Optimization of CFTR gating through evolution of its extracellular loops

Marton Simon and László Csanády

Corresponding Author(s): Marton Simon, Semmelweis University

Review Timeline:

Submission Date:	September 15, 2022
Editorial Decision:	October 21, 2022
Revision Received:	December 2, 2022
Editorial Decision:	January 12, 2023
Revision Received:	January 16, 2023

Editor: Joseph Mindell

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1085/jgp.202213264

October 21, 2022

Mr. Marton A. Simon
Semmelweis University
Biochemistry
Tuzolto street 37-47.
Budapest H-1094
Hungary

Re: 202213264

Dear Mr. Simon,

Thank you for submitting your manuscript, entitled "Central role of a conserved serine residue across CFTR molecular evolution" to JGP. Your manuscript has now been seen by 3 reviewers, whose comments are appended below. You will see that the reviewers have raised several concerns that should be addressed prior to further consideration of the manuscript at JGP. In particular, Reviewer # 1 raises important concerns about differences between the electron density and the atomic models used to guide your mutations as well as about the differences between some of your electro physiological results and those in the literature and whether they may stem from incomplete control of phosphylation. Reviewer #2 also raises important questions about the analysis of bursting behavior, which are essential to address.

We would be pleased to receive a suitably revised manuscript that addresses these concerns, which will be re-reviewed, most likely by some or all of the original referees. Based on the scope of the requested changes, we typically anticipate that the revision process will take no longer than 6 months, however, we understand you may need additional time to work on your resubmission to JGP. We therefore ask that you simply keep us informed as to a realistic submission timeline that is appropriate for your particular circumstances. In addition, please do not hesitate to contact me (via the editorial office) if you feel that a discussion of the reviewers' and editors' comments would be helpful.

Please submit your revised manuscript via the link below along with a point-by-point letter that details your responses to the editors' and reviewers' comments, as well as a copy of the text with alterations highlighted (boldfaced or underlined). If the article is eventually accepted, it would include a 'revised date' as well as submitted and accepted dates. If we do not receive the revised manuscript within one year, we will regard the article as having been withdrawn. We would be willing to receive a revision of the manuscript at a later time, but the manuscript will then be treated as a new submission, with a new manuscript number.

Please pay particular attention to recent changes to our instructions to authors in sections: Data presentation, Blinding and randomization and Statistical analysis, under Materials and Methods, as shown here: https://rupress.org/jgp/pages/submission-guidelines#prepare. Re-review will be contingent on inclusion of the required information (including for data added during revision) and demonstration of the experimental reproducibility of the results (i.e., all experimental data verified in at least 2 independent experiments).

Please note, JGP now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts (when applicable). This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. If your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.
Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JGP, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jgp/pages/submission-guidelines#revised

When revising your manuscript, please be sure it is a double-spaced MS Word file and that it includes editable tables, if appropriate.

Please submit your revised manuscript via this link:
Link Not Available

Thank you for the opportunity to consider your manuscript.

Sincerely,

Joseph A. Mindell, M.D., Ph.D.
On behalf of Journal of General Physiology

Journal of General Physiology's mission is to publish mechanistic and quantitative molecular and cellular physiology of the highest quality; to provide a best-in-class author experience; and to nurture future generations of independent researchers.

Reviewer #1 (Comments to the Authors):

In the manuscript by Simon and Csanady, the authors investigated the functional roles of several potential hydrogen-bond pairs in CFTR gating particularly in a condition where ATP hydrolysis is abolished (by mutating the conserved glutamate, E1371 in hCFTR and E1372 in zCFTR). They first inspected the cryo-EM structures of hCFTR and zCFTR in their unphosphorylated as well as phosphorylated ATP-bound forms. By employing mutant cycle analysis with their electrophysiological data, they derived a simplified kinetic model to explain how changes of these hydrogen-bond pairs cause functional improvement of CFTR during evolution from zebrafish to human. This type of study, when carried out properly, can indeed provide exquisite insights into the molecular mechanism of CFTR protein evolution. There are, however, a few major issues that need to be addressed before we can assess the validity of current studies.

One major concern is the use of the modeled structures of zCFTR and hCFTR as the basis of all experimental designs in the current study. The authors should take a close look at the original cryo-EM data (i.e., the electron density map of each structure), instead of totally relying on the derived models for specific amino acid positioning. In the electron density maps of zCFTR and hCFTR, the reviewer noted that the extracellular regions of interest in this work show poorly resolved electron densities for amino acid main/side chain assigning. For example, the authors proposed that in OF-hCFTR the side chain of R117 and the carbonyl oxygen of E1124 can form a hydrogen bond (Fig. 1D), but the density for the side chain of R117 is not accurately assigned based on the density in that region (Model: 6MSM, electron density map: EMD-9230). Another example is in Fig. 1E where the authors claimed there is no hydrogen bonding between R118 and D1132 in zCFTR, but if they examine the original electron density from which the model 5W81 is built (EMD-8782), neither residue shows clear electron density for side-chain assignment. This is exactly why Zhang et al. used alanine for these positions when building their model. This problem is not limited to the above-discussed region. For instance, the paired residues of S109 and N120 (Fig. 3D) may or may not form a hydrogen bond as the model depicted because the electron density around N120 is not even good enough to assign the backbone/main chain of those amino acids, not to mention the side chains (Model: 5W81, electron density map: EMD-8782). Thus, it is crucial for the authors to take all these into consideration if they want to give credence to their claim.

