Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is “None”, please specify “None” as your response. “Not applicable” is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report must be completed using MS Word. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Temple University – of the Commonwealth System of Higher Education

2. **Reporting Period (start and end date of grant award period):** 01/1/2009 -12/31/2012

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Germaine Calicat, MLA

4. **Grant Contact Person’s Telephone Number:** 215.204.7655

5. **Grant SAP Number:** 4100047651

6. **Project Number and Title of Research Project:** 25 - Understanding Drug Binding to the M2 Channel of the Influenza A Virus

7. **Start and End Date of Research Project:** 12/1/2009 – 11/20/2012

8. **Name of Principal Investigator for the Research Project:** Michael L. Klein, PhD

9. **Research Project Expenses.**

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 335,630.78

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of all persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).
<table>
<thead>
<tr>
<th>Last Name</th>
<th>Position Title</th>
<th>% of Effort on Project</th>
<th>Cost</th>
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<tr>
<td>Klein</td>
<td>Principal Investigator</td>
<td>18% Yr4</td>
<td>52,748.36</td>
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<tr>
<td>DeVane</td>
<td>Faculty Appointment</td>
<td>25% Yr3</td>
<td>11,146.04</td>
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<tr>
<td>Fiorin</td>
<td>Post Doc/Faculty Appt</td>
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<td>Dong</td>
<td>Post Doc</td>
<td>100% Yr4</td>
<td>56,875.67</td>
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9(C) Provide the names of all persons who worked on this research project, but who were not supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

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<tr>
<th>Last Name</th>
<th>Position Title</th>
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9(D) Provide a list of all scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

<table>
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<tr>
<th>Type of Scientific Equipment</th>
<th>Value Derived</th>
<th>Cost</th>
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10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_________ No____ X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_________ No____ X_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds
to be awarded (column E). If the grant was not funded, insert “not funded” in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

<table>
<thead>
<tr>
<th>A. Title of research project on grant application</th>
<th>B. Funding agency (check those that apply)</th>
<th>C. Month and Year Submitted</th>
<th>D. Amount of funds requested:</th>
<th>E. Amount of funds to be awarded:</th>
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<td>☐ NIH</td>
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<td>☐ Other federal (specify: ______)</td>
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<td>☐ Nonfederal source (specify: _)</td>
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11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes ___ X _____ No _________

If yes, please describe your plans:

We plan to apply for an NIH R01 grant that will build on the preliminary results obtained so far that deal with the M2 channel of the influenza A virus and various other ion channels.

12. **Future of Research Project.** What are the future plans for this research project?

The future plans are to pull together extensive preliminary results on various ion channels and build a competitive R01 grant in the next few months.

13. **New Investigator Training and Development.** Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes ________  No _____ X ______

If yes, how many students? Please specify in the tables below:
14. **Recruitment of Out-of–State Researchers.** Did you bring researchers into Pennsylvania to carry out this research project?

Yes_________ No____X______

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. **Impact on Research Capacity and Quality.** Did the health research project enhance the quality and/or capacity of research at your institution?

Yes_____X____ No__________

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

The support enabled our group to carry out studies of various voltage gated ion channels; research that might also lead to an NIH R01 proposal in the near future.

16. **Collaboration, business and community involvement.**

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?
Yes _____ X _____ No _________

If yes, please describe the collaborations:

We have an experimental collaborator William F. DeGrado (UCSF). Our project has been involved in developing tools to study ion channels using computer simulation methods. These methods are likely to be useful to study the M2 channel of the influenza A virus and other simple oligomeric ion channels such as the Sodium and Potassium voltage gated bacterial channels.

16(B) Did the research project result in commercial development of any research products?

Yes _____ X _____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes _____ X _____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.
List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.
Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project’s strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee’s written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (β) should not print as boxes (☐) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Project goals, objectives and specific aims
The original goals and objectives were to help develop drugs that inhibit the M2 proton channel of the influenza A virus. Specifically, the Klein group was to develop computational methodologies to complement ongoing experiments in the lab by Professor William F. DeGrado (UCSF). The part of the project covered by the Pennsylvania Department of Health was solely the computational methods. We specifically focused on the following areas:
- Mechanism of proton conduction by the histidine amino acids in the sequence of the M2 proton channel.
  The work on this subject has been summarized in three research articles: the first focuses on the histidines as an isolated system, and is slated to appear in an upcoming issue of Chemical Physics. The second paper focuses on the histidines within the M2 protein in its non-conductive form, and is to be submitted to the Journal of Physical Chemistry Letters: due to its relatively limited length, we include the complete text and figures. The third paper is currently in preparation, and focuses on the conductive form of the M2 protein: we include a short summary of its contents.

- Structure and functionality of naturally occurring mutants of the M2 channel.
  Most in vitro data have been measured on the Udorn strain of the influenza A virus, or of its drug-resistant mutations S31N and V27A. Other viral strains such as Weybridge and Rostock also carry mutations of the asparate residue D44, whose effects on drug inhibition are not yet understood. We carried out simulations of the D44A, D44C and D44N mutations, and compared them against experimentally measured pH:current profiles. The research paper on these results is to be submitted to the journal Biochemistry.

