Cryo-EM reveals conformational variability of the oligomeric VCC β-barrel pore in a lipid bilayer

Nayanika Sengupta, Anish Mondal, Suman Mishra, Kausik Chattopadhyay, and Somnath Dutta

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March 21, 2021

Re: JCB manuscript #202102035

Dr. Somnath Dutta
Indian Institute of Science Bangalore
Molecular Biophysics Unit
CV Raman Road
Bangalore, Karnataka 560012
India

Dear Dr. Dutta,

Thank you for submitting your manuscript entitled "Capturing the oligomeric β-barrel pore (VCC) in the lipid environment using single-particle cryo-EM." The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that reviewers consider the structure of a VCC in a lipid bilayer to be of potential interest, but are not convinced that the claims in the paper are supported at the current resolution. While we cannot offer to consider your manuscript further in its present form, if it were possible to obtain a reconstruction at higher resolution and provide better support for the conclusions, such a study might be of interest.

Although your manuscript is intriguing, we feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. Given interest in the topic, we would be open to resubmission to JCB of a significantly revised and extended manuscript that fully addresses the reviewers' concerns and is subject to further peer-review. If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission.

If you wish to expedite publication of the current data, it may be best to pursue publication at another journal. Our journal office will transfer your reviewer comments to another journal upon request.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

James Hurley, PhD
Monitoring Editor

Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This represents the first high resolution structure of VCC in the presence of a lipid bilayer. Further, the lipid bilayer in this case was a liposome, providing a somewhat more native-like environment than a lipid nanodisc might. The work seems to have been performed well, following standard practices, and the resolution estimate seems reasonable. While other high resolution structures exist, this is the first, in-which the interactions with the lipid bilayer and impact of the bilayer on the conformation of the protein could be investigated, making it of interest to a fairly broad audience of structural biologists, as well as those studying the system itself.

However, unfortunately the majority of the figures in the manuscript are not well designed, and it is virtually impossible to see any structural details at the level of the claims made in the written manuscript. Furthermore, this resolution (4.5 Å) is a bit marginal for some of the claimed observations, so a visual representation of the claimed observations is critical to assess the veracity of the claims. To properly review the manuscript, the figures need to be redesigned with focused displays of electron density with modeling in the regions of interest to identify what the authors are claiming to see. If the authors claim to see sidechains interacting with lipid, showing the specific density is necessary, both to clarify the meaning of as well as to validate the statement. It is worth noting that the manuscript is also in need of editing, as there are grammatical problems throughout.

Some comments on specific figures:

Figure 2- While the windowed out 2-D views are nice, they are windowed too tightly, so it is difficult to see the bilayer fully, and some of the TM density is nearly cut off.

Figure 3 -
* Surely the density in B is a lipid monolayer, not bilayer?
* What is going on with the density curving up around the edges of the VCC?
* It seems that the bottom monolayer may have been overly masked similar to the comment on figure 2? It would be good to see a somewhat larger region of bilayer surrounding the channel
* While the rendering is sufficient to show general agreement with the model, it also extends considerably outside the model in places, or appears to. If the model is converted to electron density and rendered, how well do the surfaces match?

Figure 4-
* I cannot relate the observed densities to the protein at all, particularly in E. I don't see any density which clearly seems to match the modeled structure. There is just a mass of confused lipid density, which, aside from any regions which are strongly interacting with the protein, is likely to be a random superposition of fluid lipid molecules.
* 4.5 Å resolution is quite marginal for real-space model improvement in Phenix to be trusted.

Remodeling hydrophobicity assumes that there have been trustworthy rearrangements of the sidechains.
* While it is possible to assess motions of large domains which are smaller than the claimed resolution. Claiming a 0.8 Å shift seems difficult to believe. How can you even claim to identify the edge of the membrane with 0.8 Å accuracy? The membrane typically has significant surface fluctuations. I just don't understand this claim at all.

Figure 5-
* This is the one figure which claims to show some close-ups of side chain density, but the way it's presented I don't really see any match between the model and the cryoEM isosurface at all. if there really is a good density match here, it is presented exceptionally poorly.

Figure 6-
* "The cryoEM model" is presumably the result of taking the crystal structure and performing real-space refinement on it? Again, this is a marginal procedure at 4.5 Å resolution, and would normally strongly bias towards the force-field rather than the cryoEM density. The large motions of whole loops are likely reliable.
* I have a hard time picturing how these models are positioned within the oligomeric map. It would be useful to have a thumbnail of the whole map with the displayed portion highlighted.