My second major issue is on their electrophysiological data. The relaxation time constant (or the burst duration) estimated for E1371S-hCFTR is ~3-fold shorter than reported previously (Bompadre et al., 2005; Zhou et al., 2006); whereas a longer time constant for E1372S-zCFTR was seen, contradicting a previous study that shows 4-fold shorter relaxation time constant when comparing E1371Q-hCFTR and E1372Q-zCFTR (Yu et al., 2016). These discrepancies cannot be attributed to differences in the expression system especially the time constant for E1371Q-hCFTR is virtually identical when the channel is expressed in oocytes or mammalian cells (Vergani et al., 2005; Yu et al., 2016). This reviewer noted that their experimental protocol entails only one-minute exposure of the patch to PKA and ATP, and hence wondered if this short exposure is sufficient for effective control of the phosphorylation level, which is known a main factor affecting CFTR gating. Therefore, it is important for the authors to show experiments that can effectively eliminate this concern in order to make fair comparison not only with what is in the literature, but also with data for various mutants in the current study. Another major difference noted is the channel behavior within a burst between E1372S-zCFTR in the current study (e.g., Fig. 2C) and E1371Q-hCFTR (Fig. 4B in Zhang et al., 2018). Could this difference also be attributed to difference in phosphorylation?

My third major concern is the inconsistency between reported intraburst Po and the raw current trace. Specifically, the intraburst Po of E1372S-zCFTR was estimated as 0.27 (page 18 and Table 1), but the raw current trace in Fig. 2D cannot be consistent with this value. Furthermore, the current traces in Fig. 4A clearly show a higher activity for zS109A-N120A compared to zE1372S, but the kinetic parameters in Table 1 show otherwise. Accurate numbers are particularly important for both relaxation analysis and intraburst kinetic analysis as they are used for mutant cycle analysis, the results of which are the basis for all their mechanistic interpretations.

Some minor issues:
P3. Class II mutation such as delF508 requires a combination regimen of two correctors and a potentiator to be effective in clinical treatment. Please cite proper references.
P4 (also in page 13). It is perhaps premature to designate Cs state to the solved IF conformation of CFTR. Afterall, this configuration represents a pre-phosphorylated state, while Cs is functionally defined as a post-phosphorylated closed state.
P5. The last part of the first paragraph needs clarification. Are these from original reports? Or the authors actually analyzed the

structures? Besides, all solved OF configurations, human or zebrafish CFTR, fail to show a patent pore. The authors should be more cautious here as well as in page 20 (Fig. 7) in assigning the open state.
P7 last sentence and P10 lower section. The validity of the claims depends on if the burst duration of E1371S-hCFTR is accurately assessed as described above.
P9. Is the difference in current decay between zE1372S and ZR118H statistically significant?
P14. Why do you think converting S108 to alanine can change the backbone carbonyl position?
P17. The single-channel amplitude of hS108A-I119N seems larger. Please verify.

Some minor suggestions:
The authors may want to avoid using the same title for sections in the result and figures.
Overall, the manuscript will also be improved by citing more references especially from others.

Reviewer #2 (Comments to the Authors):

In this manuscript Simon and Csanády investigate the functional role of a serine in an extracellular loop of the CFTR chloride channel, the protein that is mutated in cystic fibrosis. The authors note that two CFTR orthologues with available structural information, hCFTR and zCFTR, display marked functional differences despite overall structural similarities. They identify a network of interactions in the first extracellular loop (ECL) that stabilize different conformations of hCFTR and zCFTR. Specifically, the open state of hCFTR is stabilized by a hydrogen bond formed by R117 whereas in zCFTR the flickery closed state, rather than the open state, is stabilized by a hydrogen bond between the neighboring N120 and S109 residues. Interestingly, the S108A mutation impairs gating of hCFTR in a manner similar to R117H but the effects of the two are not additive, suggesting these residues interact in the open state.

Overall, this is a well-written manuscript that addresses an interesting problem. The results are largely well-grounded in the data.

I have only one major concern, that the authors should clarify or address.
For hCFTR the bursting behavior is clear from the single channel traces, as long-lived bursts can be clearly identified. For zCFTR however, the single channel traces do not reveal clearly defined bursts. Rather, the channels are very flickery and only short lived open states are visible from the traces. While it is possible that this has been addressed in past work, I think it is critical to clearly illustrate and define what is a burst of the last open channel in zCFTR so that a reader can follow the analysis. This is especially important since so much of the analysis reported here critically depends on the quantification of the mean lifetimes and Keq of the bursts in the two constructs. For example, in the trace in Fig2C zE1372S, how do the authors distinguish between the last and the last-but-one bursts?

Related to the point above, in several traces the opening of several channels is visible (e.g. in Fig. 4A zN120A, zS109A-zN120A; in Fig. 5D and 6D hS108A). Wouldn't this affect estimates of Keq, if not of τburst, which is determined from macroscopic currents. How did the authors correct for this?

While I like the evolutionary portion of the discussion, I think it should be shortened and tightened. As it stands, I found it a bit too speculative and teleological.

Reviewer #3 (Comments to the Authors):

The manuscript by Simon and Csanády attempts to understand the role of a specific residue in evolution of function within CFTR, largely by taking advantage of a combination of structural analysis and sequence analysis across evolutionarily distanced orthologs: human and zebrafish. The authors are commended for the scientific aspects of this work and presentation. Scientifically, this manuscript is wonderful. I do have several concerns about the writing. Correcting these defects will enable a greater impact on the field.

1. The Abstract is not written for a general audience, even just within the ion channel field. The very last sentence of the Abstract, I think, is a bit of a major leap, since it implies that all of the differences in gating between these orthologs comes down to a single pocket of residues studied here.