Below, we include a summary of each of the four research papers mentioned above.
Proton affinity of the histidine-tryptophan cluster motif from the influenza A virus from ab initio molecular dynamics

Arindam Bankura, Michael L. Klein, Vincenzo Carnevale,
Institute for Computational Molecular Science, Temple University, Philadelphia, Pennsylvania 19122, United States

Abstract
Ab initio molecular dynamics calculations have been used to compare and contrast the deprotonation reaction of a histidine residue in aqueous solution with the situation arising in a histidine-tryptophan cluster. The latter is used as a model of the proton storage unit present in the pore of the M2 proton conducting ion channel. We compute potentials of mean force for the dissociation of a proton from the Nδ and N∊ positions of the imidazole group to estimate the pKa’s. Anticipating our results, we will see that the estimated pKa for the first protonation event of the M2 channel is in good agreement with experimental estimates. Surprisingly, despite the fact that the histidine is partially desolvated in the M2 channel, the affinity for protons is similar to that of a histidine in aqueous solution. Importantly, the electrostatic environment provided by the indoles is responsible for the stabilization of the charged imidazolium.

The full article is accessible at:
http://dx.doi.org/10.1016/j.chemphys.2013.03.006

Multiple configurations for the proton-conducting groups of the M2 channel from the influenza A virus

Hao Dong, Giacomo Fiorin, Michael L. Klein
Institute for Computational Molecular Science, Temple University, 1900 North 12th Street, Philadelphia, Pennsylvania 19122, United States

Abstract
The M2 proton channel is a homo-tetrameric proton channel embedded in the membrane of the influenza A virus. Its conduction of protons is regulated by pH, reaching peak rates just below pH 7. The four His37 amino acids located in the transmembrane domain are essential in the mechanism of conduction, and therefore the object of intensive structural investigations. Despite strong similarities in the structures of the protein’s backbone, the His37 tetrad has been observed at neutral pH to adopt two distinct configurations, labeled the “histidine-box” and the “dimer-of-dimers”. The two configurations suggest competing models, but could also cover instead complementary roles in a unified conduction mechanism. We used molecular dynamics (MD) simulations based on density functional theory (DFT) to simulate the channel at sub-nanosecond time scales, and a classical treatment to cover sub-microsecond time scales and to provide a rough estimate for the energy barriers beyond that. Both configurations are stable, and there are no indications that the system could be kinetically trapped in either one. We also estimated the energy required to remove one proton from either configuration: in both cases, this energy is too high for spontaneous
proton conduction, consistently with their proposed occurrence at the low-current regime. These data suggest a multi-configuration model, which can reconnect experimental data obtained at different conditions.

The diffusion of protons (H\(^+\)) through water solutions is a ubiquitous phenomenon with unique characteristics among those involving atomic ions. Few examples exist of controlled proton diffusion around neutral pH conditions, one of which comes from biology: the M2 protein from the influenza A virus is a tetrameric channel embedded in the viral membrane, conducting protons from the exterior of a viral particle into its lumen.\(^1,\,\!^2\) The conduction is modulated by the presence of a lower pH level at the exterior: after entering a host cell, a viral particle acidifies and thereby releases its viral genetic material into the host cell. Therefore, significant efforts are devoted to understand the mechanism of proton conduction through a lipid membrane.\(^3\,\,^5\)

Extensive experimental\(^6\,\,^14\) and computational\(^15\,\,^20\) work has been devoted to study the structure of the tetrameric M2 channel protein. Not surprisingly, the structure of its backbone is sensitive to pH, and an allosteric mechanism was hypothesized to model pH regulation.\(^8\,\,^10\,\,^20\) At the hinge of such mechanism are the functionally essential histidine amino acids, required for pH-regulated conduction of protons and other cations\(^21\,\,^23\) When histidine is present in the M2 amino acid sequence at position 37 (His37), the conduction rate is highly non-linear with the proton concentration, suggesting that the two mechanisms of gating and conduction are intimately connected.\(^21\,\,^22\) To understand both mechanisms, knowledge of the structural ensemble of the His37 tetrad is paramount.

