

Peer Review File

Structural basis of adenine nucleotides regulation and neurodegenerative pathology in ClC-3 exchanger



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

CIC-3 is a chloride/proton exchanger that functions in intracellular acidic organelles. Mutations of endosomal CIC-3 result in severe neurodegeneration, exemplifying its physiological importance. The authors determined cryo-EM structures of apo CIC-3 and CIC-3 bound to different nucleotides, presumably acting as potentiators or antagonists, and of a CIC-3 mutant. However, essentially no conformational changes can be observed between the apo and ligand-bound structures that might account for the functional effects of nucleotides that the authors and others reported. In general, no rigorous experimental support is provided for the conclusions made and the interpretation of the structural data is not well founded. Therefore, no fundamental mechanistic insights could be gained despite the cryo-EM structures presented.

Line 52: My understanding from the citation is that CIC-3-5 activity is modulated by cytosolic adenine nucleotides but there is no indication that they are activated by ATP. Please reword.

Fig. 1l, m, q: For the comparison of whole-cell current magnitudes, please quantify the expression and surface expression level of the investigated constructs with an internal loading control.

All whole-cell recordings: Please normalize the current magnitude to the membrane capacitance (pA/pF).

Line 116-119, 128: The steady-state current is a function of open probability, conductance, and expression level. An increase in the current size alone cannot distinguish between these three possibilities. Please reword accordingly and take this into account in the interpretation.

Fig. 2a-d: Please provide the representative time courses for the current increase.

Line 142: Please change the description 'bona fide agonist' as the data merely suggest a modulation of CIC-3 activity by nucleotides.

Fig. 2: Please provide a closeup densities of the described ATP binding site directly comparing the apo and ATP, ADP, and AMP-bound maps superposed with the models. In particular, a panel for the densities of the described ATP binding site of the apo model should be presented. It is important to show that there is no density in the apo map using a similar contour setting and relative to the protein density.

Fig. 2: Please provide a superposition of the backbone of the described ATP binding site directly comparing the conformations of the apo and ATP, ADP, and AMP-bound models, instead of only showing the sidechains and loops. It is important to be able to correlate the observed nucleotide effects with their corresponding structures.

To confirm that AMP, ADP, and AMP-PNP bind to CIC-3 but do not have any effect and to illustrate their approximate affinities, please include a concentration-response relation each for AMP, ADP, and AMP-PNP showing that these nucleotides can antagonise the current increase induced by ATP in a concentration-dependent manner and in a manner consistent with competitive antagonism.

Fig. 2e and p: There is a duplication of the data shown. Please address this.

Extended Data Fig. 8: Please show the raw traces for both the blank immobilization and with CIC-3 immobilization in addition to the corrected traces for panels a, c, and e for all ligand concentrations. Please also show the fitted curves of the SPR traces.

Line 209-210, Fig. 3g and h: I am not sure at this resolution whether the densities would allow any interpretation of the subtle changes in the sidechain orientations shown in Fig. 3g and h. The backbone is essentially unchanged in all cases. Please show the densities for the models to support this argument or rectify this statement/paragraph for correctness.

Fig. 3j-n. Please show the densities for these potential sidechain movements for all models to support the described changes or rectify the corresponding statements/paragraph for correctness.

Line 243, Line 293-294: The authors suggest a potential increase in ion transport by ATP. As mentioned above, an increase in the current size alone cannot distinguish between an effect on open probability or conductance even in the same patch/cell. Please provide direct experimental evidence for an increase in ion transport or reword accordingly and take this into account in the interpretation.

Fig. 4c and d: Please include data showing that the effect of pH 5.0 on I607T, especially on the appearance of the inward current, is reversible upon switching back to pH 7.3.

Line 292-294: The described affinity is based on SPR with very small signals and the raw data for both the background and with CIC-3 immobilization are not available. I am not sure if the available data allow the authors to make any conclusions other than that the observed signals are probably due to some affinity process that happens to be in the same range. Please reword and acknowledge the limitation of the currently presented data.

Line 292-294: The reported structural data do not show at all any conformational changes between the apo, ATP, ADP, and AMP-bound models and thus do not provide any mechanistic insights into the described effects. Please reword and acknowledge the limitation of the currently presented data.

Abstract: Please remove the description of conformational changes concerning nucleotide regulation as there is essentially no rearrangement at the level of the protein backbone amongst the presented models. Please also reword ion occupancy changes since the occupancy of the ions did not change.

Electrophysiology methods: Please include the series resistance and compensation, and state whether these are consistent between all comparisons that were made.

Reviewer #2 (Remarks to the Author):

In this manuscript Wan et al use cryoEM, electrophysiology and MD simulations to

investigate the structure of CLC-3 and its regulation by adenosine nucleotides. They find that ATP, ADP and AMP bind to a conserved site in the CBS domain. Their structures show that only ATP can interact with residues in the N- and C-terminal regions, suggesting these interactions are important for transducing the signal from the CBS domains to the transmembrane region. Notably, the three structures show that CLC-3 undergoes ATP-dependent local conformational changes within the Cl⁻ and H⁺ pathways as well as global rearrangements near the dimer interface. Indeed, a disease causing mutation, I607T, causes and rearrangement where the two protomers move towards each other to give rise to a more compact dimer architecture.