1a. It is misleading to state that the zebrafish and human orthologs represent "two ends of CFTR molecular evolution", in part because the lamprey version of CFTR is evolutionarily much more distant from the human than is the zebrafish.

1b. It also is a bit odd to suggest that these orthologs possess different gating properties "consistent with structural differences" since this implies that you can infer differences in gating from studying the structures. That's not really true.

1c. The "outward-facing" concept is not defined in the Abstract.

Fig. 1. Again, OF is not defined. Please check spelling of "pale".

Page 5, top para. You state that hCFTR is the "current endpoint of CFTR molecular evolution." What makes that the case? What can you state to support this designation?

Page 5, top para. I have no idea what you mean by "the mechanical development" that occurred during CFTR evolution.

Page 6, top para. Given that you will make a comparison to the work of Hwang who studies CFTR in mammalian cells, it would be good to mention very early in this paragraph that you have studied CFTR in Xenopus oocytes.

Page 6, top para. What do you think might be the mechanism underlying the difference between your study and Hwang's RE zCFTR? Please suggest a potential basis for the different observations.

Page 7, top. You say that the B state of zCFTR seems to be rather stable. Compared to what?

Fig. 2 legend. You don't tell us what you mean by "-20/-40 mV" in this case, or similar cases elsewhere in the paper.

Page 9, top. You state that zR118H mutation resulted in "a slower current decay". Are there any statistics to say that this is actually different?

Page 10, middle. You state that because the unitary amplitude of last openings was smaller than 0.3 pA, intraburst kinetics could not be examined reliably. Isn't this even more related to the apparent brevity of openings, rather than the amplitude?

Page 10, bottom, to 11, top. Rather a weird transition. Given the writing in the prior paragraph, why not describe the effect of mutations a hS108, first?

Page 11, second para. More appropriate would be to say that "... as one amino acid OFTEN takes part in multiple interactions,...".

Page 11, bottom. Great paragraph!

Page 21, top. Isn't it also possible that mutations at this site are embryonic lethal?

Page 21, second para. Really a great paragraph.

Page 23, bottom. You state that statistical significance of interaction energies were calculated by t test. But, where are those statistical differences indicated? Literally, none of the figures or Table 1 have any indication of statistically significant differences.

Page 24, top. "Infield" is an incomplete reference.

Fig. S2. Really need to pay attention to appropriate capitalization of genus names and lack of capitalization of species names.

Responses to Reviewers

We thank all three Reviewers for their careful evaluation and insightful comments. We have performed additional experiments to clarify the reason for appararent discrepancies between our study and previous reports (new Fig. 3C-D) and to consolidate the quantitative gating parameters for several of our constructs. These experiments have provided further support for our mechanistic conclusions which remain unchanged. On the other hand, as a result of slight adjustments of the absolute values of some of the rates, fits to the C_s-C_f-O and C_s-O-C_f scheme now provide less convincing bias for the former scheme. Thus, as that issue is peripheral to our main conclusions, we have omitted original Fig. S4 and toned down the discussion of this topic. Finally, we have provided more detailed explanations for several aspects of the work pointed out to be unclear by the Reviewers. Responses to each individual comment are detailed below.

Reviewer #1 (Comments to the Authors):

In the manuscript by Simon and Csanady, the authors investigated the functional roles of several potential hydrogen-bond pairs in CFTR gating particularly in a condition where ATP hydrolysis is abolished (by mutating the conserved glutamate, E1371 in hCFTR and E1372 in zCFTR). They first inspected the cryo-EM structures of hCFTR and zCFTR in their unphosphorylated as well as phosphorylated ATP-bound forms. By employing mutant cycle analysis with their electrophysiological data, they derived a simplified kinetic model to explain how changes of these hydrogen-bond pairs cause functional improvement of CFTR during evolution from zebrafish to human. This type of study, when carried out properly, can indeed provide exquisite insights into the molecular mechanism of CFTR protein evolution. There are, however, a few major issues that need to be addressed before we can assess the validity of current studies.

One major concern is the use of the modeled structures of zCFTR and hCFTR as the basis of all experimental designs in the current study. The authors should take a close look at the original cryo-EM data (i.e., the electron density map of each structure), instead of totally relying on the derived models for specific amino acid positioning. In the electron density maps of zCFTR and hCFTR, the reviewer noted that the extracellular regions of interest in this work show poorly resolved electron densities for amino acid main/side chain assigning. For example, the authors proposed that in OF-hCFTR the side chain of R117 and the carbonyl oxygen of E1124 can form a hydrogen bond (Fig. 1D), but the density for the side chain of R117 is not accurately assigned based on the density in that region (Model: 6MSM, electron density map: EMD-9230).

We agree with the Reviewer. Generally speaking, the electron density of the extracellular regions of all human (6msm, 5uak) and zebrafish (5w81, 5uar) structures are poorly represented. Specifically, for Fig 1D, which depicts the hR117 – E1124 interaction, we have discussed this issue in our previous article (Simon et al., eLife 2021). Based on Fig 1 – Fig Suppl. 1

(Simon et al., eLife 2021), both loops are well represented, nevertheless density for the R117 side chain is visible only down to the delta carbon. Importantly, in the same study the existence and relevance of the hR117-hE1124 H-bond was functionally validated.

Another example is in Fig. 1E where the authors claimed there is no hydrogen bonding between R118 and D1132 in zCFTR, but if they examine the original electron density from which the model 5W81 is built (EMD-8782), neither residue shows clear electron density for side-chain assignment. This is exactly why Zhang et al. used alanine for these positions when building their model.