One of the first atomic-resolution experiments specifically aimed at the His37 tetrad measured the magnetic chemical shifts of their nitrogen atoms with solid-state nuclear magnetic resonance (SSNMR) spectroscopy. Based on the magnitude of the shifts, a “dimer-of-dimers” arrangement was proposed for the four imidazole groups of His37,\(^6\) in which one charged histidine forms a strong hydrogen bond (HB) with an adjacent neutral histidine. Based on this hypothesis the structure of the His37 tetrad was further refined by additional SSNMR measurements on the protein backbone and by quantum mechanical calculations (PDB entry: 2L0J).\(^14\) Instead, a high resolution X-ray crystallographic structure (PDB entry: 3LBW, 1.65 Å) showed a tightly packed “histidine-box” structure,\(^10\) with the four imidazoles forming HBs with adjacent water molecules rather than with each other.\(^6\,\,^14\) Extensive experimental work emerged to support either the “dimer-of-dimers”\(^24\,\,^26\) or the “histidine-box”\(^24\,\,^29\) configuration, suggesting that the two are mutually exclusive. However, it is possible that the two coexist at neutral pH, and that their relative equilibrium is finely tuned by experimental conditions such as pH, temperature, and the composition of the viral membrane mimic.

We tested this hypothesis by using methods spanning multiple time scales. Firstly, we studied the stability of the two alternative configurations, performing molecular dynamics (MD) simulations of the protein embedded in fully hydrated phospholipid bilayers. To accurately model the structure of the proton-binding chemical groups, we used a quantum mechanical / molecular mechanical (QM/MM) methodology, explicitly calculating the electronic structure of His37, the adjacent Trp41, as well as the neighboring water molecules. We suggest that at fixed protonation, both configurations represent stable states of the M2 channel. Secondly, we calculated the energy required to remove a proton from His37 to the C-terminal side, and found that the deprotonation at a 2+ charged state is energetically
unfavorable in both models. Finally, we estimated an upper bound (up to 10 kcal/mol) to the energy barriers for the transition between the two models, using MD simulations with enhanced sampling. Based on the results shown below, we propose that the protein populates an ensemble of multiple configurations, which can account for both the structural and the thermodynamic measurements.

To model the equilibrium structure of the protein backbone and the distribution of water molecules, we embedded the high-resolution X-ray structure\textsuperscript{10} in a hydrated lipid bilayer and performed two long MD simulations with the His37 and Trp41 constrained in either configuration. We then extracted two uncorrelated snapshots from each trajectory, and used them to initialize two QM/MM MD simulations (four in total). For each simulation we then accumulated \( \sim 25 \) ps of time (100 ps in total). Within the time explored, both models appear to represent stable minima for the free energy of the system. The root mean square deviations (RMSDs) of the backbone C\( \alpha \) atoms saturate quickly at 0.6 Å, indicating that the protein backbone had been fully relaxed in the pre-equilibration steps (Fig. 1A). To detect specific transitions of the His37 tetrad between the two configurations, we calculated a principal component \( \lambda \) (see Supporting Information) along the vector connecting the two configurations: \( \lambda = 0 \) represents the “dimer-of-dimers” configuration and \( \lambda = 1 \) the “histidine-box”. During all simulations, \( \lambda \)-values remained stable within 0.1 from their initial values (Fig. 1B). To detect more subtle changes, we then monitored the key structural motifs of either configuration.

Based on chemical shift measurements, in the “dimer-of-dimers” model each pair of histidines was proposed to form a low barrier hydrogen bond (LBHB),\textsuperscript{6} with the shared proton freely moving between the donor and the acceptor.\textsuperscript{30} Our simulations confirm the short bond length of the “dimer-of-dimers” model, \( \sim 2.75 \) Å, and explicitly show multiple proton “jumps” between the donor and the acceptor nitrogens (Fig. 2). Although the proton was treated as a classical particle, a high rate of jumps strongly indicates a rather delocalized wave function.\textsuperscript{31} We did not observe a strong preference for either the N\( \delta \) position (\( \pi \) tautomer) or the N\( \varepsilon \) position (\( \tau \) tautomer): the relative occupancies are 39\% and 61\%, respectively. This suggests that the structure of the histidine cluster tunes the p\( K_a \)-s of the two sites to be approximately equal, providing optimal conditions to form LBHBs. Furthermore, the LBHBs appear explicitly to be robust structural motifs: even though one LBHB pair temporarily broke in one of the simulations, it quickly restored in \( \sim 2 \) ps.

