Peer Review File

Manuscript Title: Prestin's Conformational Cycle Underlies Outer Hair Cell Electromotility

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

This manuscript by Bavi and colleagues describes the structure of the outer-hair-cell motor protein, Prestin, in six different states using cryoEM. These include structures in the presence of chloride or sulfate anions as well as in the salicylate-inhibited state. The mutagenesis analysis of TM6 and the electrostatics calculations the support a molecular mechanism linking anion and membrane potential conditions to the Prestin conformations. Overall, by comparing the structures, they infer a series of voltage- and ligand-induced conformational changes that they show would provide associated changes to the lipid bilayer that can largely explain the electromotility of outer hair cells. Overall, this is a very exciting study, connecting the behavior of the Prestin motor protein, its lipid environment, chemical ligands, and a physical stimulus (membrane potential), to a cellular behavior. The data are strong and generally well presented, and support the conclusions put forth by the authors.

The manuscript has several similarities to a recent publication in Cell from the Gouaux group (Ge et al., 2021, Cell 184, 1–11). There are some differences between the two studies. For example, in the chloride-based structure, density for chloride is resolved in the structure from the Gouaux, but not in the structures obtained by the Perozo group. The Perozo study includes interesting observations and results about the glycine-rich TM6 helix playing a role in influencing the shape of the lipid bilayer as it also changes conformation in the different Prestin states. The salicylate-bound structure is inferred to be expanded in the Gouaux study, but somewhat intermediate here. Yet the structures from both groups are similar, and the general mechanism proposed are gratifyingly largely in agreement.

One difference in interpretation is that the Gouaux group favors a largely intrinsic voltage sensor mechanism, whereas the Perozo group favors a combination of intrinsic and extrinsic (bound anion) sensor. In that respect, I found the analysis of electrostatics and charge transfer (Ext Fig 8) quite interesting and helpful in understanding the proposed mechanism. It also distinguishes their work from the recently published Gouaux study, providing a more complete understanding of how a combination of intrinsic elements and the anions can combine to sense and respond to the membrane potential. The authors should consider moving part of this figure into one of the main figures.

In the first two paragraphs of the section starting at the bottom of p. 2, the authors make some inferences about the temporal order of the different conformations that they observed. The language should be carefully edited to make explicit what is known and what is inferred. For example, "an Intermediate conformation preceding the Up state" – the data do not demonstrate "preceding", this is inferred (which is described a bit more clearly at the beginning of the next paragraph). So, the temporal ordering is a model (that does make a lot of sense) put forth by the authors. Similarly, "This inability to complete a canonical transport cycle rationalizes…" – the authors do not observe a complete cycle, but do not provide foolproof evidence that it does not, cannot exist. This could be better explained.

Technical comments:

In the validation reports for the "Inhibited I", "Down I", and "Down II" structures, the "Map-Model Overlay" and "Atom inclusion" sections of the validation reports are clearly problematic. However, the maps and coordinate files provided by the authors look ok; but they should make sure that all PDB and map submissions are accurately completed.

The manuscript should include a table of data and refinement statistics for the 6 structures.

Minor comments:
Abstract, line 25: "by immobilizing locking" – the authors likely intended only one of these two verbs.
p. 2 line 23: it would be useful to include the % similarity or % identity to human Prestin.
p. 2 line 26: the second sentence of the paragra, describing Fig 1a, is missing references.
p. 3 line 43: "this density" – the "this" has no prior reference; what density do the authors mean? (Presumably the salicylate density?)
p. 4 line 42: I don't think the reference to Fig. 3b is correct?
Ext Fig 3: in part (a), are residues 460-505 of one or both subunits aligned?
Ext Fig 5: Please explain what Δz is in the heat maps in part (b).
The authors should carefully check the manuscript throughout for typos, including the figure legends. A few I noted:
"ATCC" is misspelled twice in the "Cell lines" section of the methods.
p. 7 line 15: "diameter" is misspelled.
p. 7 line 22: "wild-type" is misspelled.

Referee #2 (Remarks to the Author):

In the present manuscript, combining structural determination and functional characterizations, Perozo and colleagues have determined the cryo-EM structures of dolphin Prestin in six distinct states, allowing them to analyze in detail the coupling between structural rearrangements of the proposed voltage sensor and the change of protein-membrane interface, which provides important molecular insights into the unique electromotility property of Prestin. Overall, the study has been well designed and the data and analysis is of high quality. In fact, the structural information is highly consistent to an online paper describing the structural studies of human Prestin (Ge et al., Cell 2021). By comparing the present study with the published one, one discrepant interpretation concerns the voltage sensor. In the present study, the authors suggest that the anion together with its coordinating fixed charges acts as a dynamic voltage sensor. R399 is a key voltage-sensing residue. In contrast, Ge et al. have argued that the bound chloride ion does not function as extrinsic voltage sensor (R399 does not directly interact with the bound chloride ion), instead multiple charged residues distributed at the intracellular and extracellular membrane interfaces might serve as voltage sensor. Given the discrepancy, it will be important to clarity the nature of the bona fide voltage sensor. One potential experiment for the author to consider is to determine the structure of a R399-mutated prestin. If R399 indeed serves as the voltage-sensing residue, its neutralization might lead to a down-state (expanded state) even in the presence of chloride.

