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Proton Transport through the Influenza A M2 Channel: Three-Dimensional Reference Interaction Site Model Study

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Abstract: The three-dimensional distribution function (DF) and the potential of mean force (PMF) of water and hydronium ions in five protonated states of the influenza A M2 channel are calculated by means of the three-dimensional reference interaction site model (3D-RISM) theory in order to clarify the proton conduction mechanism of the channel. Each protonated state, denoted as *i*H, where $i = 0-4$, has a different number of protonated histidines, from 0 to 4. The DF of water in each state exhibits closed structures of 0H, 1H, and 2H and open structures in 3H and 4H. In the closed form, the DF and PMF indicate that hydronium ions are excluded from the channel. In contrast, the ion can distribute throughout the opened channel. The barrier in PMF of 3H, $\sim 3-5$ kJ/mol, is lower than that of 4H, $5-7$ kJ/mol, indicating that 3H has higher permeability to protons. On the basis of the radial DFs of water and hydronium ions around the imidazole rings of His37, we propose a new mechanism of proton transfer through the gating region of the channel. In this process, a hydronium ion hands a proton to a non-protonated histidine through a hydrogen bond between them, and then the other protonated histidine releases a proton to a water molecule via a hydrogen bond. The process transfers a proton effectively from one water molecule to another.

1. Introduction

In early 2009, a new strain of H1N1 influenza A virus spread over many countries and has become a pandemic. It is an urgent task to understand this virus and to seek either vaccines or new drugs to cope with the infection. The M2 protein channel, which is found in the viral lipid envelope, has received a great deal of attention as a target for drug development due to its important role in proton transport and viral replication.[1,2] It is known that a drug family called "amantadines" is effective against influenza A by blocking the M2 channel, which blocks proton conduction through the membrane and consequently causes inhibition of viral replication.[3] However, the underlying mechanism of the proton blockade activity by amantadines is not yet clear. In addition, many new strains of influenza virus are resistant to amantadines. Therefore, understanding the mechanism of proton transport through the virus membrane is one of the central issues for drug design.

The M2 channel forms a homotetramer, each monomer comprising 97 amino acid residues. The monomer includes three domains, the 24-residue N-terminal extracellular domain, the 19-residue transmembrane domain, and the 54-residue cytoplasmic domain.[4] Several electrophysiological results have shown that the M2 channel is highly selective for protons and its gating is controlled by pH.[5−9] A number of researchers have explored the relationship between functions and molecular structures of the M2 channel and have indicated the important role of His37 in its gating mechanism. Pinto and co-workers have demonstrated that mutants of the M2 channel with the His residue substituted by Gly, Ser, or Thr lose their proton selectivity and that the selectivity is restored upon addition of imidazole; this implies that the imidazole group plays an important role in proton selectivity.[10] Concerning the pH-controlled gating mechanism, Hu et al. have made important suggestions based on their experiments that the pK_a's associated with the four histidines in the gating region are different from one another and that the open forms are dominated by triply

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and quadruply protonated histidine.[11] These experimental results suggest that His37 acts as a pH sensor switch to turn the gate "on" and "off" and as a selective filter to allow the permeation of protons but not other cations. There is another suggestion based on experiments indicating that Trp41 also has an important role in the gating: namely, a protein in which Trp41 is replaced by residues with smaller size, Ala, Cys, or Phe, has higher proton conductivity compared with its wild type.[12] UV Raman spectra show the interaction between the protonated imidazole of His37 and the indole of Trp41, a cation$-\pi$ interaction.[13,14] These investigations suggest that the indole of Trp41 functions to occlude the channel pore. This residue behaves as a door to "open" or "close" the pore, which is controlled by protonated His due to the cation$-\pi$ interaction.