Overall, this is an excellent manuscript. The experiments are carefully planned, well executed and interpreted. The results provide new insights into the function and regulation of the CLC-3 transporters. I think the manuscript is a strong candidate for publication in Nature Communications, and have only minor suggestions.

Fig 1 Is it possible to color code the CAVAR profiles in panels i-j, n-o to directly visualize the areas with constructions? In the current version, it seems the pore is wide enough to allow Cl⁻ passage throughout, but the superposition in j and o shows this is not the case.

How do you know the density shown in Fig 1o is a water and not a Cl⁻ ion? The argument that mutating the nearby residues impairs current is weak, as the mutations could impair Cl⁻ transport rather than H⁺ transport, or gating. This should be discussed.

Fig. 1-2, would it be possible to report currents as I-V curves (as shown in i.e. Fig. 4) rather than ramps? For proteins with voltage dependent kinetics ramps are less informative on the mechanistic origin of the effects (i.e. slower activation kinetics or reduced currents would give similar phenotypes in the ramp currents but be distinguishable in the I-Vs).

Does the I607T mutation cause a rotation of the protomers as seen in the recently reported CLC-7 conformation (<https://doi.org/10.52396/JUSTC-2022-0172>)? Or is it just a contraction? In either case, it would be important to add a figure and discuss what happens to the ion permeation pathway and occupancy in I607T. Since this is a gain of function mutant, the structures could represent some conformations towards an activated state.

Minor

Line 63, sever should be severe

Reviewer #3 (Remarks to the Author):

The authors have determined the atomistic structures of CLC-3 and the disease-causing mutant in their apo-state, as well as in complex with ATP, ADP, and AMP. These findings constitute a valuable addition to the existing structure series of ClC channels. However, before the manuscript can be accepted, I have the following comments and concerns:

1. The authors hypothesized that the electrostatic interactions between the negatively charged gamma phosphate in ATP and the positively charged K798 and H89 residues are critical for ATP activation of ClC-3. Have the authors mutated the positively charged K798 and H89 residues to neutral ones? If so, what impact did these mutations have on ATP activation?

2. The authors have conducted MD simulations of mCLC-3 with ATP and have shown a stable binding pose. Have the authors also performed simulations with ADP and AMP? Do

these simulations suggest different binding poses, and do they exhibit variations in their interaction patterns and flexibility in the binding?

3. Furthermore, the main text describing the dynamic network analysis is quite concise and may be difficult to understand in detail. The authors claim that they observed a mutual information pathway starting from the ATP binding pocket in the CBS domains to the vicinity of Cl⁻ ions observed in cryo-EM. This observation is indeed intriguing, but did the authors attempt to mutate the key residues within this pathway? Additionally, have the authors conducted control simulations on mCIC-3 in complex with ADP and AMP, where the same pathway was either not observed or disrupted? Without this analysis, the validity of the MD simulations may not be sufficient.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

ClC-3 is a chloride/proton exchanger that functions in intracellular acidic organelles. Mutations of endosomal ClC-3 result in severe neurodegeneration, exemplifying its physiological importance. The authors determined cryo-EM structures of apo ClC-3 and ClC-3 bound to different nucleotides, presumably acting as potentiators or antagonists, and of a ClC-3 mutant. However, essentially no conformational changes can be observed between the apo and ligand-bound structures that might account for the functional effects of nucleotides that the authors and others reported. In general, no rigorous experimental support is provided for the conclusions made and the interpretation of the structural data is not well founded. Therefore, no fundamental mechanistic insights could be gained despite the cryo-EM structures presented.

Line 52: My understanding from the citation is that ClC-3-5 activity is modulated by cytosolic adenine nucleotides but there is no indication that they are activated by ATP. Please reword.

We have modified the main text as the reviewer suggested (line 58). Now we write “ClC-3, together with ClC-4 and ClC-5, have been proposed as metabolic energy sensors as they are differentially modulated by ATP, ADP or AMP”. Potentiation by ATP are used to replace ATP activation in the revised manuscript.

Fig. 1l, m, q: For the comparison of whole-cell current magnitudes, please quantify the expression and surface expression level of the investigated constructs with an internal loading control.

We have performed additional experiments to quantify the expression and surface expression level of the ClC-3 mutants. We observed that the overall expression levels of the mutants K245A, E339A and E631A were reduced as compared to that of WT (Extended Data Fig. 3e). However, with the surface biotinylation assay, we observed that the expression levels of these mutants on cell surface were at least comparable to that of the WT ClC-3 (Extended Data Fig. 3e). The surface expression levels of E339A and E631A mutants, which exhibited much reduced current density (Fig. 1q), were even higher than that of WT (Extended Data Fig. 3e), indicating these point mutations disrupted the function of ClC-3.