Figure 7-
* ResMap has an unfortunate tendency to over-estimate resolution, and is particularly unreliable in cases where fine resolution features are observed. It would be worthwhile to check this result against one of the several other standard tools used for local resolution assessment (each of which uses a different mathematical method).
* The pictured resolution ranges seem like they should produce an overall resolution significantly better than 4.5 Å if they are accurate. This raises some concerns about the local estimate. At 2.8 Å, sidechains should be fairly reliable and clearly represented. If you focus on one of the highest resolution regions of the map, is this true?
* D seems to try and show the quality of the side chain fitting, but there is so much overlap it is difficult to dell how good this really is (though at first glance it does seem quite decent). This somewhat addresses the local resolution concerns. Is the inside of the B-barrel one of the high resolution domains?

Figure S1- As commented above, the mask is so tight it is difficult to reliably see the membrane region, and the TM domains are nearly cut off, raising questions of how many CTF effects there may be due to overmasking.

Figure S4 - In the presented view it is very difficult to tell what the orientation distribution look like. You cannot tell the height of a cylinder when you are looking along its length.

Reviewer #2 (Comments to the Authors (Required)):

Sengupta et al. report the single particle cryo-EM structure of the V cholerae cytolysin (VCC) embedded in lipid bilayer of liposomes. The derived atomic model is compared with the crystallographic structure of the detergent solubilized VCC to eventually pinpoint conformational changes of the VCC in a natural lipid environment. The results presented appear preliminary, mostly because of the limited resolution of the cryoEM model, and because the figures presented fail to

convince that the experimental data support the authors' conclusions.

Figure 6 shows a comparison between the crystallographic and the reported cryoEM atomic model; the observed displacements between the two structures are mostly in the 1 to 3 Å range, which is below the overall resolution of the 4.5Å cryoEM structure. At a minimum, the experimental 3D cryoEM map should be shown in the figure, so as to appreciate the quality of the data and the relevance of the reported differences. Only for figures 7-D and S4-B are shown detailed fits between the 3D cryoEM map and the derived atomic model, but the stacking of residues in figure 7-D makes it difficult to appreciate the fitting, and in figure S4-B only a sequence of a few residues, which are not identified, are shown. The signal from the lipids is depicted in several figures (figures 3, 4, 5), which appear as noisy densities (color coded orange), however the densities for the protein, which the reader would expect to see, are not shown (figures 4-D and 4-E) or hard to see (figures 5-C and 5-D). The authors should have concentrated more on depicting the protein densities.

We would like to thank the Editor for sending our manuscript for review and for giving us an opportunity to resubmit the revised manuscript. We would also like to thank all the reviewers for taking their time and effort to review our manuscript. We are really grateful for their constructive suggestions and comments on our manuscript, which helps us to improve the manuscript. The reviewers had two major concerns 1. representation of figures and 2. Improve the resolution of the lipid membrane embedded VCC. **We have successfully addressed all the reviewers concerns in this current manuscript.** In this manuscript, we aimed at resolving liposome embedded VCC at a resolution better than our previous submission. Therefore, we performed another round of cryo-EM data collection and data processing, after which all the results presented here are a revised version of the past manuscript. The resolution of the VCC reconstruction is improved significantly and overall resolution is around 3.7-4.0 Å. This resolution is quite similar (3.9 Å) for a 3D reconstruction of AcrB in the presence of liposome, although the reconstruction was performed using Titan Krios. It is a difficult task to achieve below 3.7 Å resolution structure of biological macromolecules in the presence of real lipid environment, which are not any detergent or lipid nanodiscs. Additionally, our study is the only study of small PFT in the presence of real lipid. Furthermore, we have reprocessed the data and re-made all the figures according to reviewer's suggestions.

By addressing these comments, we feel the quality and impact of our manuscript have significantly improved. Nevertheless, these improvements and new figures do not affect, and modify our main conclusion of this manuscript. These have strengthened our conclusion that we are able to resolve various states of the VCC in the lipid environment at atomic resolution. Furthermore, according to reviewers' suggestions, most of the figures are modified and represented properly. Therefore, this will help readers/users to understand the manuscript and implement this protocol in their research. Furthermore, the editor's support is extremely helpful to improve the manuscript.

Reply to Reviewers

Reviewer 1:

Figure 2- While the windowed out 2-D views are nice, they are windowed too tightly, so it is difficult to see the bilayer fully, and some of the TM density is nearly cut off.