Referee #3 (Remarks to the Author):

This manuscript reports the structure of Dolphin Prestin in six distinct states using single particle cryo-EM. Different states are described that are tunable by anions and salicylate completes with the anion binding site to inhibit prestin locking it in a novel conformation.

This report coincides with the publication this month of another manuscript by Ge et al. Cell, Sept 02, 2021, which reports the structure of human prestin via cryo-EM analysis. Similar findings are reported with similar cryo-EM structure and different states associated with binding of anions and salicylate.

This manuscript differs in that it includes more functional data and an analysis of evolutionary conserved regions that play an important role in Prestin function in mammals. For example, different glycine residues are identified that are conserved across all vertebrates and are located at the hinge site of TM6. Such feature was not picked up by the recently published manuscript and they reveal another important region of the protein. To determine the importance of these residues in Prestin function, Bali et al. evaluate the function of different mutants by analyzing the

non-linear capacitance associated with each prestin. Additionally, the study from Bali et al. provides important insights regarding prestin voltage sensor.

Overall, this report offers a complementary view of the structure of prestin and a better understanding of the structural transitions that are associated with somatic motility. It is largely in agreement with the recent report from Ge et al. and as such reinforces the models proposed in both studies.

Comments:

1 -It is unclear why the authors choose to work on the Dolphin prestin instead of the human or mouse prestin for example. Please clarify.
2- Page 2, ln 44: the concentrations are mistakenly reported here. From the methods section, it appears that the experiment involves replacing externally ~144 mM Cl- with 10 mM Cl-/ 115mM Na2SO42- and internally ~144mM Cl- with 10mM Cl/ 130mM Na2SO42-. Also, it is unclear why TEA is used for the Cl based experiments but absent for the sulfate-based measurements.
3-Page 3, Ln 39: Please confirm the chloride concentration for salicylate experiments as it seems unusually high: "[360mM] Cl- or SO2- [120mM]" (also in methods).
4- The supplemental video showing electromotility in HEK cell is not very helpful. It's hard for the viewer to identify the "large-voltage driven movements" described in the text (Page 2, ln 27)
5- Several typos are noted in the supplemental document. Please review. Mm instead of mM; "Wilde" instead of "wild"… etc
6- Page 2, ln 26 … for other mammalian systems. Add references.
7- Page 2, ln 24. Define NLC (first time it is used)
8- Page 2, ln 25. Confirm the unit for the voltage sensitivity. Shouldn't this be dQ/ dV?
8- Fig 1 Legend ".Cell movement is b. Electromotility" : Remove "Cell movement"

Author Rebuttals to Initial Comments:

Reviewer #1;

The authors should consider moving part of this figure into one of the main figures.

Response:

We completely agree and appreciate the Reviewer's suggestion. In fact, we tried to include parts of these calculations in the main text in consultation with the nature Editor. However, we are already over the journal limit both in terms of number of figures and word counts.

In the first two paragraphs of the section starting at the bottom of p. 2, the authors make some inferences about the temporal order of the different conformations that they observed. The language should be carefully edited to make explicit what is known and what is inferred. For example, "an Intermediate conformation preceding the Up state" – the data do not demonstrate "preceding", this is inferred (which is described a bit more clearly at the beginning of the next paragraph). So, the temporal ordering is a model (that does make a lot of sense) put forth by the authors. Similarly, "This inability to complete a canonical transport cycle rationalizes…" – the authors do not observe a complete cycle, but do not provide foolproof evidence that it does not, cannot exist. This could be better explained.

Response:

We fully agree with the reviewer. We have now rephrased the text accordingly to:

"Even when the Prestin voltage sensor is its most compact (sensor Up) conformation, the anion-binding site is still not exposed to the extracellular side, preventing release of anions to the extracellular space. While it is possible that even more compact states might be discovered, we speculate that Prestin is unable to reach a truly "outward-facing" conformation (in a canonical elevator cycle). This hypothesis rationalizes why mammalian Prestin does not efficiently transport anions, unlike other members of the SLC26 family."

Technical comments:

In the validation reports for the "Inhibited I", "Down I", and "Down II" structures, the "Map-Model Overlay" and "Atom inclusion" sections of the validation reports are clearly problematic. However, the maps and coordinate files provided by the authors look ok; but they should make sure that all PDB and map submissions are accurately completed.

Response:

We thank the reviewer for their eagle-eyed comment. We have now solved that issue, as evident in the new validation reports updated in the previously shared folder:

The manuscript should include a table of data and refinement statistics for the 6 structures.

Response:

Added.

Minor comments:

Abstract, line 25: "by immobilizing locking" – the authors likely intended only one of these two verbs.

Response:

Corrected.

p. 2 line 23: it would be useful to include the % similarity or % identity to human Prestin.