Moreover, we performed fluorescence co-localization assay to further examine the expression profile of these mutants. All the point mutants, which were fused with GFP as in patch-clamp recordings, exhibited clear co-localization with the cell membrane labeled with the fluorescent membrane dye DiD, indicating that they were expressed on the cell membrane (Extended Data Fig. 3f).

All whole-cell recordings: Please normalize the current magnitude to the membrane capacitance (pA/pF).

We have normalized the current magnitude to the membrane capacitance as the reviewer

suggested in the revised figures, such as Fig. 1l, 1m and 1q.

Line 116-119, 128: The steady-state current is a function of open probability, conductance, and expression level. An increase in the current size alone cannot distinguish between these three possibilities. Please reword accordingly and take this into account in the interpretation.

We agree with the reviewer and modified the text by adding “However, as the steady-state current level is a function of three factors (open probability, conductance, and expression level), the changes in current ratio we observed in these mutants here and mutants later reported in this study may be caused by any or a combination of these three factors.” (line 136 to 139).

Fig. 2a-d: Please provide the representative time courses for the current increase.

We have added the representative time courses for the current increase in Extended Data Fig. 5 as the reviewer suggested.

Line 142: Please change the description ‘bona fide agonist’ as the data merely suggest a modulation of CIC-3 activity by nucleotides.

We have replaced “bona fide agonist” with “potentiator” (line 167).

Fig. 2: Please provide a closeup densities of the described ATP binding site directly comparing the apo and ATP, ADP, and AMP-bound maps superposed with the models. In particular, a panel for the densities of the described ATP binding site of the apo model should be presented. It is important to show that there is no density in the apo map using a similar contour setting and relative to the protein density.

We have added the Extended Data Fig. 8a to 8e to show the closeup views of densities of the described ATP binding site to directly compare the apo and ATP, ADP, and AMP-bound maps superposed with the models. We contoured all the maps shown in this figure to the level of 0.243. No density is observed in the apo state map but the density of ATP is clearly visible (Extended Data Fig. 8a and 8b).

Fig. 2: Please provide a superposition of the backbone of the described ATP binding site directly comparing the conformations of the apo and ATP, ADP, and AMP-bound models, instead of only showing the sidechains and loops. It is important to be able to correlate the observed nucleotide effects with their corresponding structures.

We have provided the superposition of the backbone of the described ATP binding site in the revised Fig. 2k, 2n and 2o as the reviewer suggested.

To confirm that AMP, ADP, and AMP-PNP bind to CIC-3 but do not have any effect and to illustrate their approximate affinities, please include a concentration-response relation each for AMP, ADP, and AMP-PNP showing that these nucleotides can antagonise the current increase

induced by ATP in a concentration-dependent manner and in a manner consistent with competitive antagonism.

We have added more data in the Fig. 2p to show that these molecules can antagonize ATP-potentiated current in a concentration-dependent manner. For instance, 1 mM AMP barely reduced ATP-potentiated current, but 5 mM or 10 mM significantly reduced the current.

Fig. 2e and p: There is a duplication of the data shown. Please address this.

We have modified Fig. 2e and 2p to avoid duplication of data. Now Fig. 2e shows current potentiation by ATP and ATPys, but not by ADP or AMP; Fig. 2p shows the antagonization of ATP potentiation.

Extended Data Fig. 8: Please show the raw traces for both the blank immobilization and with CIC-3 immobilization in addition to the corrected traces for panels a, c, and e for all ligand concentrations. Please also show the fitted curves of the SPR traces.

We have added more panels (Extended Data Fig. 8g to 8l) to show the raw traces for both the blank immobilization and with CIC-3 immobilization. As we measured the SPR signal from the steady state level of responses to calculate the binding affinities, where high concentrations of ligands exhibited saturated response amplitude, no kinetics measurements (therefore no fittings of the response kinetics) were performed.

Line 209-210, Fig. 3g and h: I am not sure at this resolution whether the densities would allow any interpretation of the subtle changes in the sidechain orientations shown in Fig. 3g and h. The backbone is essentially unchanged in all cases. Please show the densities for the models to support this argument or rectify this statement/paragraph for correctness.

We have added more panels in Extended Data Fig. 8f and 8g to show the density of sidechain in S239, S453, Y630, K281 and E282 in the apo and ATP bound state contoured at the same level of 0.243.

We agree with the reviewer that the conformational changes in these sidechains are subtle, so that we have modified the text accordingly (line 238), for instance, we acknowledge that “such as K281, E282, S239, S453 and Y630, showed slightly altered sidechain orientation”. We also removed description on the distance changes in residue pairs.

Fig. 3j-n. Please show the densities for these potential sidechain movements for all models to support the described changes or rectify the corresponding statements/paragraph for correctness.