Answer: We thank the reviewer for their suggestion and have thus increased the mask diameter for better visibility of the lipid and transmembrane density. The 2D views have been updated in the Main Figure 2B and Figure S1A. Furthermore, this is the first time any **small pore forming toxin** is characterized in the presence of real lipid membrane using **single particle cryo-EM**. When pore forming toxin binds to liposome, most of the time toxin binds randomly and we have no control of this biological process. Many VCC particles are closely packed on the liposome. Thus, it is a bit difficult to isolate single VCC particles from adjacent VCC molecules. Therefore, it is difficult to use extremely big box size. We have used a moderate box size to process data. Additionally, **there are very few single particle cryo-EM studies**

performed in the presence of real lipid environment. Most of the studies (even GPCRs, PFTs, Channel proteins) were performed in the presence of detergent or lipid nanodiscs. As per our knowledge, only one channel protein AcrB and few pore forming toxins such as Pneumolysin, YenTcA, MPEG1, Gasdermin A3, have been resolved in the presence of real lipid membrane using single particle analysis (Cryo-EM analysis of a membrane protein embedded in the liposome). Sometimes it is extremely difficult to visualize very strong density of lipid when small biological samples are imaged in the presence of real liposome. However, in our current reconstruction, lipid bilayer is clearly visible in 2D class averages and 3D reconstruction. And we appreciate reviewer's suggestion to increase the mask diameter. Increased mask diameter helped to visualize the lipid membrane properly in 2D and 3D structure.

To address the concern sufficiently, we have also made a modified supplementary figure, Figure S1, where class averages with different side views are shown. The bilayer lipid density and the TM channel in between indicates how the PFT is bound in the liposome membrane. We also show that including a large area of lipid during 3D reconstruction lowers the global resolution (Figure S1 A) therefore in calculating the 3D structure, the volume of lipid included was limited keeping the mask reasonably loose.

Figure3-
*Surely the density in B is a lipid monolayer, not bilayer?
*What is going on with the density curving up around the edges of the VCC?
* It seems that the bottom monolayer may have been overly masked similar to the comment on figure 2? It would be good to see a somewhat larger region of bilayer surrounding the channel
* While the rendering is sufficient to show general agreement with the model, it also extends considerably outside the model in places, or appears to. If the model is converted to electron density and rendered, how well do the surfaces match?

Answer: We want to thank the reviewer for his valuable suggestions. Based on the valuable suggestions of the reviewers, we aimed at resolving liposome embedded VCC at a resolution better than our previous submission. We used 200 kV Talos Arctica for cryo-EM data collection and we were very particular about picking/selecting particles for 3D reconstruction. We only selected those particles which are directly associated big liposome. Thus, we decided more cryo-EM dataset is required to achieve high-resolution structure. However, we went for another round of data collection and another ~1500 cryo-EM images are collected for image processing. Furthermore, around 4,00,000 extra particles are added to the old dataset to improve the resolution of the VCC. The resolution of the fully lipid embedded structure has significantly improved, and it is 4.0 Å (previous resolution was 4.5 Å) at 0.143 FSC. Therefore, after data processing, all the results presented here, are revised and represented again in this version of the past manuscript.
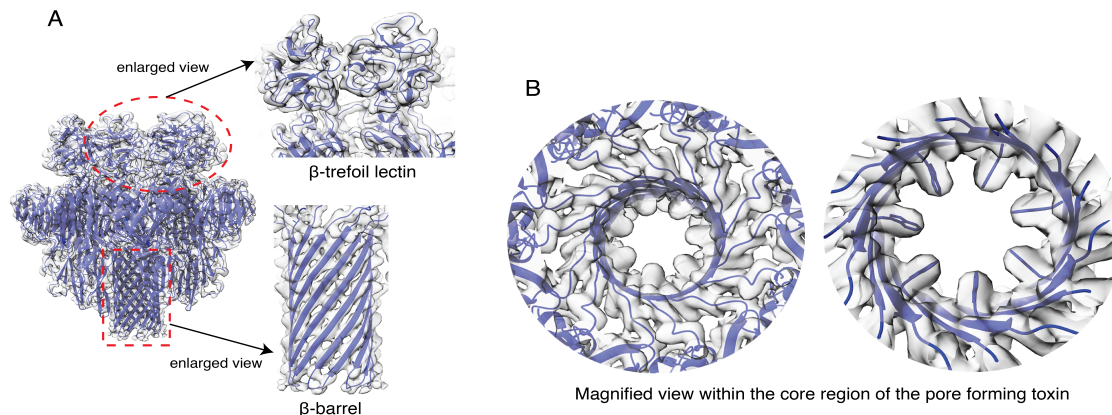
To answer the first query, we agree that it was a typing error on our part. We made a new figure, Figure 3 with the revised results and have corrected all typing errors.

