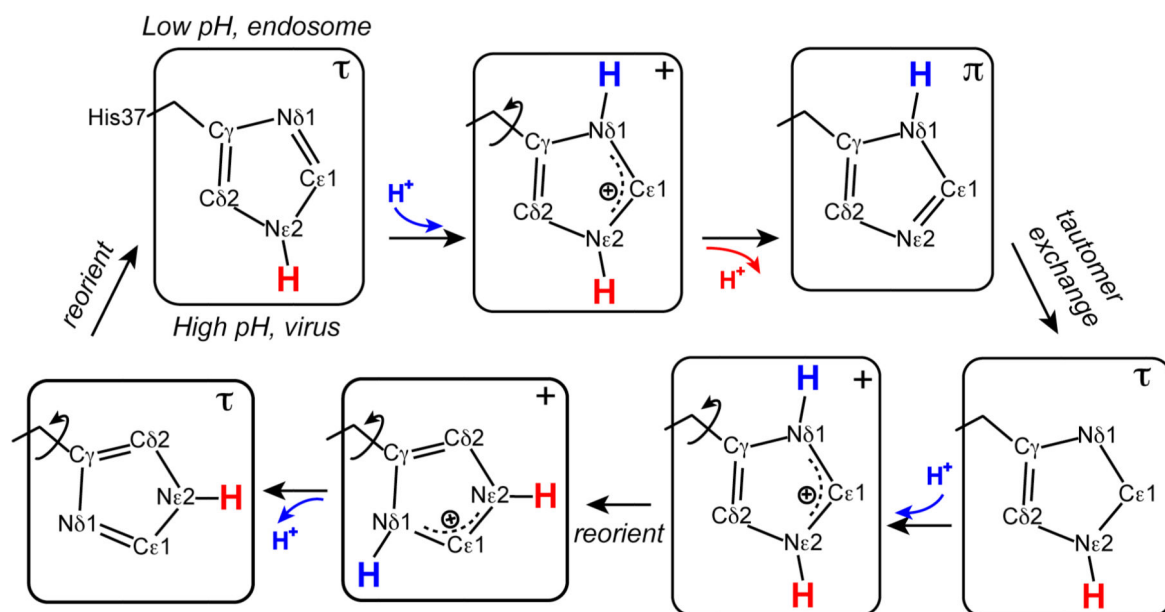


Figure 10.

Proposed mechanism of His37-water proton exchange for S31N and WT M2 channels. His37 residues undergo rapid interconversion among τ , π , and cationic states during proton transfer with water. Ring reorientations facilitate this proton shuttling by pointing the unprotonated nitrogen towards the low pH exterior.

Table 1

^{15}N and ^{13}C chemical shifts (ppm) of individual His37 in S31N-M2 bound to the virus-mimetic membrane.

Site	τ tautomer	π tautomer	Cation	observed $\tau : \pi$: cation 53 : 24 : 23 exchange
Ca	55.4	54.8	54.8	55.1 ^a
C β	30.1	27.9	28.4	29.2
C γ	136	126	132	133
N δ 1	251	170	170	213
Ce1	134	134	134	134 ^a
Ne2	159	251	176	185
C δ 2	114	124	117	118

^aThese are predicted exchange-averaged chemical shifts that are not resolved from unexchange peaks.