Different techniques, including solid-state NMR (ssNMR), solution NMR, and X-ray, have revealed dramatically different and controversial results for the structures of M2 channels.[15−17] Thus, molecular dynamics (MD) studies of the M2 channel, which simulated the M2 channel on the basis of different structure sources, show dissimilar results. The MD studies using the structures from X-ray and solution NMR demonstrated the second gating at Val27:[18−20] the channel will close and disrupt the water profile at Val27. However, the MD studies based on the structures from ssNMR have never found the narrowest region at Val27.[18,21] The experimental studies based on the mutagenesis are not conclusive concerning whether Val27 plays a role in gating. Holsinger et al. showed that the mutation of Val27 to Ala, Ser, or Thr does not change the current of protons.[22] On the other hand, recent work by Pinto and co-workers indicates that replacement of Val27 by Ala or Asp increases the activity, while replacement by Ser, Thy, Gly, Lys, Arg, Phe, or Trp decreases the activity of proton transport.[23] Even replacement of Val27 by a smaller residue such as Ala or Gly shows either an increase or a decrease in the proton current. In addition, the MD simulations based on the X-ray structure are inconsistent among themselves. The study by Arkin and Leonov, in which the X-ray structure was directly applied to the simulation, showed that the channel always closes at Val27 in neutral, bi-protonation, and tri-protonation states of His37.[18] On the other hand, Khurana et al. have not used the whole structure of the channel; they have chosen only a single transmembrane (TM) domain and duplicated the other three TM domains.[19] They found that at high pH the channel is in the $Open_{out}-Close_{in}$ state (the channel is open at the Val27 gate and closed at His37), while at low pH the channel is in the $Close_{out}-Open_{in}$ state (the channel is closed at the Val27 gate and open at His37). However, this result seems to be problematic, because the starting structure they have used, known as the D4 model, is closed at both the Val27 and His37 gates, and no evidence supports this model.

In contrast to the MD studies based on the X-ray structure, all of the MD results based on the ssNMR structure are consistent among each other, showing that the channel will close around the His37/Trp41 gate at low protonation state and open at high protonation state.[18,21,24,25] The groups of Arkin and Hannongbua have shown that the accessibility of water to the channel pore is enhanced with increasing number of protonated states of histidine, and the channel is closed at low protonation state at the His37 and Trp41 regions.[21,25] Voth and co-authors used the alternative simulation methodology to calculate the potential of mean force (PMF) and the diffusion coefficient of proton in the channels. Their results have shown that the 3H state has the highest proton permeability,[24] which agrees with the suggestion from the studies based on ssNMR that the channel becomes activated at the triple protonation state of His37.[11]

In this report, we present an alternative approach to the problem based on the three-dimensional reference interaction site model (3D-RISM) theory.[26] The 3D-RISM theory is a statistical mechanics theory for molecular liquids. The theory has scored great success in exploring a variety of solvation processes occurring in biosystems, especially the "molecular recognition" process.[27−33] The method describes the molecular recognition or binding of guest molecules trapped inside a cavity of a protein molecule in terms of the three-dimensional (3D) distribution of the ligands. Using this method, we investigate the 3D distribution of water molecules as well as hydronium ions, a model of protons, inside the M2 channel with different protonated states in order to clarify the molecular mechanism of gating and proton conduction in the channel. Considering the consistency of the previous MD studies based on the ssNMR structure, we adopt the protein structure from the MD simulation, which has been carried out previously by two of the authors,[25] for our calculations on the distribution of water and proton in the M2 channel.

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2. Methods

The detailed formulation of the 3D-RISM theory is provided in the references.[26,30] Here, only a brief interpretation of the theory is provided in order to help readers to understand the "physical" aspect of the theory.

Let us consider the average density of water molecules at a position around a protein molecule. When the position is far from the protein molecule, so as to be regarded as in the bulk, the density will be constant, the same as in the pure liquid. On the other hand, when it is near the protein, the density will be "perturbed" significantly by the field due to the protein and will be different from that in the bulk, depending on the strength of the perturbation. The 3D-RISM theory can be interpreted as a "nonlinear" perturbation theory as follows.

Let us denote the constant density of solvent atom γ at the bulk, the density near the protein, and the density response to the perturbation as ρ_γ, $\rho_\gamma(\mathbf{r})$, and $\Delta\rho_\gamma(\mathbf{r})$, respectively. Then, the statement made above can be expressed as

$$\rho_\gamma(\mathbf{r}) = \rho_\gamma + \Delta\rho_\gamma(\mathbf{r}) \tag{1}$$