In the “histidine-box” configuration, despite the tight packing there are no direct HBs between the histidines: instead, all histidines form HBs with the adjacent clusters of water molecules,\textsuperscript{10} and the overall histidine-water HB network is well conserved also in our simulations. The distribution of water molecules fits well to the electron density determined in the X-ray structure.\textsuperscript{10} A few HB distances changed with respect to the X-ray structure: the two outermost waters of the “entry cluster”, separated by \( \sim 2.6 \) Å in the X-ray structure,\textsuperscript{10} featured an average O…O distance of \( \sim 2.8 \) Å during our simulations. The N\( \delta \)1…O distance between histidines and water is \( \sim 2.8 \) Å for all four histidines in the X-ray structure: in our simulations, one charged histidine reduced its HB distance to \( \sim 2.7 \) Å, while the remaining three increased to 3.0-3.1 Å. HB distances with the two waters at the N\( \varepsilon \)2 positions did not differ from the X-ray structure beyond the observed fluctuations. However, the magnitude of such fluctuations is larger than that of the six waters at the N\( \delta \)1 positions, a fact consistent with the \( ^{15} \text{N}-^{1} \text{H} \) dipolar couplings measured by SSNMR.\textsuperscript{12}
Finally, the neighboring Trp41 side chains are another structural motif for both configurations, as shown by the high resolution structures, and Raman spectroscopy. At pH < 6, when 3 or 4 histidines are thought to be charged, a cation-π interaction has been suggested between each charged histidine and the indole of the neighboring Trp41. At the 2+ charge state here studied, such cation-π interactions are obviously weaker, due to charge delocalization. Nevertheless, these are preserved during our simulations: in the “dimer-of-dimers”, the distance and angle between the histidine’s N-H and the indole remain at their equilibrium values; in the “histidine-box”... These may play a role in stabilizing the rotameric state of Trp41, which is the “tp” rotamer (χ₁ = 180°, χ₂ = −90°) in the “dimer-of-dimers”, and the “tm” rotamer (χ₁ = 180°, χ₂ = 90°) in the “histidine-box”. If additional protons bind to the histidines, cation-π interactions can become prominent in both models, reaching the threshold to be detected by Raman spectroscopy.

Because the presence of water through the channel is the prerequisite for proton conduction, we monitored the water distribution and pore size during all simulations. In both configurations, two constriction regions could be identified along the channel’s pore (Fig. 3): one is lined by His37 and Trp41, responsible for gating and conduction. The other constriction region is hydrophobic, formed by Val27 at the outer end of the channel, and shown in previous simulations to partially hinder the water flow through the channel. While both regions are narrow in either configuration, the “dimer-of-dimers” configuration features a wider hydrophobic portion (1.6 Å) and a narrower hydrophilic portion (0.9 Å): the “histidine-box” has instead 1.0 Å and 1.3 Å, respectively. This alternation is reminiscent of the allosteric mechanism of proton conduction proposed for the M2 channel, where pH regulates the dynamical equilibrium between two states, labeled “open-out-closed-in” and “closed-out-open-in”.

Having not observed spontaneous loss of protons, we then proceeded to calculate deprotonation energies in either configuration. To this end we computed the difference between the final structure of the QM/MM MD simulations, and a structure where a proton was removed from the Nε2 position of one charged histidine and bound to the adjacent water molecule to form a hydronium ion (H₃O⁺). We then optimized both structures, for each configuration. The energy difference in the “dimer-of-dimers” reaches ~40 kcal/mol, which compares well with the estimated LBHB formation energy of ~31 kcal/mol in gas phase. In the “histidine-box” configuration, the calculated energy difference between the two is ~12 kcal/mol, which is high enough to prevent fast spontaneous release of protons. The relatively high energies are most likely to attribute to the presence of only two acceptor molecules: when one of them is transformed into a H₃O⁺, the lack of a complete solvation shell destabilizes the product. These results indicate that the proton release, bringing the histidine tetrad from a 2+ to a 1+ charge state, is a kinetically unfavorable event, if not thermodynamically. This is supported by the first and second pKa constants of the His37 tetrad, which were both estimated at 8.2 by SSNMR, compared to a third pKa of 6.3. Therefore, the M2 channel at neutral pH is considered to be at the resting, non-conducting state. Finally, the calculated energy differences explicitly show the ability of M2 to confine excess charge in a low dielectric environment such as the lipid membrane: the delocalization of charge throughout the proton-binding groups enhances this ability.

Having established that both the “dimer-of-dimers” and the “histidine-box” are stable configurations of the system, we finally tried to evaluate the time scale of conversion between the two. A direct computation of a kinetic constant is currently not possible, because
the large collections of states associated to the protein backbone and to the water distribution are greatly under-sampled by sub-nanosecond and probably even sub-microsecond MD simulations. This task is further complicated by a predictably small free energy difference between the two configurations. Therefore, we performed an approximate calculation only to evaluate the order of magnitude of the energies involved, using the classical CHARMM27 force field\textsuperscript{37}, a metadynamics biasing potential acting on the two RMSD functions of the His37 and Trp41 tetrads, respectively. Based on these calculations, we estimate that the highest energy required to transition between the “dimer-of-dimers” and the “histidine-box” configurations is about 10 kcal/mol. Due to the classical force field approach, this number does not account for the LBHBs, characteristic of the “dimer-of-dimers” configuration, and therefore it is probably overestimated. In between the two configurations, we did not observe significant energy barriers: this is consistent with a putative transition pathway composed only by individual ring flips, rather than one concerted motion. Based on this assumption, we predict that the time scale of conversion between the “dimer-of-dimers” and the “histidine-box” is larger than or equal to the time of a ring flip, measured by SSNMR to be approximately microseconds.\textsuperscript{12} Due to the enhanced sampling protocol, we were able to access some high-energy configurations (Fig. 4).