As previously mentioned, we have re-processed the entire dataset and in the current Figure 3, we have shown the intermediate structures we obtained at high resolution from the particle set. In correlation with our past manuscript, we still find the similar direction of heterogeneity in the VCC oligomers. All the figures have been represented such that the protein density can be distinguished from the lipid density.

In our current manuscript, we have increased the mask diameter to comfortably contain the TM density within the mask and remove any possibility of over masking that could lead to missing density. All structures have been refined using a soft mask created in RELION 3.1 according to the suggested values. However, we noticed that including a large layer of lipid within the reconstruction hampers the global resolution and makes it difficult to analyze the details of the protein structure (Figure S1 A). To maximize the structural information, a soft solvent mask was applied. **Moreover, the reliability of the intermediate structural states have now been addressed by using cryoSPARC 3D variability assessment which predicts motion in the particles.** As 3DVA is performed without the use of any solvent mask, the correlation of direction of movement with the results from RELION 3.1, support our observation that the maps do not appear different because of overly masked regions (Figure S3A) and video files (S7 and S8).

In our current reconstruction, we observe good agreement of the map and the model. This is also supported by a high EM Ringer score of 2.81 when the map is fitted with the PHENIX real space refined model of VCC heptamer.

To appreciate the map and model fitting, we have included a new figure, Figure 4 where the fitting of the secondary structural elements has been highlighted. Figure 4 is attached here Figure 4

A

enlarged view

β-trefoil lectin

enlarged view

β-barrel

B

Magnified view within the core region of the pore forming toxin

Figure4-

* I cannot relate the observed densities to the protein at all, particularly in E. I don't see any density which clearly seems to match the modeled structure. There is just a mass of confused lipid density, which, aside from any regions which are strongly interacting with the protein, is likely to be a random superposition of fluid lipid molecules.
* 4.5 Å resolution is quite marginal for real-space model improvement in Phenix to be trusted. Remodeling hydrophobicity assumes that there have been trustworthy rearrangements of the sidechains.
* While it is possible to assess motions of large domains which are smaller than the claimed resolution. Claiming a 0.8 Å shift seems difficult to believe. How can you even claim to identify the edge of the membrane with 0.8 Å accuracy? The membrane typically has significant surface fluctuations. I just don't understand this claim at all.

Figure5-

* This is the one figure which claims to show some close-ups of side chain density, but the way it's presented I don't really see any match between the model and the cryoEM isosurface at all. if there really is a good density match here, it is presented exceptionally poorly.

Answer: We are sincerely thankful to the reviewer for their constructive input. Based on the suggestions, we have altered the representation of lipid-protein interaction that we observe through our reconstruction. After re-processing data, we have obtained an improvement of 0.5 Å in the global resolution. The map and the real-space model correlate well. Moreover, we observe good match between the crystal structure and the real-space model. On the basis of this observation and the suggestion of the reviewer, we have refrained from claiming any changes in the hydrophobicity profile of the protein in a lipid bilayer environment.

We are thankful to the reviewer for pointing out the mistake we made in the figure legend. We apologies for the error of wrongly writing **nm as Å** which led to a misunderstanding of the results. We would also like to explain that the displacement mentioned here was not that of the lipid. We agree that the membrane surface is very dynamic, which also is one of the reasons why we observe distinct structural intermediates depending on the extent of lipid protein interaction. However, over several attempts of reconstructing the bilayer embedded map of VCC, we have consistently seen the appearance of lipid densities around various aromatic and aliphatic residues in the membrane proximal rim domain (updated Figure 5). **The previous crystal structure study had not obtained any lipid densities, but it was predicted based on sequence alignment that Leu238 plays an important role in interacting with cholesterol. In our structure we are able to observe lipid densities at Leu238 and also around other residues higher up away from the membrane surface. Moreover, it was also predicted that the Ala360 and Leu361 residues in an adjacent loop may also have an important role to play in membrane binding (De et. al, PNAS, 2011). Building upon this hypothesis, we analyzed our structure calculated from a membrane bound state and noticed that indeed Ala360 and Leu361 are intermingled with lipid densities. At that particular threshold of viewing the volume (Figure 5D), we observe lipid densities till Val363. From such appearance of lipid densities, we proposed the distance from Leu361**

and Val363 (which is most distant from lipid densities at a reliable threshold of viewing the map). This distance is nearly 0.8 nm and hence we propose that in a native environment, the VCC molecule could possibly be almost 1 nm additionally embedded within lipid than was previously predicted. In order to rectify a past typographical error, we have represented the distance as Figure 5F and corrected the figure legend with the correct unit of distance. As the real-space model correlates strongly with the crystal model, we hypothesize that the observed lipid protein interaction could be reported with reliability.