We have added more panels in Extended Data Fig. 8h to 8m to show the density of sidechain in K230, R418 and K521 in the apo and ATP or AMP bound state contoured at the same level of 0.118; the density of sidechain in K245, M589, E339 and E631 in the apo and ATP or AMP bound state contoured at the same level of 0.166.

We can clearly observe that the sidechain in K230 and E521 showed conformational changes in apo, AMP or ATP bound state (Extended Data Fig. 8h to 8j). Sidechain in E631 also showed clear conformational changes (Extended Data Fig. 8k to 8m). For other residues, the changes in sidechain orientation are less prominent. We have modified the text accordingly.

Line 243, Line 293-294: The authors suggest a potential increase in ion transport by ATP. As mentioned above, an increase in the current size alone cannot distinguish between an effect on open probability or conductance even in the same patch/cell. Please provide direct experimental evidence for an increase in ion transport or reword accordingly and take this into account in the interpretation.

We agree with the reviewer and modified the text by adding “Nevertheless, the changes in Cl⁻ ion transport we observed in these mutants here and mutants later reported in this study may be caused by any or a combination of changes in open probability, conductance, and expression level of CIC-3.” (line 252 to 254). In discussion, we also added “However, the increase in Cl⁻ ion transport by ATP may be caused by any or a combination of changes in open probability, conductance, and expression level of CIC-3.” (line 331 to 333).

Fig. 4c and d: Please include data showing that the effect of pH 5.0 on I607T, especially on the appearance of the inward current, is reversible upon switching back to pH 7.3.

We have added Extended Data Fig. 16 to show that on I607T, the appearance of the inward current induced by pH 5.0 is reversible upon switching back to pH 7.3, while the current of WT CIC-3 was not potentiated by pH 5.0 (line 278 to 279).

Line 292-294: The described affinity is based on SPR with very small signals and the raw data for both the background and with CIC-3 immobilization are not available. I am not sure if the available data allow the authors to make any conclusions other than that the observed signals are probably due to some affinity process that happens to be in the same range. Please reword and acknowledge the limitation of the currently presented data.

We intend to clarify that as ATP, ADP and AMP are small molecules with the molecular weight much smaller than that of CIC-3 protein, the value of Response Unit measured from SPR is expected to be smaller than the cases where two proteins with similar molecular weight interact in SPR. For instance, in our previous study of the small molecule tanshinone binding to the EZH2 protein, the Response Unit values were also less than 10 (Huang Y, Yu S H, Zhen W X, et al. Tanshinone I, a new EZH2 inhibitor restricts normal and malignant hematopoiesis through upregulation of MMP9 and ABCG2. *Theranostics*. 2021, 11 (14) : 6891-6904).

Nevertheless, we agree with the reviewer that the currently presented data is indeed small in response amplitude, so that we have added more discussion to acknowledge such a limitation (“The binding affinity measurements were based on the relatively small response signal from the SPR experiments, so that such a limitation should be taken into the interpretation of differences in

binding affinities of ATP, ADP or AMP”, line 333 to 336).

Line 292-294: The reported structural data do not show at all any conformational changes between the apo, ATP, ADP, and AMP-bound models and thus do not provide any mechanistic insights into the described effects. Please reword and acknowledge the limitation of the currently presented data.

We have modified the discussion on conformational changes by first acknowledging the slight conformational changes, and then reworded the text. Now the revised discussion is “Though conformational changes, especially in the backbone of CLC-3 protein, between the apo, ATP, ADP, and AMP-bound state are relatively small, we observed that ATP binds to CLC-3 with a higher affinity and induced changes in ion occupancy between apo and ATP-bound state, which may facilitate the transport of Cl^- and H^+ ions. However, the increase in Cl^- ion transport by ATP may be caused by any or a combination of changes in open probability, conductance, and expression level of CLC-3. The binding affinity measurements were based on the relatively small response signal from the SPR experiments, so that such limitations should be taken into the interpretation of differences in binding affinities of ATP, ADP or AMP.” (line 327 to 336).

Abstract: Please remove the description of conformational changes concerning nucleotide regulation as there is essentially no rearrangement at the level of the protein backbone amongst the presented models. Please also reword ion occupancy changes since the occupancy of the ions did not change.

We have removed the “differentially induced conformational” and reworded ion occupancy changes as the reviewer suggested in the Abstract. The ion occupancy has indeed changed when the apo state and ATP-bound state are compared (Fig. 3d, 3e and 3f), so now we write “we revealed how the adenine nucleotides binds to CLC-3 and changes in ion occupancy between apo and ATP-bound state.” in the Abstract (line 36 to 37).

Electrophysiology methods: Please include the series resistance and compensation, and state whether these are consistent between all comparisons that were made.

The series resistance was about 25 M Ω under our whole-cell patch-clamp settings. We have compensated the series resistance by 60-70% in the PatchMaster software. We used consistent series resistance compensation in all whole-cell recordings. We have added this information to the revised Methods session.