Response:

Added to page 2, Line 24 : "Dolphin Prestin has ~ 94 % identity (~97% similarity) to human Prestin."

p. 2 line 26: the second sentence of the paragra, describing Fig 1a, is missing references.

Response:

The references have been added now.

p. 3 line 43: “this density” – the “this” has no prior reference; what density do the authors mean? (Presumably the salicylate density?)

Response:

Corrected, “ … salicylate density helps confirm the overall nature …).

p. 4 line 42: I don’t think the reference to Fig. 3b is correct?

Response:

Removed.

Ext Fig 3: in part (a), are residues 460-505 of one or both subunits aligned?

Response:

Only one subunit. Now added to the figure legend.

Ext Fig 5: Please explain what Δz is in the heat maps in part (b).

Response:

Now it has been defined and added to the figure legend.

The authors should carefully check the manuscript throughout for typos, including the figure legends. A few I noted:“ATCC” is misspelled twice in the “Cell lines” section of the methods.

Response:

We proofread the manuscript and corrected all the typos.

p. 7 line 15: “diameter” is misspelled.

Response:

Corrected.

p. 7 line 22: “wild-type” is misspelled.

Response:

Corrected.

Reviewer #2;

One potential experiment for the author to consider is to determine the structure of a R399-mutated prestin. If R399 indeed serves as the voltage-sensing residue, its neutralization might lead to a down-state (expanded state) even in the presence of chloride.

Response:

This is, of course, an excellent idea. However, we are unable to include this additional structure in the present manuscript for several reasons: 1- While we and other groups have functional information on a few R399 mutants, the biochemistry will need to be developed from the scratch, with no guarantees that it will lead to a structure in the short term. 2- We know that this mutation (R399Q) is not voltage sensitive, but the thermodynamic state that this mutation resides in is currently unclear. Therefore, it isn't clear that a single structure will fully address the questions regarding the composite nature of the voltage sensor, as the reviewer suggests, since these structures will likely need to be carried out in different anions as well to reach reliable conclusions. Whether or not this mutation renders Prestin insensitive to anion type will require additional effort that is beyond the scope of the present manuscript. The next chapters in this long-term investigation of electromotility are forthcoming, for sure, but they will require an additional commitment of effort and (especially) time.

Reviewer #3;

1 -It is unclear why the authors choose to work on the Dolphin prestin instead of the human or mouse prestin for example. Please clarify.

Response:

This is now been clarified in our method section, page 1 of the Extended Data;

"To access a biochemically stable Prestin preparation suitable for single particle cryo-EM, a variety of orthologs were explored. We hypothesized that those adapted to detect high-frequency signals (i.e., bats and whales) might exemplify particularly robust systems. An initial screening by fluorescence-detected size-exclusion chromatography (FSEC) led to the identification of a Prestin candidate from the bottlenose dolphin (Tursiops truncatus)."

Response: We have also compared the identity to of dolphin Prestin to human Prestin, as requested by reviewer #1. Page 2, Line 24 : "Dolphin Prestin has ~ 94 % identity (~97% similarity) to human Prestin."

2- Page 2, ln 44: the concentrations are mistakenly reported here. From the methods section, it appears that the experiment involves replacing externally ~144 mM Cl- with 10 mM Cl-/

115mM Na2SO42- and internally ~144mM Cl- with 10mM Cl/ 130mM Na2SO42-. Also, it is unclear why TEA is used for the Cl based experiments but absent for the sulfate-based measurements.

Response:

We thank the reviewer for noticing this typo. Indeed the concentration of SO_4^{2-} in both side are similar. We corrected this to 115 mM (SO_4^{2-}) in both part in the method section. We did not use TEA in the Sulphate-based buffer because we did not observe as much leak currents due to K+ channels as we see when used Cl-.

3-Page 3, Ln 39: Please confirm the chloride concentration for salicylate experiments as it seems unusually high: "[360mM] Cl- or SO2- [120mM]" (also in methods).

Response:

This is correct. It is known that Prestin's affinity for Cl^- is low. We used high Cl^- concentrations only in in our purifications (not in patch-clamp experiments), to make sure that we have the most compact (sensor up) state during structure determination. This has now been further clarified in the methods.

4- The supplemental video showing electromotility in HEK cell is not very helpful. It's hard for the viewer to identify the "large-voltage driven movements" described in the text (Page 2, ln 27).

Response:

We have now included subtitles to make the movie clear.

5- Several typos are noted in the supplemental document. Please review. Mm instead of mM; "Wilde" instead of "wild"… etc

Response:

Thank you. We have now further proofread the Extended data and have corrected those typos.

6- Page 2, ln 26 … for other mammalian systems. Add references.

Response:

Added.

7- Page 2, ln 24. Define NLC (first time it is used).

Response:

Defined.

8- Page 2, ln 25. Confirm the unit for the voltage sensitivity. Shouldn't this be dQ/ dV?

Response:

Confirmed, [1/mV] is correct. And to avoid any confusion, we have now included this in our methods as well.

8- Fig 1 Legend ".Cell movement is b. Electromotility" : Remove "Cell movement".

Response:

Removed.