The distribution of the $\chi_1$ and $\chi_2$ angles of His37 is shown in Fig. 4. In both charged and neutral His37 the bulk of the values of the $\chi_1$ angle are around 180º, the value observed in all experimental structures.\textsuperscript{7,8,10,11,14} The value of $\chi_2$ is more widely distributed: in our simulation, by effect of the biasing potential it spans reversibly multiple values throughout the entire 360º period. In particular, $\chi_2$ covers values characteristic of both the “dimer-of-dimers” and “histidine-box” configurations with comparable frequencies. In addition, we also observed values characteristic of the low pH structures,\textsuperscript{8} which feature an expanded protein backbone: however, the protein backbone was held restrained during our simulations (RMSD < 1.5 Å). This suggests that the His37 can sample both the neutral pH configurations, and even the low pH one, with limited backbone expansion and relatively low free energy cost.

**Conclusion**

In summary, in present work, we investigated the conformational stability, the deprotonation energy and the transformation of two competing models for the His37 tetrad: the “histidine-box” and the “dimer-of-dimers”. Both were found to be stable in sub-nanosecond time scale by QM/MM MD simulations, and their unique characteristics are well reproduced. The proton release from His37 to a water molecule on the intraviral side is energetically unfavorable in both models, consistently with the low current at neutral pH conditions. The energy cost associated with converting between the two configurations is comparable to that of individual ring flips. Based on the above data, we proposed a multi-configuration model, in which both the “dimer-of-dimers” and the “histidine-box” configurations can coexist at neutral pH, but have different probabilities of proton uptake or release. This conclusion sets the grounds for a highly detailed description of the proton conduction mechanism.

**ACKNOWLEDGMENTS**

This work was supported by the National Institute of Health through grant U01-AI-74571 (PI: William F. DeGrado, UCSF), the Commonwealth of Pennsylvania Health Research Formula Fund, and by the National Science Foundation through XSEDE allocation TG-MCA93S020.
ASSOCIATED CONTENT
Additional computational details, and figures on the hydrogen bond distance, the water density, the side chain
dihedral angles, and the 2D free energy profile, are available free of charge at http://pubs.acs.org.

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Author Contributions
§Contributed equally to this work

REFERENCES


(29) Hong, M.; Fritzsching, K. J.; Williams, J. K. Journal of the American Chemical Society 2012, 134, 14753.


FIGURES:
Fig 1. Two His37 configurations in the M2 protein at the 2+ state and their stabilities. (a) the “dimer-of-dimers” configuration (PDB entry: 2L0J); (b) the “histidine-box” configuration (PDB entry: 3LBW); (c) RMSDs of the backbone Ca atoms in the simulations. In all systems, the RMSDs are less than 0.6 Å, indicating the limited fluctuation of the overall structure; (d) Stability of the His37 sidechain. In both (c) and (d), two parallel simulations of the “dimer-of-dimers” model are colored in red and green, and two parallel simulations of the “histidine-box” model are in blue and pink.
Fig 2. Correlation between the hydrogen bond distance and the population of proton in the two parallel “dimer-of-dimers” model simulations. Inset graph shows the frequent jump of proton within a dimer in the “dimer-of-dimers” model by monitoring the hydrogen bonding involved Nd-H (red) and Ne-H (green) distances.

Fig 3. Pore size of the two configurations. (a) Averaged radius of the pore in the “dimer-of-dimers” (red line) and the “histidine-box” (blue line) models. The pore dimension of the “dimer-of-dimers” model (b) and the “histidine-box” model (c) were generated by the HOLE program. The backbone is shown in silver ribbon, and side chains of Val27, His37 and Trp41 are shown in orange, yellow and magenta, respectively, in stick mode.
**Fig 4.** $\chi_1$-$\chi_2$ angles in the enhanced sampling simulation for the charged histidines. “H-box” indicates the “histidine-box” structure, “d-o-d” the “dimer-of-dimers”, “low-pH” the low pH configuration.

**Probing the deprotonation mechanism of the His37 tetrad in the M2 channel from the influenza A virus via molecular simulation**

Hao Dong, Michael L. Klein, Giacomo Fiorin
Institute for Computational Molecular Science, Temple University, 1900 North 12th Street, Philadelphia, Pennsylvania 19122, United States

The activity of the M2 proton channel of the influenza A virus is controlled by pH. The configuration of His37, a key residue in the M2 transmembrane four-helix bundle, determines the gating mechanism of the channel. We previously compared the energetics and dynamics of two configurations of the doubly protonated state, namely a four-fold symmetric histidine box and a two-fold symmetric dimer-of-dimers, and proposed a multi-configuration model. Here, we further study configurations of the His37 tetrad and its subsequent deprotonation in the triply protonated state via QMMM/MD simulations, starting with the aforementioned configurations. Protons transfer from one charged His37 to a neighboring water cluster at the C-terminal side of the channel. With limited backbone expansion, for both configurations, the free energy barriers for proton release to the viral interior at low pH are much lower than their counterparts at the neutral pH. The effects of counterions on the deprotonation are discussed.