The density response to the perturbation can be expressed on the basis of 3D-RISM theory as

$$\Delta\rho_\gamma(\mathbf{r}) = \sum_\alpha \int \chi_{\gamma\alpha}(\mathbf{r}, \mathbf{r}')\rho_\gamma c_\alpha(\mathbf{r}')\, d\mathbf{r}' \tag{2}$$

where $c_\alpha(\mathbf{r}')$ is the "re-normalized" perturbation due to the protein, for which several approximate equations have been devised. For example, the HNC approximation takes the following expression:

$$c_\gamma(\mathbf{r}) = \exp[-\beta u_\gamma(\mathbf{r}) + h_\gamma(\mathbf{r}) - c_\gamma(\mathbf{r})] - [h_\gamma(\mathbf{r}) - c_\gamma(\mathbf{r})] - 1 \tag{3}$$

In this expression, $u_\gamma(\mathbf{r})$ is the direct interaction potential exerted on water molecules from protein, and $h_\gamma(\mathbf{r})$ is the density fluctuation of water at position $\mathbf{r}$, normalized by the bulk density, namely,

$$h_\gamma(\mathbf{r}) = \Delta\rho_\gamma(\mathbf{r})/\rho \tag{4}$$

The three-dimensional distribution function (3D-DF) used in this study is defined from $h_\gamma(\mathbf{r})$ by

$$g(\mathbf{r}) = h(\mathbf{r}) + 1 \tag{5}$$

It is not only the direct interaction $u_\gamma(\mathbf{r})$ with protein that perturbs the density of water at a certain position but also interactions with water molecules at the other positions, whose density is also perturbed by the existence of the same protein. Such "indirect" perturbations are renormalized into the terms including $h_\gamma(\mathbf{r}) - c_\gamma(\mathbf{r})$. Such renormalization makes the perturbation highly "nonlinear".

The response function $\chi_{\gamma\alpha}(\mathbf{r},\mathbf{r}')$ is equivalent to the density pair correlation function of pure or bulk solvent,

$$\rho^2\chi_{\alpha\gamma}(\mathbf{r}, \mathbf{r}') = \langle \delta\rho_\alpha^{(0)}(\mathbf{r})\, \delta\rho_\gamma^{(0)}(\mathbf{r}')\rangle \tag{6}$$

where $\delta\rho_\sigma^{(0)}(\mathbf{r})$ is the density fluctuation of atom σ in the pure liquid, defined by $\delta\rho_\sigma^{(0)}(\mathbf{r}) = \rho_\sigma^{(0)}(\mathbf{r}) - \rho_\sigma$. This response function can be obtained in advance from the one-dimensional RISM theory.

The various initial structures of the proton-selective M2 channel of five different protonation states (PS) on the histidine residue His37 were extracted from the previous MD simulations which were based on the ssNMR structure (Figure 1);[15,25] 11 conformations for each protonation state were chosen. The proton states are non-PS (0H), single PS (1H), double PS at diagonal position (2H), triple PS (3H), and quadruple PS (4H). For the calculation of solvent distribution with the 3D-RISM theory, all the coordinates related to solvent environment, water, ions, and lipids were removed, so that only those associated with the M2 helix bundle were used as the input of the protein coordinates. The protein was immersed in an aqueous solution of 0.01 M HCl, in which electrolytes are mimicked by a mixture of chloride and hydronium ions as in the previous studies.[31,32] For closing the 3D-RISM equation, we chose the KH approximation for its advantage of rapid convergence.[34,35] The PMFs were calculated by integrating 3D-DFs along the channel axis.[32] The potential parameter and structure of solvent molecules were adopted from previous work.[31,32] Amber-99 was employed to obtain potential parameters of the protein, M2.[36] 3D-RISM and KH-closure equations were solved on a grid of 128^3 points in a cubic supercell of 64 Å^3.