Indeed, zD1132 and zR118 show no clear electron density in the phosphorylated zebrafish structure. However, the presence of a H bond between them can be excluded simply based on distance arguments. Modelling the arginine side chain using either Pymol or Missense3D, the H-donating amino groups are too far (approximately 8 Å) from any H-accepting group in ECL1. Thus, despite the poor density of zR118 and zD1132, it is highly unlikely that a H bond should be present in the state captured by the cryo-EM structure.

This problem is not limited to the above-discussed region. For instance, the paired residues of S109 and N120 (Fig. 3D) may or may not form a hydrogen bond as the model depicted because the electron density around N120 is not even good enough to assign the backbone/main chain of those amino acids, not to mention the side chains (Model: 5W81, electron density map: EMD-8782). Thus, it is crucial for the authors to take all these into consideration if they want to give credence to their claim.

We understand the Reviewer's concern, and apologize if our wording was unclear. The resolution of these extracellular regions is indeed poor in both electrondensity maps (EMD-9230, EMD-8782). Moreover, even in well resolved protein regions charged side chains typically produce poor cryo-EM signals. For these reasons, the modeled side-chain interactions depicted in Fig. 4D must be interpreted with caution. However, that does not mean that inspection of these structures should not be used as a starting point for identifying potential functionally relevant state-dependent interactions. The aim of functional studies like those presented here is exactly to validate/disprove the existence and functional relevance of such putative interactions using rigorous thermodynamic measurements. That is what has been done here, and the results of our thermodynamic mutant cycles provide functional evidence for the putative interactions suggested by the structural models. We have clarified these issues in both Results (p10 bottom - p11 top) and Discussion (p15 bottom - p16 top).

My second major issue is on their electrophysiological data. The relaxation time constant (or the burst duration) estimated for E1371S-hCFTR is ~3-fold shorter than reported previously (Bompadre et al., 2005; Zhou et al., 2006);

Our mean burst duration (τ_{burst}) for hE1371S correlates well with previous studies performed under identical conditions, i.e., measuring current relaxation of pre-phosphorylated hE1371S channels following exposure to ATP alone (Csanády et al. PNAS 2010, τ_{burst} = ~28 s (N = 16); Csanády et al. JGP 2013, τ_{burst} ~28 s (N = 41)).

The experiments by Bompadre et al. and Zhou et al. referenced by the Reviewer cannot be compared with the current study, as they were performed under non-identical conditions: in those studies relaxations following simultaneous removal of ATP+PKA were analyzed. It is well documented that under non-hydrolytic conditions channels opened by PKA+ATP close slower compared to those opened by ATP alone: for K1250A channels τ=83 s (Vergani et al., JGP 2003) vs. ~25 s (Csanády et al. PNAS 2010; Csanády et al. JGP 2013), for WT channels locked by AMPPNP τ=~50 s (Vergani et al., JGP 2003) vs. <20 s (Csanády et al. JGP 2013) under the two conditions (all parameters obtained in *Xenopus* oocytes). Indeed, in our present experiments on hS1371S, analyzing the relaxations upon simultaneous removal of ATP+PKA following the initial phosphorylation period, we obtain a time constant of τ=81$\pm$19 s (N=5).

The reason for this phenomenon is unknown, but is unlikely to reflect differences in phosphorylation levels (see below, also, cf., Mihályi et al. PNAS 2020) – in any case, this issue is irrelevant to the questions addressed in the current study. What matters for assessing energetic effects of ECL mutations is to observe mutant and background constructs under identical conditions. Here, consistent with our previous study (Simon et al., 2021), mutational effects on gating were all assessed on pre-phosphorylated channels in the presence of ATP alone.

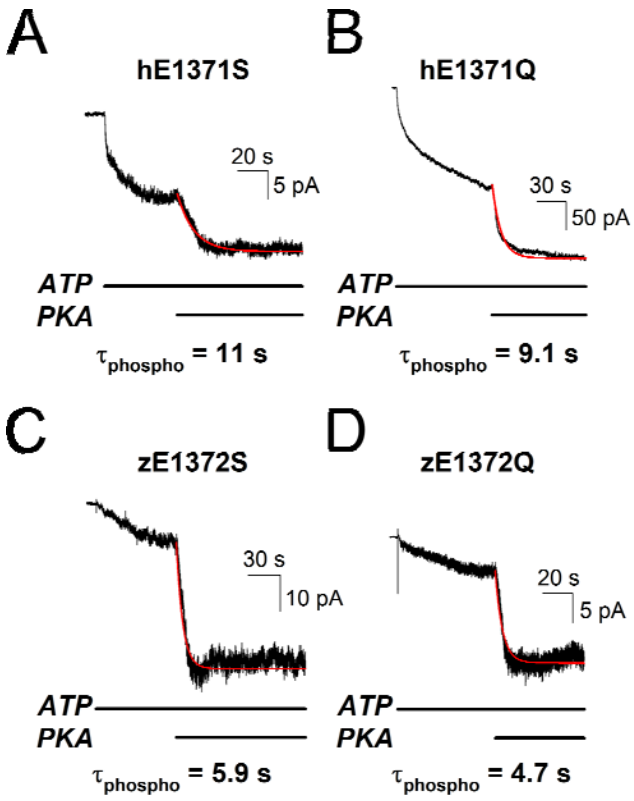
... a longer time constant for E1372S-zCFTR was seen, contradicting a previous study that shows 4-fold shorter relaxation time constant when comparing E1371Q-hCFTR and E1372Q-zCFTR (Yu et al., 2016). These discrepancies cannot be attributed to differences in the expression system especially the time constant for E1371Q-hCFTR is virtually identical when the channel is expressed in oocytes or mammalian cells (Vergani et al., 2005; Yu et al., 2016).