(The manuscript is currently in preparation: results presented in the 2013 American Chemical Society meeting in New Orleans by Hao Dong.)
Functional Model for the Dependence of Activation and Rectification of the M2 Ion Channel of Influenza Virus upon a Residue at the C-terminus of the Transmembrane

Chunlong Ma, Giacomo Fiorin, Vincenzo Carnevale, Robert A. Lamb, Michael L. Klein, William F. DeGrado, and Lawrence H. Pinto

Channel gating and proton conductance of the influenza A M2 channel result from complex pH-dependent interactions involving the pore-lining residues at position 37, 41, and 44. Channels bearing different residues at position 44 (D44 in the Udorn strain) show significantly different conductances. Here we investigate the role of this residue by relating physiological measurements performed on three different subtypes of the influenza A virus (Udorn, Weybridge and Rostock) and on several synthetic mutants with the results from molecular dynamics simulations. We observed that mutations D44N, D44A, and D44C, which show a larger conductance than D44, induce a conformational change to the structure of M2 similar to that produced by low pH conditions. As a result, the release of protons to the virion interior is facilitated. Moreover, these mutants show a reverse flow of protons similar to that observed in W41F. Our calculations show that mutations at position 44 are able to affect the structural stability of the hydrophobic seal resulting from the interacting indole groups of W41. We conclude that the chemical nature of a single residue at position 44 is able to determine two important properties of the M2 proton channel.

The H37xxxW41 motif (or HxxxW motif) of the M2 proton channel pore is essential for its gating and proton selectivity [1-3]. Accordingly, H37 and W41 are the only pore-lining amino acids conserved among the strains of influenza A virus [4], and synthetic mutants of either residue are non-functional. Mutations of the pore-lining residues 27 and 31 confer resistance against inhibitors [5], but only moderately affect the channel’s proton conductance [6]. Mutation of G34, one helical turn from H37, produces either a non-functional channel, or one very similar to the WT protein [7]. Finally, mutations of D44 produce the only functional channels with markedly different conduction properties from the WT channel (Udorn strain), and are found in several other strains of the influenza A virus, such as the Weybridge and Rostock strains. Therefore, key to the design of new multi-strain inhibitors is to model the structures of M2 carrying any or all of the naturally occurring mutations. Recent studies showed that mutation of D44 to other residues resulted in a proton-selective amantadine-sensitive channel with enhanced conductance in the physiological pH range [7, 8], suggesting that D44 interact with the HxxxW motif to regulate both the pH dependence and channel gating. Many high-resolution structures of M2TM have been published in recent years [9-16] showing D44 interacting with the HxxxW motif, either directly via a hydrogen bond [14] or indirectly via a small cluster of water molecules [9]. Together with collaborator Lawrence Pinto at Northwestern University, we performed MD simulations and physiological measurements on both the WT channel and various D44 mutants. The results presented here are also discussed in a research article, to be submitted for publication.

Electrophysiology experiments by Pinto and coworkers showed that synthetic mutants with the D44A and D44C mutations which feature a higher conductance in the physiological pH range (5-8) than M2 proteins possessing D44. However, when the pH was lowered below 5, saturation of proton current was observed at higher pH than the WT for each mutated protein.
We then modeled and analyzed the structures of the three mutants D44A, D44C, D44N, and
R45C of Udorn A/M2 by molecular dynamics (MD) simulations in a hydrated lipid bilayer.
The protein functional unit was a tetramer of the transmembrane helix region of A/M2
(M2TM - residues 25–46). We initialized the WT protein at four different protonation states
of the H37 tetrad (+1, +2, +3, +4), and the three mutants D44A, D44C, and D44N at the +2
state. All proteins were initialized using the 1.65 Å resolution X-ray structure of the M2TM
[9]. We observed that mutations from D44 induce a conformational change to a structure of
M2TM which is very similar to the open structure induced at low pH conditions [9, 14, 16,
17]. The conformational change was observed within tens of nanoseconds (Figure 1), the
same time scale at which we previously observed pH-induced conformational changes [9,
17]. To quantify the magnitude of the conformational compared to that induced by pH, we
computed a principal component, $p$, defined as the backbone structure projected on the
transition vector between the high pH and low pH structures. Values of the principal
component $p$ range from 0 at the high pH / intermediate pH backbone structure [9, 14] to 1 at
the low pH backbone structure [16]. We observed (Figure 2) that all three D44 mutations
(D44A, D44C and the naturally occurring D44N) induce conformational changes that are
close in magnitude ($p$ ranging from 0.6 to 0.9) to that induced by setting a +4 total charge on
the H37 side chains ($p \approx 1$).