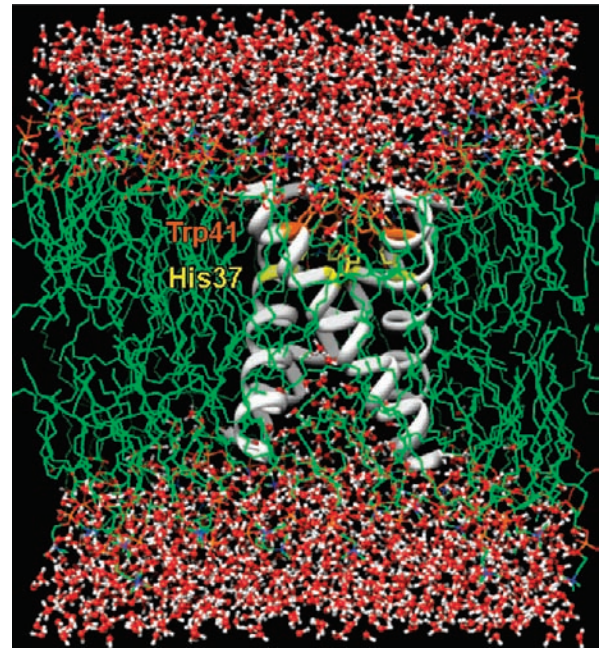

Figure 1. Simulation of the M2 tetramer in pre-equilibrated POPC lipid bilayer and modeled water. The His37-selective filter and Trp41 gating residues are also shown in yellow and orange, respectively.

3. Results and Discussion

3.1. Distribution of Water in the M2 Channel. To investigate the mechanism of proton transfer in the M2 channel, we consider the distribution of water inside the channel as a function of the number of protonated state (PSs) of the histidine tetrad, from non-PS (0H) to quadruple PS (4H), which are regulated by changing the pH. For each state, we randomly pick coordinates for the M2 protein from a trajectory of the MD simulation which was carried out previously.[25] Because the results of various conformations in the same protonated state are similar among themselves, (see Supporting Information), one representative result for each protonated state is shown. The 3D-DF of water with $g(\mathbf{r}) > 1$ in the five different protonated states of the M2 channel is depicted in Figure 2. The figure indicates that the accessibility of water (cyan in Figure 2) to the channel pore increases with the protonated state of the channel in the order 0H < 1H < 2H < 3H < 4H. This result can be explained readily in terms of the pore diameter, which is enhanced due to the electrostatic repulsion among the protonated histidines His37. The structural change originated at His37 propagates to the bulky

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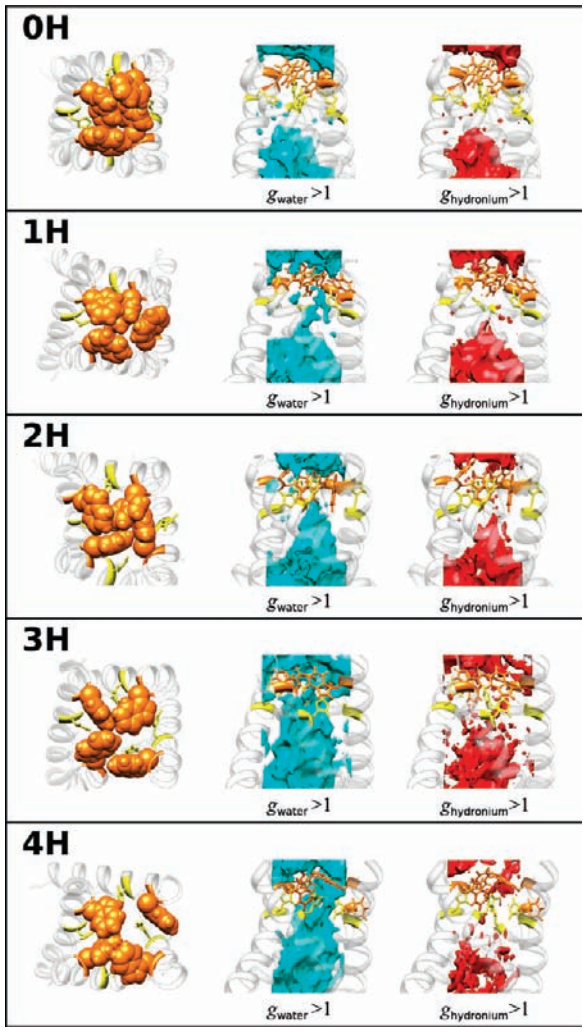

Figure 2. 3D-DFs of water in the channel (cyan) and hydronium ion (red), with $g(\mathbf{r}) > 1$. Molecular structure of the channel gating region is also depicted at the left for protonation different states of the His37: Trp41, orange; His37, yellow.