We agree with the Reviewer that such a discrepancy cannot be explained by the different model systems – as shown in Fig. 3A-B, we find little impact of the expression system on CFTR gating. To evaluate the alternative possibility that the different substitutions used in the two studies underlie the seemingly discrepant results we constructed hE1371Q and zE1372Q mutants and tested mean burst durations in macroscopic inside-out patch clamp recordings in oocytes. For hE1371Q τ_{burst} was 470 ± 65 (N = 4), while for zE1372Q τ_{burst} was 78 ± 6.7 (N = 6), reproducing the differences reported in the earlier studies (Yu et al., 2016; Zhang et al., 2018). Thus, replacement of the catalytic glutamate with a glutamine results in bursts which for hCFTR are >10-fold longer, but for zCFTR not significantly longer (p=0.092), compared to those of the respective serine mutants. The fact that different substitutions of the catalytic glutamate differentially affect burst stability in zCFTR and hCFTR suggest important differences in the network of interdomain interactions that stabilize the NBD dimer interface. We now dedicate a separate section "*Different substitutions of the catalytic glutamate differentially affect burst stability in zCFTR and hCFTR*" for the discussion of these new interesting results, which are summarized in new Fig. 3C-D.

This reviewer noted that their experimental protocol entails only one-minute exposure of the patch to PKA and ATP, and hence wondered if this short exposure is sufficient for effective control of the phosphorylation level, which is known a main factor affecting CFTR gating.

Therefore, it is important for the authors to show experiments that can effectively eliminate this concern in order to make fair comparison not only with what is in the literature, but also with data for various mutants in the current study.

Thank you for this insightful comment, but the apparent discrepancy is fully explained by the differential effects of the Q vs. S mutations on the two orthologues (see above). In our experiments the time constants for current activation upon initial exposure to 2 mM ATP + 300 nM bovine PKA were ~10-20 s, and the ~1 min exposure to PKA was sufficient to reach steady state in each case (see figure below). This fact is now stated in Methods (p6, 2nd par).



Another major difference noted is the channel behavior within a burst between E1372S-zCFTR in the current study (e.g., Fig. 2C) and E1371Q-hCFTR (Fig. 4B in Zhang et al., 2018). Could this difference also be attributed to difference in phosphorylation?

The intraburst P_o of zE1372S measured here (0.29) is close to the previously reported value for zE1372Q (~0.35). Furthermore, we also observe that the open- and closed-time distributions are complex, as reported by Zhang et al., 2018 (that is why we restricted analysis of intraburst gating to extraction of P_o). This results in substantial data heterogeneity, and we admit that the trace chosen in the original figure, was a poor representation of average behaviour. It has now been replaced by a more suitable record (Figs. 2C and 5C).

My third major concern is the inconsistency between reported intraburst Po and the raw current trace. Specifically, the intraburst Po of E1372S-zCFTR was estimated as 0.27 (page 18

and Table 1), but the raw current trace in Fig. 2D cannot be consistent with this value. Furthermore, the current traces in Fig. 4A clearly show a higher activity for zS109A-N120A compared to zE1372S, but the kinetic parameters in Table 1 show otherwise. Accurate numbers are particularly important for both relaxation analysis and intraburst kinetic analysis as they are used for mutant cycle analysis, the results of which are the basis for all their mechanistic interpretations.

We thank the Reviewer for pointing out these inconsistencies. As discussed above, the trace shown for zE1372S indeed poorly represented mean intraburst P_o for that construct. It has now been replaced. In addition, given the heterogeneity in intraburst gating of zCFTR, we have solidified the mutant cycle shown in Fig. 5A-C (original Fig. 4A-C) by increasing the numbers of experiments for the corners of the cycle. This expanded data set resulted in some readjustments of the measured gating parameters (summarized in Table 1), but otherwise reinforced our original conclusion: strong energetic coupling between the two positions.

Some minor issues:

P3. Class II mutation such as delF508 requires a combination regimen of two correctors and a potentiator to be effective in clinical treatment. Please cite proper references.

Thank you, the text has been corrected and proper references cited.

P4 (also in page 13). It is perhaps premature to designate Cs state to the solved IF conformation of CFTR. Afterall, this configuration represents a pre-phosphorylated state, while Cs is functionally defined as a post-phosphorylated closed state.

We agree and have added the following clarification (p4, top): "*Of note, the currently available cryo-electronmicroscopy (cryo-EM) structures of IF and OF CFTR (PDB: 5UAK, 6MSM) are not precise representations of the C_s and B states, respectively, as the former was obtained from unphosphorylated CFTR in the absence of ATP, while in the latter structure the external end of the pore is too narrow to accommodate a hydrated chloride ion. Nevertheless, to date, these structures are the best available models for gating-associated conformational changes of the channel pore.*"

P5. The last part of the first paragraph needs clarification. Are these from original reports? Or the authors actually analyzed the structures?

These statements about differences between ECLs and NBD site 1 of zCFTR vs. hCFTR are taken from the original reports that have analyzed these features in detail. We have repositioned the citations to these two papers

to make this more clear.

Besides, all solved OF configurations, human or zebrafish CFTR, fail to show a patent pore. The authors should be more cautious here as well as in page 20 (Fig. 7) in assigning the open state.

We agree, and explicitly state this fact (see above).