Reviewer #2 (Remarks to the Author):

In this manuscript Wan et al use cryoEM, electrophysiology and MD simulations to investigate the structure of CLC-3 and its regulation by adenosine nucleotides. They find that ATP, ADP and AMP bind to a conserved site in the CBS domain. Their structures show that only ATP can interact with residues in the N- and C-terminal regions, suggesting these interactions are important for transducing the signal from the CBS domains to the transmembrane region. Notably, the three

structures show that CLC-3 undergoes ATP-dependent local conformational changes within the Cl⁻ and H⁺ pathways as well as global rearrangements near the dimer interface. Indeed, a disease causing mutation, I607T, causes a rearrangement where the two protomers move towards each other to give rise to a more compact dimer architecture.

Overall, this is an excellent manuscript. The experiments are carefully planned, well executed and interpreted. The results provide new insights into the function and regulation of the CLC-3 transporters. I think the manuscript is a strong candidate for publication in Nature Communications, and have only minor suggestions.

We appreciate the reviewer's support and suggestions. We have revised the manuscript accordingly.

Fig 1 Is it possible to color code the CAVAR profiles in panels i-j, n-o to directly visualize the areas with constrictions? In the current version, it seems the pore is wide enough to allow Cl⁻ passage throughout, but the superposition in j and o shows this is not the case.

We color coded the CAVAR profiles as the reviewer suggested. Now orange color has been applied for the region where pore radius less than 1.0 Å, blue or yellow have been applied to regions with a radius greater than 1.0 Å for the Cl⁻ or H⁺ pathway, respectively.

How do you know the density shown in Fig 1o is a water and not a Cl⁻ ion? The argument that mutating the nearby residues impairs current is weak, as the mutations could impair Cl⁻ transport rather than H⁺ transport, or gating. This should be discussed.

We agree with the reviewer that based on map only, we cannot definitely claim that the density in Fig. 1o is a water but not a chloride ion. However, as many previous studies have suggested that water molecules are likely to present here (please see Fig. 1A-B and Fig. 2B of <https://www.pnas.org/doi/full/10.1073/pnas.1317890111> ; Fig 6-Fig S1 and Fig. 8A-C of <https://elifesciences.org/articles/53479> ; <https://www.pnas.org/doi/full/10.1073/pnas.2113141118> and Fig. R1 below), we reasoned that most likely this density is a water. As we cannot rule out the possibility of other entities for this density, we have revised the main text accordingly (line 131 to 132).