indole rings of Trp41, which actually play the role of gating. The results suggest that there are two distinct states in the channel conformation, or "open" and "closed" forms. The 0H, 1H, and 2H forms are considered as closed forms, since water distributions are not observed at the selective filter regions of His37 (yellow stick in Figure 2) and at the gating region with the Trp41 residues (orange sphere and stick in Figure 2). On the other hand, the 3H and 4H forms, with a continuous water distribution along the pore, are identified as the open forms (Figure 2). In addition, the narrowest region in the 3D-DF of water in the channel, also the narrowest region of the pore, is located at the Trp41 region, which is regarded to play a role of "gating". From the experimental results, Trp41 not only blocks inward proton transportation when the pH of the exterior is high but also blocks outward proton transportation when the interior environment is acidic.[12] The results suggest that the indole moiety of Trp41 should completely block water to reach His37 from the viral interior when the channel is closed, which is consistent with our results.

The PMFs of water along the channel of various conformations in each protonation state corresponding to the DF are shown in Figure 3, in which the high barriers are found only in the 0H, 1H, and 2H states. It is obvious that in the closed form water cannot overcome the high barrier due to the steric hindrance between the channel atoms and water molecules. On the other hand, the PMF of water in the 3H and 4H states is negative along the entire channel pore, which indicates that water molecules in the channel are more stable than those in the bulk and that water permeates through the channel. The results are in harmony with previous theoretical studies carried out by different methods.[21,24,25]

3.2. Distribution of Hydronium Ions in the M2 Channel and Gating Mechanism. The 3D distributions of hydronium ions inside the channels with $g(\mathbf{r}) > 1$ are also depicted in Figure 2. For clarification, PMFs of the ion in various structures of the M2 channel in each protonated state are shown in the Figure 3.

In the three states of closed gate, or 0H, 1H, and 2H, hydronium ions exhibit behavior similar to that of water, but with lower distribution and higher barrier in PMFs compared to those of water (Figure 3). This indicates that a hydronium ion, or a proton, cannot distribute in the channel and is prevented from transporting across the channel. The results are consistent with those for the closed conformations of the M2 channel at high pH values reported in many experimental and theoretical studies.[5–9,24]

In contrast to the distributions of water, the distributions of hydronium ion in the 3H and 4H states do not look much different from those in 0H to 2H. The difference is apparent, however, if one looks at Figure 3, where the PMFs of the hydronium ion in 3H and 4H exhibit entirely different behavior than those in 0H, 1H, and 2H. Protons in the 0H, 1H, and 2H forms have extremely high barriers, for the same reason as in the case of water, and no chance to get into the gating region of the channel, while the barrier heights in the 3H and 4H forms are just 2−3 and 5−7 kJ/mol, respectively, comparative to the thermal energy and are able to be overcome by the thermal fluctuation of the protein conformation. There is another interesting observation in the figure. The barrier height for protons is higher in 4H than in 3H, which is against the heuristic argument based on the pore size around the gating region.

All these observations suggest two competing factors working on the proton distribution as the protonated state of the channel is increased from 0H to 4H. One of those is the channel opening due to the increased repulsion among the protonated His residues, which tends to enhance the distribution of protons as well as water in the channel. The other factor is the electrostatic repulsion between protons and the protonated His residues, which will reduce the proton distribution with increasing number of protonated His. The two effects balance in the 3H state to maximize the proton distribution in the channel. These results are consistent with those reported by Voth et al.[24]

3.3. Conduction of Hydronium Ions in the M2 Channel. Two models have been proposed to explain the mechanism of proton transport through the M2 channel. The first and the simplest model is called the "gating" or "shutter" mechanism, claiming that water can penetrate through the channel and form a proton wire whenever the gate is opened.[24,37] A proton is transported from the hydronium ion to a neighboring water molecule through the H-bond train, or Grotthuss mechanism.[38] The model requires a well-defined hydrogen-bonded train of water molecules inside the channel in order to form a double-well potential with a shallow barrier in between, through which a proton may tunnel.