P7 last sentence and P10 lower section. The validity of the claims depends on if the burst duration of E1371S-hCFTR is accurately assessed as described above.

Our mean burst durations are as accurately assessed as those in any other study: from fits to single exponentials. Simple visual inspection of the raw data traces (Figs. 2A, 3A) show that our extracted time constants (Figs. 2B, 3B) are reasonable.

On the other hand, we agree that claims such as "rather stable" are poorly defined. We therefore now explicitly state "*the B state of zE1372S seems as stable as that of hE1371S*" (p9). Furthermore, we now explain that – regardless of which catalytic site mutant most closely represents the prehydrolytic state of a WT zCFTR channel – non-hydrolytic closing rate is >100-fold slower than hydrolytic closing rate, suggesting strong coupling between ATP hydrolysis and channel gating already in the zebrafish orthologue (p15): "*given the reported* τ_{burst} *of ~0.7 s of WT zCFTR (Zhang et al., 2018a), the observed ~100-fold longer* τ_{burst} *of non-hydrolytic mutants (Fig. 2B, 2D gray) reports that the gating (burst-interburst) cycle is strictly coupled to ATP hydrolysis already in the zebrafish orthologue.*"

P9. Is the difference in current decay between zE1372S and ZR118H statistically significant?

No, they are not ($p = 0.27$), as now explicitly stated (p10).

P14. Why do you think converting S108 to alanine can change the backbone carbonyl position?

hS108 is the terminal residue of helix TM1. In the modeled structure of phosphorylated hCFTR (6msm) the side chain of hS108 forms a H bond with the backbone carbonyl group of hI105, located in the last helical turn of TM1, thereby stabilizing the conformation of the initial part of ECL1. Although the electron density in this region poorly supports the modeled structure, there is no alternative conformation in which this residue could be fitted into the density. We thus hypothesize that by perturbing this H bond, mutation hS108A destabilizes the positioning of the hS108 backbone. However, in the absence of tools to test it, we decided not to include such a speculative hypothesis into the manuscript.

P17. The single-channel amplitude of hS108A-I119N seems larger. Please verify.

It is indeed larger, as the hE1371S, hS108A, hR117H channels were recorded at -80 mV, while the hS108A-I119N trace was obtained on -120 mV. This is now indicated in the figure legend.

Some minor suggestions:

The authors may want to avoid using the same title for sections in the result and figures.

Thank you for your comment. The titles of the figures have been modified.

Overall, the manuscript will also be improved by citing more references especially from others.

We have been trying to carefully include citations to earlier reports from all laboratories, whenever relevant to the present study. In the revised manuscript we have added 8 additional citations. However, we feel that discussing all previous studies in which our background construct was tested under experimental conditions different from ours is irrelevant to the current study and would distract the focus of the reader from the aims of the present work.

Reviewer #2 (Comments to the Authors):

In this manuscript Simon and Csanády investigate the functional role of a serine in an extracellular loop of the CFTR chloride channel, the protein that is mutated in cystic fibrosis. The authors note that two CFTR orthologues with available structural information, hCFTR and zCFTR, display marked functional differences despite overall structural similarities. They identify a network of interactions in the first extracellular loop (ECL) that stabilize different conformations of hCFTR and zCFTR. Specifically, the open state of hCFTR is stabilized by a hydrogen bond formed by R117 whereas in zCFTR the flickery closed state, rather than the open state, is stabilized by a hydrogen bond between the neighboring N120 and S109 residues. Interestingly, the S108A mutation impairs gating of hCFTR in a manner similar to R117H but the effects of the two are not additive, suggesting these residues interact in the open state.

Overall, this is a well-written manuscript that addresses an interesting problem. The results are largely well-grounded in the data.

I have only one major concern, that the authors should clarify or address. For hCFTR the bursting behavior is clear from the single channel traces, as long-lived bursts can be clearly identified. For zCFTR however, the single channel traces do not reveal clearly defined bursts. Rather, the channels are very flickery and only short lived open states are visible from the traces. While it is possible that this has been addressed in past work, I think it is critical to clearly illustrate and define what is a burst of the last open channel in zCFTR so that a reader can follow the analysis. This is especially important since so much of the analysis reported here critically depends on the quantification of the mean lifetimes and Keq of the bursts in the two constructs. For example, in the trace in Fig2C zE1372S, how do the authors distinguish between the last and the last-but-one bursts?

The Reviewer's well-founded concern is centered on two issues: (i) discerning flickery from interburst closures, and (ii) discerning the activities of the last-but-one and the last channel.

As to the first issue, flickery closures are operationally defined here as those from which the pore may reopen without the binding of a new ATP molecule from the bulk solution, in contrast to interburst closures which involve nucleotide exchange. (This approach is justified from a structural perspective in that it separates closures during which the NBD dimer, harbouring two occluded ATP molecules, remains tight from closures in which the dimer interface disengages and the nucleotide in catalytic site 2 becomes rapidly exchangeable.) As these segments of recording are obtained under continuous superfusion with an ATP-free solution, reopening from an interburst closure is not possible. (Contamination by extremely infrequent spontaneous re-openings of unliganded channels can be safely neglected.)

As to the second issue, we analyzed segments of record following the last observed superimposed channel opening. Indeed, for low-P_o constructs this approach does not eliminate the possibility that two channels are still active during some initial part of the analyzed segment even if they do not open simultaneously. However, such a scenario predicts a sudden irreversible drop in apparent P_o, at the time point of final closure of the last-but-one channel somewhere within the analyzed segment. Because we did not observe such trends upon visual inspection, we believe that for each of the studied constructs the majority of total "last-channel" recording time must have indeed documented gating of a single active channel. I.e., a potential overestimation of intraburst P_o should be minimal.