Figure 1. Root mean square deviations (RMSDs) of M2TM from the initial X-ray structure
[9], plotted as a function of time and computed for different subset of atoms: all heavy atoms
except for the mutated side chain at position 44 (blue), all backbone atoms (black), all heavy
atoms of the H37 (orange) and W41 side chains (purple).
Figure 2. Structural models of the three mutants of M2TM (D44A, D44C and D44N), showed in the upper part of the graph, compared to the intermediate pH [9] and low pH structures [16] of WT-M2TM, shown at left and right sides. The protein backbone is drawn as ribbons, while the pore-lining side chains (residues 27, 31, 34, 37, 41 and 44) and the carbonyl groups interacting with water molecules are represented as sticks. The density of water, averaged over each MD trajectory, is shown as a purple transparent diagram. Points indicate average simulated values of the principal component \( p \) in the direction between the two experimental structures; error bars indicate standard deviations. Water densities drawn within the two WT-M2TM structures were calculated at the +2 and +4 charge states, respectively. All simulations were initialized at the intermediate pH structure [9].

Our results have produced a molecular rationale for the most important consequence of the D44 mutations, which is an alteration in the saturation behavior of the channel at low pH. At pH under pH 5, all four H37 side chains are protonated, according to the current estimates of the pK_a, and all mutants of M2TM feature a similar structure, which is closed at the outward-facing end of the channel. In this situation, proton uptake may become the rate-limiting step, as a conformational transition toward the intermediate pH structure [9] is required for protons to diffuse to the H37 residues. Based on our analysis, WT-M2 may retain a higher fraction of intermediate pH structure than its D44 mutants for values of pH below which proton conduction occurs. This could account for the saturation of the synthetic mutants at about pH 5. This saturation is also seen in naturally occurring strains (Weybridge and Rostock) bearing the D44N mutation.

Remarkably, the effects of the D44 mutations on the structure and dynamics of the HxxxW motif are similar to those induced by protonation of the histidines from the +2 to the +3 or +4 charged states. However, the mutation does not cause a conformational change of the backbone as large as that induced by a change from high to low pH_out. Therefore the amino acid at position 44 does not appear to interact with the HxxxW motif simply by tuning the bundle structure, but rather via interactions mediated by pore waters. These pore-bound water molecules participate in regulating gating and conductance [1-3].
These results are crucial to the development of new inhibitors: because the equilibrium is shifted towards the low pH structure [16] in strains carrying mutations of D44, a broad spectrum inhibitor must either stabilize the intermediate pH structure, or be able to inhibit the low pH structure, as amantadine does against the WT channel. Furthermore, our computational protocol has proven successful in explaining the phenotypes associated with naturally occurring mutations, and provides an essential tool in the rational design of influenza inhibitors.

References

(The manuscript is in preparation, and will be submitted to Biochemistry within weeks).

18. **Extent of Clinical Activities Initiated and Completed.** Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be “No.”

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

- [ ] Yes
- [x] No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

- [ ] Yes
- [x] No

If “Yes” to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both “No.”)

18(C) How many hospital and health care professionals were involved in the research project?

______Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

______Number of subjects originally targeted to be included in the study
______Number of subjects enrolled in the study

**Note:** Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?
Gender:
_____ Males
_____ Females
_____ Unknown

Ethnicity:
_____ Latinos or Hispanics
_____ Not Latinos or Hispanics
_____ Unknown

Race:
_____ American Indian or Alaska Native
_____ Asian
_____ Blacks or African American
_____ Native Hawaiian or Other Pacific Islander
_____ White
_____ Other, specify: ____________________________
_____ Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____ Yes
X No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____ Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. Include only those publications that acknowledge the
Pennsylvania Department of Health as a funding source (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the “Cognition and MRI in Older Adults” research project (Project 1), and two publications for PI Zhang for the “Lung Cancer” research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

<table>
<thead>
<tr>
<th>Title of Journal Article:</th>
<th>Authors:</th>
<th>Name of Peer-reviewed Publication:</th>
<th>Month and Year Submitted:</th>
<th>Publication Status (check appropriate box below):</th>
</tr>
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<tr>
<td>1. Proton affinity of the histidine-tryptophan cluster motif from the influenza A virus from ab initio molecular dynamics</td>
<td>Arindam Bankura, Michael L. Klein, Vincenzo Carnevale</td>
<td>Chemical Physics</td>
<td>November 2012</td>
<td>☐Submitted ☐Accepted ■Published</td>
</tr>
</tbody>
</table>

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____ X_____ No________

If yes, please describe your plans:

(See also section 17) After the first article accepted in Chemical Physics, three more manuscripts are being prepared for submission. Two are at an advanced stage of preparation: the first deals with the inactive state of the M2 channel, is being submitted to Journal of Physical Chemistry Letters, and is included entirely within section 17 of this report. A second one, dealing with mutations of the M2 channel, is to be submitted to Biochemistry: a long summary is included in section 17. An additional manuscript is currently in preparation for
the active state of the channel (short summary included).

**21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project.**
Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert “None”; do not use “Not applicable.” Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

Our work impacts the prediction of resistance to new drugs. We calculated the structure of the target protein and of several of its mutants, including the protons that are very commonly hidden to experimental approaches, but fundamental in the viral infection mechanism. The calculated structures of the M2 channel and of its mutants can be used by our lab and by other computational scientists to predict the efficacy of new drugs.