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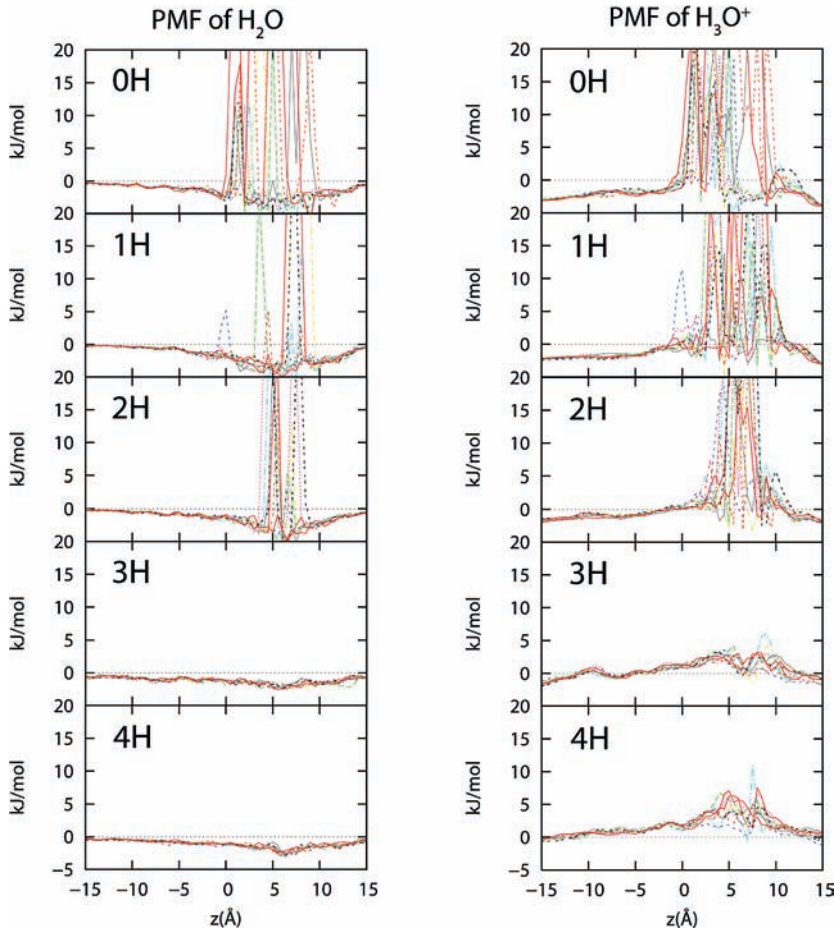

Figure 3. Potential of mean force of water and hydronium ion in each state. A line represents the PMF for a conformation in the state.

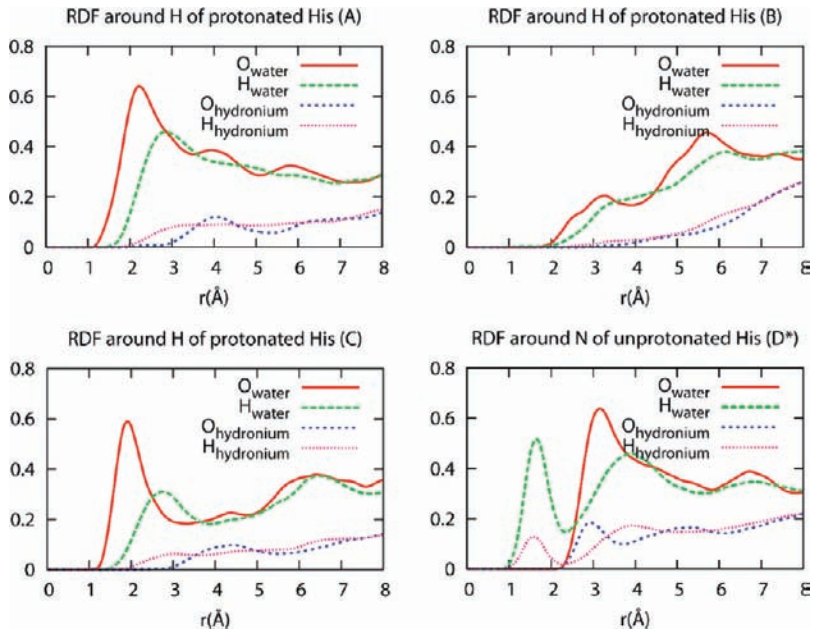

Figure 4. Radial distribution functions of oxygen and hydrogen of water and hydronium ion around the nitrogen of unprotoneted imidazole and hydrogen of protonated imidazole: A, B, C and D in parentheses denote each helix, and an asterisk denotes an unprotonated histidine loop.