These considerations are now explained more clearly in the Methods section (p6 bottom – p7 top).

Related to the point above, in several traces the opening of several channels is visible (e.g. in Fig. 4A zN120A, zS109A-zN120A; in Fig. 5D and 6D hS108A). Wouldn't this affect estimates of Keq, if not of τburst, which is determined from macroscopic currents. How did the authors correct for this?

The traces show final ~1-minute segments of recording, and in some cases the initial parts indeed still show activity of multiple channels. However, for each recording only the segment following the last superimposed channel opening was analyzed. To make this more clear, we now highlight for each trace the analyzed segment using a black line (see Fig 2C, 4A, 5D, 6A).

While I like the evolutionary portion of the discussion, I think it should be shortened and tightened. As it stands, I found it a bit too speculative and teleological.

We have tightened this paragraph by restricting the discussion to facts that are directly supported by our data.

Reviewer #3 (Comments to the Authors):

The manuscript by Simon and Csanády attempts to understand the role of a specific residue in evolution of function within CFTR, largely by taking advantage of a combination of structural analysis and sequence analysis across evolutionarily distanced orthologs: human and zebrafish. The authors are commended for the scientific aspects of this work and presentation. Scientifically, this manuscript is wonderful. I do have several concerns about the writing. Correcting these defects will enable a greater impact on the field.

We thank the Reviewer for the constructive suggestions for improving clarity and accessibility of our manuscript. Following these suggestions we have made substantial revisions to the text.

1. The Abstract is not written for a general audience, even just within the ion channel field. The very last sentence of the Abstract, I think, is a bit of a major leap, since it implies that all of the differences in gating between these orthologs comes down to a single pocket of residues studied here.

We have reworded the entire Abstract. The last sentence now says "an example for how gating mechanism was optimized".

1a. It is misleading to state that the zebrafish and human orthologs represent "two ends of CFTR molecular evolution", in part because the lamprey version of CFTR is evolutionarily much more distant from the human than is the zebrafish.

Corrected to "The human channel (hCFTR) and the distant zebrafish orthologue (zCFTR)"

1b. It also is a bit odd to suggest that these orthologs possess different gating properties "consistent with structural differences" since this implies that you can infer differences in gating from studying the structures. That's not really true.

Replaced by "display differences both in their gating properties and structures"

1c. The "outward-facing" concept is not defined in the Abstract.

Inward- and outward-facing has been replaced by "unphosphorylated apo-" and "phosphorylated ATP-bound".

Fig. 1. Again, OF is not defined.

IF and OF are now explained in the third paragraph of the Introduction and OF is also fdefined in the legend for Fig. 1.

Please check spelling of "pale".

Corrected, thank you.

Page 5, top para. You state that hCFTR is the "current endpoint of CFTR molecular evolution." What makes that the case? What can you state to support this designation?

We agree with the Reviewer that such a designation is problematic, as CFTR of any extant mammalian species might equally qualify for it. To date, CFTR has been studied in several other vertebrates, and unlike in humans, in which both reduced and excessive CFTR activity leads to disease, proper CFTR function appears less important in many other species. In that sense, human CFTR might have been optimized by stronger evolutionary pressure. Nevertheless, we now say "one of the current endpoints".

Page 5, top para. I have no idea what you mean by "the mechanical development" that occurred during CFTR evolution

Replaced by "structural development".

Page 6, top para. Given that you will make a comparison to the work of Hwang who studies CFTR in mammalian cells, it would be good to mention very early in this paragraph that you have studied CFTR in Xenopus oocytes.

A completely novel section "*Different substitutions of the catalytic glutamate differentially affect burst stability in zCFTR and hCFTR*" is now devoted to discussing the reason for the apparent discrepancies with earlier work from the Hwang group. That section starts with a comparison of expression systems.

Page 6, top para. What do you think might be the mechanism underlying the difference between your study and Hwang's RE zCFTR? Please suggest a potential basis for the different observations.

A separate section and figure panels 3A-D are now devoted to clarifying this issue. We find little impact of the expression system on CFTR gating. Instead, we find that the different substitutions used in the two studies underlie the seemingly discrepant results. We constructed hE1371Q and zE1372Q mutants and tested mean burst durations in macroscopic inside-out patch clamp recordings in oocytes. For hE1371Q τ_{burst} was 470 ± 65 (N = 4), while for zE1372Q τ_{burst} was 78 ± 6.7 (N = 6), reproducing the differences reported in the earlier studies (Yu et al., 2016; Zhang et al., 2018).

Thus, replacement of the catalytic glutamate with a glutamine results in bursts which for hCFTR are >10-fold longer, but for zCFTR not significantly longer (p=0.092), compared to those of the respective serine mutants. The fact that different substitutions of the catalytic glutamate differentially affect burst stability in zCFTR and hCFTR suggest important differences in the network of interdomain interactions that stabilize the NBD dimer interface. Although the mechanistic explanation of this phenomenon is beyond the scope of our manuscript, this finding opens up an interesting scientific question to be answered.

Page 7, top. You say that the B state of zCFTR seems to be rather stable. Compared to what?