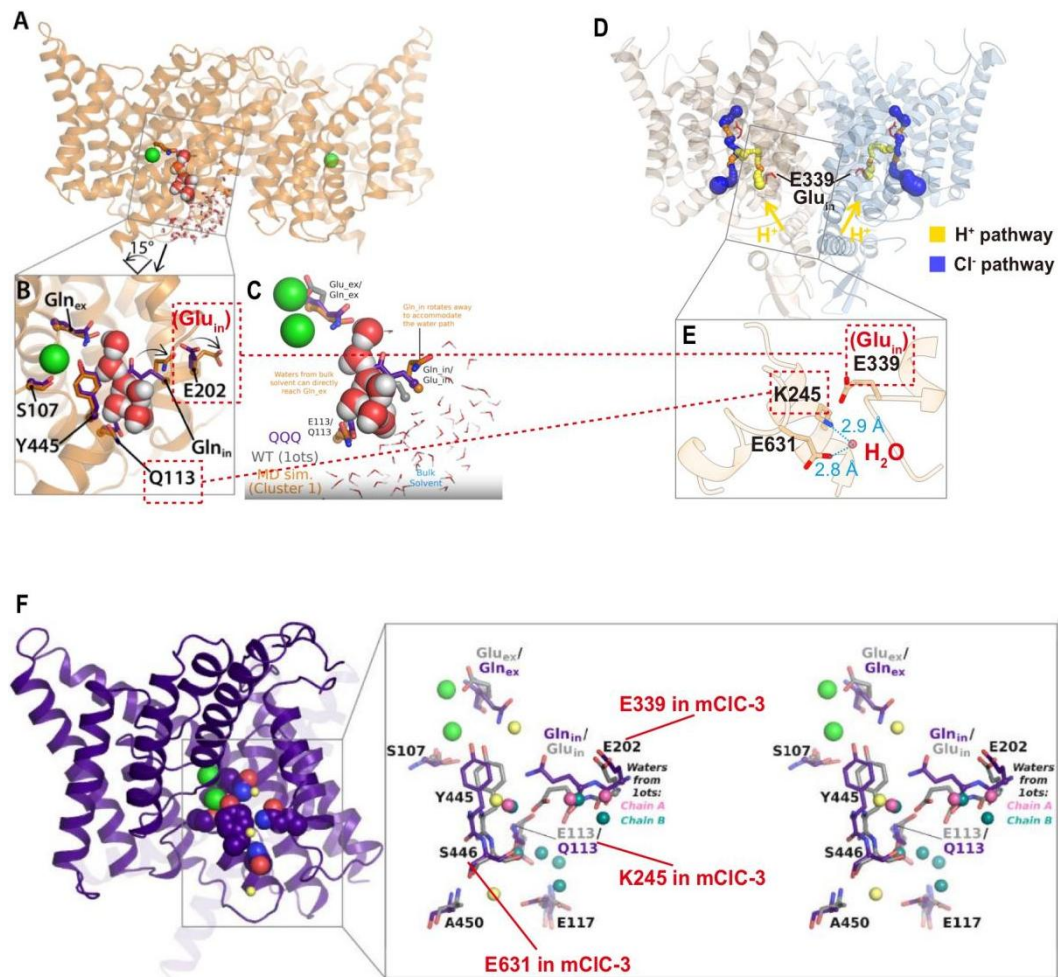


Fig. 1-2, would it be possible to report currents as I-V curves (as shown in i.e. Fig. 4) rather than ramps? For proteins with voltage dependent kinetics ramps are less informative on the mechanistic origin of the effects (i.e. slower activation kinetics or reduced currents would give similar phenotypes in the ramp currents but be distinguishable in the I-Vs).

Following the reviewer's suggestion, we have performed additional patch-clamp recordings with the voltage step protocol as I-V curves (Extended Data Fig. 6). We have observed similar current-voltage relationships in response to ATP, ATP γ s, ADP or AMP as with the voltage ramp (Fig. 2a to 2d).

Does the I607T mutation cause a rotation of the protomers as seen in the recently reported CLC-7 conformation (<https://doi.org/10.52396/JUSTC-2022-0172>)? Or is it just a contraction? In either case, it would be important to add a figure and discuss what happens to the ion permeation pathway and occupancy in I607T. Since this is a gain of function mutant, the structures could represent some conformations towards an activated state.

Upon close inspection as the reviewer suggested, we observed that in the ATP bound state, indeed the I607T mutation caused a counterclockwise rotation as compared to WT when viewed from the side or the top, so we have added one more figure (Extended Data Fig. 19) to present such

conformational changes. No such a rotation was observed in the apo state (Fig. 4e).

We also observed that the chloride ion occupancy was changed in the I607T mutant, as there were chloride ions occupying all the four S_{out} , S_{ext} , S_{cen} and S_{in} binding sites in the permeation pathway in I607T, but in the WT the S_{ext} site was not occupied (Extended Data Fig. 19). Such an increase in ion occupancy is in line with the gain-of-function phenotype of I607T when activated by ATP (Fig. 4b and 4d). We have added more description in the revised manuscript (line 290 to 296).

Minor

Line 63, sever should be severe

We appreciate the reviewer and have corrected this typo in the revised manuscript.

Reviewer #3 (Remarks to the Author):

The authors have determined the atomistic structures of CLC-3 and the disease-causing mutant in their apo-state, as well as in complex with ATP, ADP, and AMP. These findings constitute a valuable addition to the existing structure series of ClC channels. However, before the manuscript can be accepted, I have the following comments and concerns:

1. The authors hypothesized that the electrostatic interactions between the negatively charged gamma phosphate in ATP and the positively charged K798 and H89 residues are critical for ATP activation of ClC-3. Have the authors mutated the positively charged K798 and H89 residues to neutral ones? If so, what impact did these mutations have on ATP activation?

We have mutated the positively charged K798 and H89 to the neutral residues such as Ala, Gly and Leu as the reviewer suggested. As shown in the revised Fig. 2j, these mutations largely reduced ATP induced current as compared to the WT in patch-clamp recordings. Therefore, we believe that observations on these mutations support our hypothesis that the negatively charged gamma phosphate in ATP and the positively charged K798 and H89 residues are critical for ATP activation of ClC-3.

2. The authors have conducted MD simulations of mCLC-3 with ATP and have shown a stable binding pose. Have the authors also performed simulations with ADP and AMP? Do these simulations suggest different binding poses, and do they exhibit variations in their interaction patterns and flexibility in the binding?

We have performed MD simulations with ADP or AMP as the reviewer suggested. Three independent simulation trajectories were performed for each ligand. We observed that though the ensemble plots of ADP or AMP show that these ligands were still bound inside the pocket in the CBS domain (Extended Data Fig. 10d and 10f) with the phosphate groups (colored in orange and red) pointing towards H89 and K798. However, the binding poses of ADP and AMP were less stable than that of ATP as shown in their larger variation in the binding configurations in ensemble plots (Extended Data Fig. 10b, 10d and 10f), which suggests that ADP and AMP may exhibit

variations in the interaction strengths with the pocket in the CBS domain.

3. Furthermore, the main text describing the dynamic network analysis is quite concise and may be difficult to understand in detail. The authors claim that they observed a mutual information pathway starting from the ATP binding pocket in the CBS domains to the vicinity of Cl⁻ ions observed in cryo-EM. This observation is indeed intriguing, but did the authors attempt to mutate the key residues within this pathway? Additionally, have the authors conducted control simulations on mCIC-3 in complex with ADP and AMP, where the same pathway was either not observed or disrupted? Without this analysis, the validity of the MD simulations may not be sufficient.