The model is unlikely, though, if one examines the distribution of water and hydronium ion in the channel in detail. Figure 4 shows the radial distribution function (RDF) of oxygen and hydrogen of water and hydronium ions around a nitrogen and hydrogen atom of unprotonated and protonated imidazole in a His residue that is close to the channel surface (A, B, C and D in parentheses in the figure represent each helix, and * indicates unprotonated state of histidine in Figure 5). It indicates that

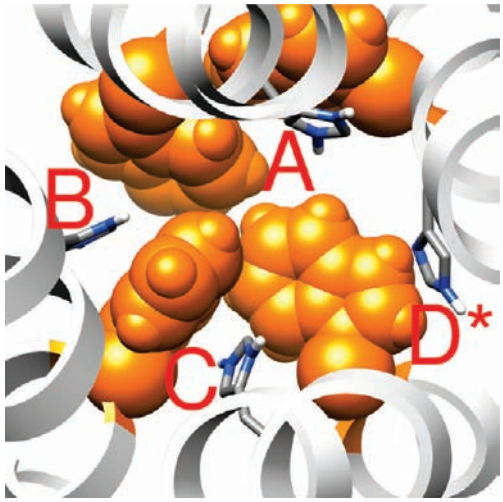

Figure 5. His37 (stick line) and Trp41 (orange sphere) in the 3H state.

hydrogen of a hydronium ion, as well as of water, is attracted to nitrogen of imidazole of non-protonated histidine (D* in Figures 4 and 5), and oxygen of water bonds to the hydrogen of protonated histidine. The hydrogen bond between imidazole and hydrogen of water or hydronium is illustrated schematically in Figure 6a. The figure strongly suggests that a water molecule or a hydronium ion in the channel is likely to make a hydrogen bond with the His residues rather than with other water molecules. Such water configurations will eliminate the possibility of the Grotthuss mechanism through a hydrogen-bonded train of water molecules. Our finding is in accord with that reported by Voth and co-workers, who also concluded that the likelihood of the Grotthuss mechanism working in the channel is low.

The second model is the so-called "shuttling" mechanism that requires at least one non-protonated histidine at the gating region, with its two nitrogen atoms pointing toward the channel pore surface.[39] A proton transfers from a hydronium ion to the nitrogen atom of imidazole; the histidine is protonated and forms a bi-protonated intermediate. The other nitrogen atom of the *same* imidazole then releases the proton to a neighboring water molecule. Finally, the process is completed by flipping of imidazole, or tautomerization, to recover the initial configuration to prepare for the next proton. However, the model seems to be questionable, because in order for a His residue to behave as a proton relay, two nitrogens of the same histidine residue should be exposed to water inside the channel pore. Such a conformation of His was not found in the MD simulation studies.[25] Accordingly, the corresponding distribution of water hydrogens was not observed in the present study.

On the basis of the results shown in Figure 4, we propose a new model for a proton-transfer mechanism in the channel which is consistent with the experimental work, suggesting that the histidine residues play essential roles in proton transport. Our model requires two histidines, protonated and non-protonated, for a proton to be transferred. The RDFs shown in Figure 4 clearly indicate that a nitrogen atom of the non-protonated imidazole is hydrogen-bonded to a hydronium ion through a hydrogen atom of the ion, while the protonated imidazole is hydrogen-bonded to water oxygen through a hydrogen atom of the imidazole. The situation is illustrated in Figure 6a. We can construct a model of proton transfer in the channel that is consistent at least with our RDF results, which is illustrated Figure 6. The proton is transported from a hydronium ion to the nitrogen of imidazole, and the other imidazole releases the proton to a nearby water molecule. These two imidazoles switch their protonation states from protonated to non-protonated and vice versa, as shown in Figure 6a,b. To complete the process, the two histidines move to the appropriate positions due to conformational fluctuations of protein and start to transfer again (Figure 6c,d). This process can occur only in the 3H state. The