**22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment.** Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert “None”; do not use “Not applicable.” Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

Our computational studies developed a quantitative model for the functional role of three amino acids of the sequence of the M2 proton channel of the influenza A virus. The first and most important is the histidine amino acid at position 37, responsible for pH-activated conduction of protons: we calculated the ensemble of structures of this amino acid at different steps of the proton conduction mechanism. We also rationalized the role of the tryptophan at position 41 and the aspartate at position 44, the latter being asparagine in several viral strains. We calculated the structure of the protein for each of the different strains, laying the groundwork for predicting potential resistance to newly developed drugs.

**23. Inventions, Patents and Commercial Development Opportunities.**

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes ________ No __X__

If “Yes” to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is “No.”)

a. Title of Invention:
b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?  
Yes_______ No______

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?  
Yes_______ No______

If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant?  
Yes_______ No______

If yes, how many licenses were granted?____________

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale?  
Yes_______ No______

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?  
Yes_______ No______ X____

If yes, please describe your plans:
Three manuscripts
24. **Key Investigator Qualifications.** Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*
Michael L. Klein, FRS
Laura H. Carnell Professor & Dean, College of Science & Technology, Temple University

Director, Institute for Computational Molecular Science,
Bio-Life Building, Suite 113; 1900 North 12th Street, Philadelphia PA 19122
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Born: London, UK; Education: B.Sc. Ph.D. (Chemistry) University of Bristol, UK

Post-doctoral Positions
CIBA-Geigy Research Fellow (Physics) University of Genoa Italy
ICI Research Fellow (Chemistry) University of Bristol UK
Research Associate (Physics) Rutgers University NJ USA

Employment
National Research Council of Canada, Ottawa, Canada 1968-1987
Associate, Senior, Principal Research Officer

University of Pennsylvania 1987-2009

Temple University 2009-present
Laura H Carnell Professor of Science; Director, Institute for Computational Molecular Science
Dean, College of Science & Technology 2013-present

Research: Pioneer in the development and application of computer simulation to understand the properties of molecular systems; research that has had broad impact in the physical and life sciences.

Mentoring: More than 100 post-doctoral and graduate students who have gone on to successful careers in academe, industry and national labs around the world, including the USA, Canada, UK, France, Germany, Italy, India, and Japan. Publications: 630+ articles; Hirsch Index = 87, with ~ 42,000 citations, growing ~3,000/year; 2 Patents Companies: Co-Founder with William F. DeGrado and others of two startup drug discovery companies (PolyMedix; InfluMedix)

Science Advisory Boards: National Labs/Universities in USA, Canada, Japan, Taiwan, India, UK, Germany, Netherlands, UAE Editorial Boards: Served more than 20 research journals; Currently: Proceedings of National Academy of Sciences, Soft Matter, Accounts of Chemical Research, Computational Materials Science, Physics Reports, Computational Science & Discovery, Chemical Theory & Computation.


Research Awards: from the American Chemical Society, American Physical Society; European Physical Society; Royal Society of Chemistry UK; Chemical Research Society of India.

Brief Biography:

Michael L. Klein received his B.Sc. and Ph.D. degrees in Chemistry from the University of Bristol UK. He was a postdoctoral fellow in Italy, U.K., and U.S.A. before joining the Chemistry Division of the NRCC in Ottawa, Canada, where he rose through the ranks from Associate to Principal Research Officer. In 1987, he returned to the United States as Professor of Chemistry at the University of Pennsylvania, where from 1993 he was the Hepburn Professor of Physical Science and Director of the Laboratory for Research on the Structure of Matter. In 2009, he moved to Temple University as the Laura H. Carnell Professor of Science and Director of the Institute for Computational Molecular Science. His research interests involve probing the structure and dynamics of macromolecular systems using computer simulation techniques; topics range from physical chemistry to soft matter, biophysics and chemical biology.

MICHAEL L. KLEIN CITATIONS (Google Scholar Citations)

http://scholar.google.com/citations?user=56ar4-cAAAAJ&hl=en
Total Citations = 47,000 Hirsch Index h = 89

- **Comparison of simple potential functions for simulating liquid water** WL Jorgensen, J Chandrasekhar, JD Madura, RW Impey, ML Klein
- **Nosé–Hoover chains: the canonical ensemble via continuous dynamics** GJ Martyna, ML Klein, M Tuckerman
  The Journal of Chemical Physics 97, 2635 (1992) Cited 1,349 times
- **Constant pressure molecular dynamics algorithms**
  GJ Martyna, DJ Tobias, ML Klein
  The Journal of Chemical Physics 101, 4177 (1994) Cited 1,044 times
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  Molecular Physics 87 (5), 1117-1157 (1996) Cited 967 times
- **Constant pressure molecular dynamics for molecular systems**
  S Nose, ML Klein