We have introduced point mutations to key residues on the mutual information pathway near the bounded Cl⁻ ions and ATP binding site, such as E282A and Y630A. These mutations led to significant changes in ion transport and ATP activation of CIC-3 (Fig. 1l and 1m, Fig. 2j, Extended Data Fig. 10g), which supported the mutual information pathway identified from simulation.

The underlying assumption for the mutual information pathway analysis is that the ligand can induce the activation of its receptor, so that this analysis is to find out the most probable pathway. However, as neither ADP nor AMP can activate CIC-3, we cannot assume that these ligands would activate the exchanger and try to find out the mutual information pathway. Therefore, no mutual information pathway analysis was performed for ADP or AMP.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

While the authors have made some changes to the original manuscript, the following comments will have to be addressed, in particular regarding the over-interpretation of the claimed sidechain motions that cannot be unambiguously resolved at the level of the presented cryo-EM densities.

1. Please indicate in all legends the background construct used for the investigated mutations.
2. Fig. 2a-d: The label 'Bath' is misleading, please revise.
3. Fig 3g-h: Please remove Fig. 3g and 3h and the corresponding discussion in the main text. These perceived motions are within the errors of model fitting as shown in the corresponding densities displayed in Fig. S8f and 8g.
4. Fig 3m-o: Again, at the level of cryo-EM densities shown in Fig. S8h-m, there is little conclusion that one can draw regarding the claimed sidechain motions described in Fig. 3m-o, except maybe for E631. Please refrain from drawing conclusions that have no experimental support and remove the speculated motions in Fig. 4p and the corresponding discussion.
5. Given the loss of the density of a part of the putative nucleotide binding site in the apo state, how does the ATP binding site compare to that in the CIC-5 nucleotide-bound cytosolic domain. Please provide a sequence alignment and a superposition of the structures of the described region.
6. Please provide a statistical comparison between the affinities obtained from SPR. In my opinion, the authors should not have used SPR for small molecule analysis given that limitations that the authors themselves have acknowledged. This is important because the authors have based their interpretation on this and then afterwards cited the limitations.

Reviewer #3 (Remarks to the Author):

My previous concerns and comments have been partially addressed by the authors. I appreciate that the authors mutated the positively charged residues K798 and H89, demonstrating their importance in ATP activation. I had also asked whether the authors mutated key residues identified from mutual information pathway analysis. However, they only mutated residues near the Cl⁻ and ATP-binding sites. This is insufficient to robustly validate the proposed pathway. Have the authors also try to mutate other residues within the pathway?

Furthermore, the authors have now also performed simulations of ADP and AMP. They aligned the poses derived from three 300 ns simulations. By only superimposing these poses, they claimed that ATP binds more tightly than ADP and AMP. A more quantitative analysis is needed to enhance the interpretation of the simulation results.

Reviewer #1 (Remarks to the Author):

While the authors have made some changes to the original manuscript, the following comments will have to be addressed, in particular regarding the over-interpretation of the claimed sidechain motions that cannot be unambiguously resolved at the level of the presented cryo-EM densities.

We deeply appreciate the reviewer's constructive comments and have revised the manuscript accordingly as described below:

1. Please indicate in all legends the background construct used for the investigated mutations.

We have added the information on the background construct used for the investigated mutations in all legends.

2. Fig. 2a-d: The label 'Bath' is misleading, please revise.

We have replaced "Bath" with the specific number of concentration of ligands in the extracellular solution.

3. Fig 3g-h: Please remove Fig. 3g and 3h and the corresponding discussion in the main text. These perceived motions are within the errors of model fitting as shown in the corresponding densities displayed in Fig. S8f and 8g.

We agree with the reviewer that the conformational changes in the original Fig. 3g and 3h may not reflect any genuine motions in the sidechains, so that we have removed Fig. 3g and 3h as the reviewer suggested. We have completely removed the description and discussion on these panels in the revised manuscript.

4. Fig 3m-o: Again, at the level of cryo-EM densities shown in Fig. S8h-m, there is little conclusion that one can draw regarding the claimed sidechain motions described in Fig. 3m-o, except maybe for E631. Please refrain from drawing conclusions that have no experimental support and remove the speculated motions in Fig. 4p and the corresponding discussion.

We have removed the sidechain conformation in M589 and K245 from Fig. 3k to 3l. As E339 is the well-known Glu_{in}, we have kept its sidechain shown but refrained from interpretation of conformational changes in E339. Accordingly, in Fig. 4p we have removed the speculated motions in S239, K245, S453, M589 and Y630. Only the well-established Glu_{out} E282, Glu_{in} E339, as well as the K521 and E631 with clear sidechain conformational changes are shown in the revised manuscript.

5. Given the loss of the density of a part of the putative nucleotide binding site in the apo

state, how does the ATP binding site compare to that in the CIC-5 nucleotide-bound cytosolic domain. Please provide a sequence alignment and a superposition of the structures of the described region.