Indeed, we agree that the claim "rather stable" is poorly defined. We now say "*the B state of zE1372S seems as stable as that of hE1371S*" (p9). Furthermore, we now explain that – regardless of which catalytic site mutant most closely represents the prehydrolytic state of a WT zCFTR channel – non-hydrolytic closing rate is >100-fold slower than hydrolytic closing rate, suggesting strong coupling between ATP hydrolysis and channel gating already in the zebrafish orthologue (p15): "*given the reported* τ_{burst} *of ~0.7 s of WT zCFTR (Zhang et al., 2018a), the observed ~100-fold longer* τ_{burst} *of non-hydrolytic mutants (Fig. 2B, 2D gray) reports that the gating (burst-interburst) cycle is strictly coupled to ATP hydrolysis already in the zebrafish orthologue.*"

Fig. 2 legend. You don't tell us what you mean by "-20/-40 mV" in this case, or similar cases elsewhere in the paper.

Thank you, these details have been added to all figure legends.

Page 9, top. You state that zR118H mutation resulted in "a slower current decay". Are there any statistics to say that this is actually different?

The difference between zE1372S and zR118H is not significant ($p = 0.27$), this now explicitly stated (p10, 2nd par)

Page 10, middle. You state that because the unitary amplitude of last openings was smaller than 0.3 pA, intraburst kinetics could not be examined reliably. Isn't this even more related to the apparent brevity of openings, rather than the amplitude?

Indeed, in addition to the reduction in unitary amplitude caused by the R118H mutation, the inherent brevity of zCFTR openings further limits the resolution of unitary events for zCFTR/R118H. We now say "Because at our recording bandwidth the unitary amplitude of last channel currents was smaller than 0.3 pA at -120 mV" (p10, 2nd par).

Page 10, bottom, to 11, top. Rather a weird transition. Given the writing in the prior paragraph, why not describe the effect of mutations a hS108, first?

Thank you for pointing out this somewhat clumsy sentence. We have reworded this and the prior paragraph in response to comments by Reviewer 1. We believe that this has smoothened the transition to the next section. We have decided to keep the order of first presenting all data on zCFTR before transitioning to data on hCFTR.

Page 11, second para. More appropriate would be to say that "... as one amino acid OFTEN takes part in multiple interactions,...".

Corrected, thank you.

Page 11, bottom. Great paragraph!

Thank you

Page 21, top. Isn't it also possible that mutations at this site are embryonic lethal?

Good point, added!

Page 21, second para. Really a great paragraph.

Thank you! In fact, the argument in favour of the C_s-C_f-O scheme based on original Fig. S4 has been weakened by the slight adjustments in the fitted rates obtained for our updated data set (more experiments added to the corners of the zN120-zS109 mutant cycle). We have therefore shortened this paragraph and omitted Fig. S4.

Page 23, bottom. You state that statistical significance of interaction energies were calculated by t test. But, where are those statistical differences indicated? Literally, none of the figures or Table 1 have any indication of statistically significant differences.

Statistical significances were calculated for all interaction free energies ($\Delta\Delta G_{int}$), the calculated p values are printed in each panel (Figs. 5C, 5F, 6C, 6F, 7C, 7F, S2C) below the value of $\Delta\Delta G_{int}$ (purple, in parentheses).

Page 24, top. "Infield" is an incomplete reference.

Corrected, thank you.

Fig. S2. Really need to pay attention to appropriate capitalization of genus names and lack of capitalization of species names.

Corrected, thank you.

January 12, 2023

Mr. Marton A. Simon
Semmelweis University
Biochemistry
Tuzolto street 37-47.
Budapest H-1094
Hungary

Re: 202213264R1

Dear Mr. Simon,

I am pleased to let you know that your manuscript, entitled "Central role of a conserved serine residue across CFTR molecular evolution" is scientifically acceptable for publication in Journal of General Physiology. Formal acceptance will follow when it is modified in accordance with the referees' remarks and our editorial policies.

Please note items that need attention are listed at the bottom of this email and on the attached marked-up pdf file. Your manuscript should be a double-spaced MS Word file and include editable tables, if appropriate.

Please submit your final files via this link:
Link Not Available

Thank you for choosing to publish your research in JGP and please feel free to contact me with any questions.

Sincerely,

Joseph A. Mindell, M.D., Ph.D.
On behalf of Journal of General Physiology

Journal of General Physiology's mission is to publish mechanistic and quantitative molecular and cellular physiology of the highest quality; to provide a best in class author experience; and to nurture future generations of independent researchers.

Manuscript formatting checklist:
- MS Word document of text(including editable tables)
- MS Word document of supplemental text, if applicable (including figure legends and editable tables)
- Brief Statement describing supplementary information (in subsection at end of Materials & Methods)
- Figures created at sufficient resolution and in acceptable format (including supplemental). If working in Illustrator, we prefer .ai or .eps file format. If working in Photoshop please use 600dpi/1000dpi .tiff or .psd file format. Minimum resolution at estimated print size: Minimum resolution for all figures is 600 dpi. For figures that contain both photographs and line art or text, 600 dpi is highly recommended. Figures containing only black and white elements (line art, no color, and no gray) should be 1,000 dpi. Maximum figure size is 7 in wide x 9 in high (17.5 x 22.8 cm) at the correct resolution. https://jgp.rupress.org/fig-vid-guidelines
- Supplemental figures conforming to same guidelines as manuscript figures (noted above)
- If images resemble one from a prior publications, the author must seek permissions (to reproduce or adapt) from the original publisher. [You can resubmit your paper while waiting to hear back from the original publisher but please keep us updated]

Reviewer #1 (Comments to the Authors):

No more comments except that the authors may want to consider giving the article a more conservative title.

Reviewer #2 (Comments to the Authors):

The authors have addressed my concerns. I have no further requests.

Reviewer #3 (Comments to the Authors):

The authors have responded well to the prior critique.
No further concerns.