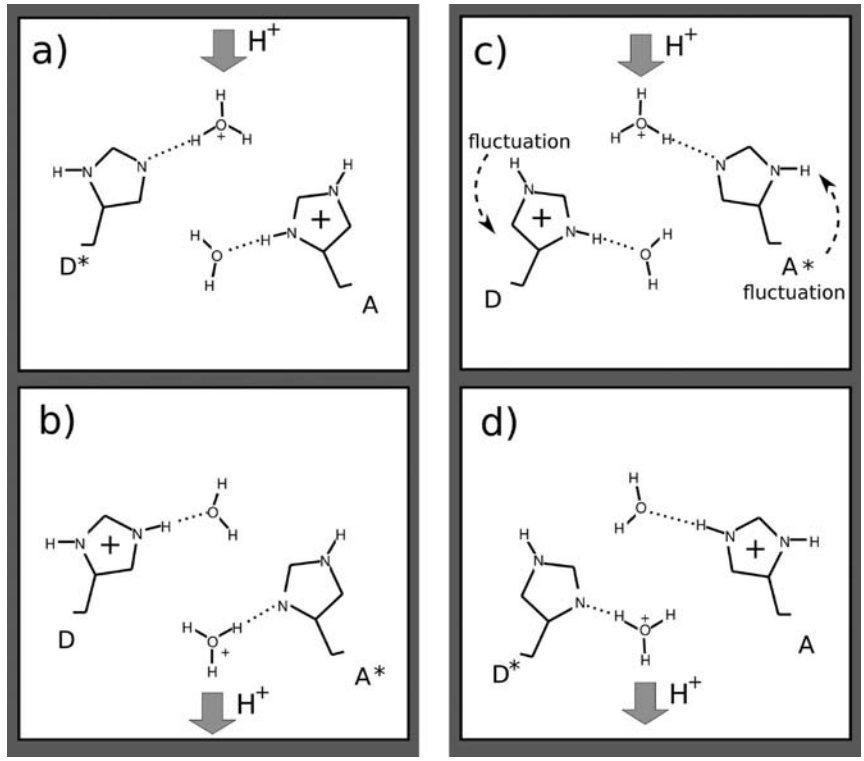

Figure 6. Schematic view of the proposed mechanism of proton transportation through the His.

first reason is that it requires at least one non-protonated histidine, bonded respectively to a water molecule and to a hydronium ion. This requirement excludes the possibility of the 4H state. Second, in the closed channels, 1H and 2H, all the Trp41 residues block water molecules from the gating area entirely, and no water molecules are available to hydrogen-bond to an imidazole at the interior channel. Consequently, a proton cannot be transported through this process in 1H, 2H, and 4H but can be in 3H.

4. Conclusions

The conduction of protons through the M2 channel in influenza viruses was investigated by means of the 3D-RISM/RISM method, the statistical mechanics theory of molecular liquids. The three-dimensional distribution functions and the potentials of mean force of the hydronium ions as well as water molecules inside the channel were calculated for five different states of the channel, which are distinguished by the number of protonated histidines (His37) at the gating region, from no histidine protonated (0H) to four histidines protonated (4H).

It was found from the calculation that the channels with zero, one, or two protonated histidines, which have been identified as "closed" forms in the previous studies, exclude both water molecules and hydronium ions from the gating region due to the steric effect of the Trp41 residues, which change conformation with the His37 residues. The results strongly indicate that neither water molecules nor protons can permeate through the channel with zero, one, or two protonated histidines. On the other hand, our results for the 3H and 4H cases indicate that water and protons can permeated through the channel, although protons have to overcome small activation barriers resulting from the residues around the gating region. The barrier turns out to be lower in the 3H case than in the 4H case. This interesting behavior was explained in terms of an interplay of two effects as the protonation level increases: enhanced pore diameter due to the increased coulomb repulsions among the protonated histidines, and enhanced coulomb repulsion between the protonated histidines and hydronium ions.

On the basis of the radial distribution functions of water and hydronium ions around the imidazole rings of His37, we have proposed a new model of proton transfer through the gating region of the channel. Our model requires two histidines, protonated and non-protonated, for a proton to be transferred. In the model, a hydronium ion hands a proton to a non-protonated histidine through a hydrogen bond between them, and then another (protonated) histidine releases a proton to a water molecule via a hydrogen bond. The process thereby transports a proton from a water molecule to another water molecule. Further study to prove this hypothesis is in progress.

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Supporting Information Available: Additional 3D-DFs of water and hydronium ion and table of rmsd's between the structures of the channel in each protonation state of His. This material is available free of charge via the Internet at http://pubs.acs.org.

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