We have added the sequence alignment between CIC-3, CIC-5 and CIC-7 in the ATP binding domain, as well as the structural superposition of these domains in the revised Extended Data Fig. 9. We observed that the ATP binding sites are in general similar in these CIC family members, with the sequence identity being 71.5% between CIC-3 and CIC-5.

6. Please provide a statistical comparison between the affinities obtained from SPR. In my opinion, the authors should not have used SPR for small molecule analysis given that limitations that the authors themselves have acknowledged. This is important because the authors have based their interpretation on this and then afterwards cited the limitations.

We agree with the reviewer so that we have removed SPR results from the revised manuscript. To investigate the binding of ATP, ADP and AMP in another approach, we investigate their binding properties in MD simulations. The RMSD plot of ATP molecule exhibited smaller deviation as compared to that of ADP or AMP (Extended Data Fig. 10g to 10i), which was consistent with the ensemble plots of these ligands (Extended Data Fig. 10b, 10d and 10f), suggesting a tighter binding of ATP. We further performed MM/PBSA calculation in the MD simulation. We observed the calculated binding energy in ATP to CIC-3 was significantly larger than that of AMP (Extended Data Fig. 10j) (line 219 to 225 in the revised manuscript).

Reviewer #3 (Remarks to the Author):

My previous concerns and comments have been partially addressed by the authors. I appreciate that the authors mutated the positively charged residues K798 and H89, demonstrating their importance in ATP activation. I had also asked whether the authors mutated key residues identified from mutual information pathway analysis. However, they only mutated residues near the Cl⁻ and ATP-binding sites. This is insufficient to robustly validate the proposed pathway. Have the authors also try to mutate other residues within the pathway?

We have introduced more point mutations (K245A, P640A, F641A, K777A and L778A, Extended Data Fig. 10k) to the mutual information pathway as the reviewer suggested. We have measured the ATP potentiation in these mutants by patch-clamp recordings, where we observed that the majority of mutations (four out of five) significantly reduced ATP potentiation (Extended Data Fig. 10l). Therefore, our additional mutagenesis study in the revised manuscript further supported the mutual information pathway proposed in the original version. (line 247 to 251 in the revised manuscript).

Furthermore, the authors have now also performed simulations of ADP and AMP. They aligned the poses derived from three 300 ns simulations. By only superimposing these poses, they claimed that ATP binds more tightly than ADP and AMP. A more quantitative analysis is needed to enhance the interpretation of the simulation results.

We have performed quantitative analysis of the MD simulations. The RMSD plot of ATP molecule exhibited smaller deviation as compared to that of ADP or AMP (Extended Data Fig. 10g to 10i), which was consistent with the ensemble plots of these ligands (Extended Data Fig. 10b, 10d and 10f), suggesting a tighter binding of ATP. We further performed MM/PBSA calculation in the MD simulation. We observed the calculated binding energy in ATP to CIC-3 was significantly larger than that of AMP (Extended Data Fig. 10j) (line 219 to 225 in the revised manuscript).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed most of my concerns.

The schematic in Fig. 4p appears to illustrate that K521 occluding the Cl⁻ exit pathway in the apo state is one of the major determinants underlying ATP potentiation. This is not supported by the functional data shown in Fig. 3j where removing the lysine sidechain in K521A does not modify ATP potentiation, as the expectation is that this would result in constitutive potentiation in the absence of ATP if occlusion was a limiting factor in the mechanism. Please revise the schematic.

Minor comment:

Fig. S5: There is an error in the label of the time axis.

Reviewer #3 (Remarks to the Author):

My previous concerns have been satisfactorily addressed by the authors.

Reviewer #1 (Remarks to the Author):

The authors have addressed most of my concerns.

We are very thankful for the support and constructive comments from Reviewer #1 and have revised the manuscript accordingly as described below:

The schematic in Fig. 4p appears to illustrate that K521 occluding the Cl⁻ exit pathway in the apo state is one of the major determinants underlying ATP potentiation. This is not supported by the functional data shown in Fig. 3j where removing the lysine sidechain in K521A does not modify ATP potentiation, as the expectation is that this would result in constitutive potentiation in the absence of ATP if occlusion was a limiting factor in the mechanism. Please revise the schematic.

We agree with the reviewer that the original schematic in Fig. 4p is somewhat misleading, so we made modifications to the schematic as requested by the reviewers. We not only rotated the sidechains of K521 in the apo state so that it does not occlude the Cl⁻ exit pathway, but also moved the sidechain of k521 away from the Cl⁻ ion to only indicate that the sidechain of K521 has rotated between the two states.

Minor comment:

Fig. S5: There is an error in the label of the time axis.

We thank the reviewer for pointing this out. We have corrected it in the manuscript from “ms” to “s” in Fig. S5.

Reviewer #3 (Remarks to the Author):

My previous concerns have been satisfactorily addressed by the authors.

We are very thankful for the support from Reviewer #3.