Considering both the comments on Figure 4 and Figure 5, we have now updated all the representations in a single figure, Figure 5. As suggested, we have focused on depicting the protein density and map-model correlation.

Figure6-
* "The cryoEM model" is presumably the result of taking the crystal structure and performing real-space refinement on it? Again, this is a marginal procedure at 4.5 Å resolution, and would normally strongly bias towards the force-field rather than the cryoEM density. The large motions   of   whole   loops   are   likely   reliable.
* I have a hard time picturing how these models are positioned within the oligomeric map. It would be useful to have a thumbnail of the whole map with the displayed portion highlighted.

Figure7-
* ResMap has an unfortunate tendency to over-estimate resolution, and is particularly unreliable in cases where fine resolution features are observed. It would be worthwhile to check this result against one of the several other standard tools used for local resolution assessment (each   of   which   uses   a   different   mathematical   method).
* The pictured resolution ranges seem like they should produce an overall resolution significantly better than 4.5 Å if they are accurate. This raises some concerns about the local estimate. At 2.8 Å, sidechains should be fairly reliable and clearly represented. If you focus on one   of   the   highest   resolution   regions   of   the   map,   is   this   true?
* D seems to try and show the quality of the side chain fitting, but there is so much overlap it is difficult to dell how good this really is (though at first glance it does seem quite decent). This somewhat addresses the local resolution concerns. Is the inside of the B-barrel one of the high resolution domains?

Answer: After refining the data, we were able to resolve the structure of membrane bound VCC at 4 Å global resolution. Real-space refinement was performed and the differences between the real-space model and the crystal model were assessed. For better representation, as highlighted by both the reviewers, we have included the thumbnail of the whole map with part of the models analyzed. We have also addressed the concern regarding ResMap and we are grateful to the reviewer for pointing out this important tendency of resolution over-estimation. This time instead, we have used Blocres to evaluate the local resolutions. Since the regions where the atomic models differ, show a displacement finer than the local resolution

estimates of that particular region (displacement is near 2 Å whereas map quality is between 3.5 to 4 Å), we have considered the movements nominal and have refrained from making claims in the manuscript.

For better depiction of side chain fitting and supporting our Blocres results, we have considered short stretches of secondary structures in chain N and updated in the current figure, Figure 6. The data from previous figures 6 and 7 have been now combined in Figure 6. Figure 6 is attached here.
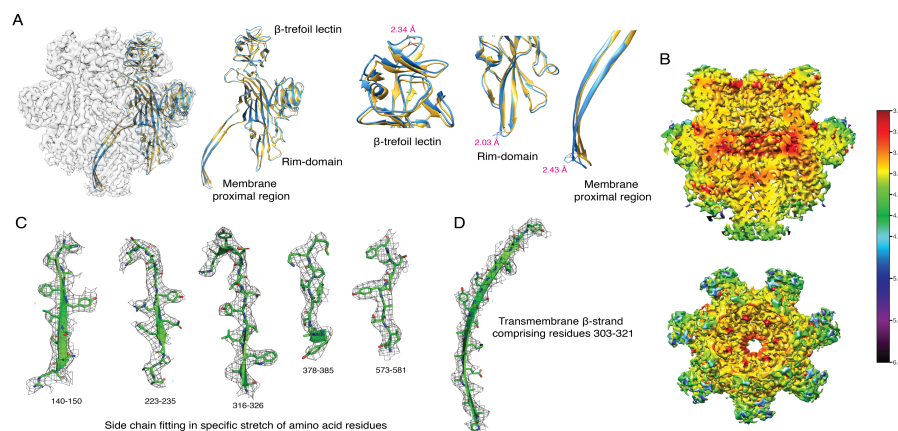

Figure 6

Answer: In order to reliably see the TM domain, we have taken the suggestion of the reviewer and enlarged the mask diameter in the 2D classes. Figure S1 now shows the different side views of the PFT obtained over a considerably large stretch of lipid where the two leaflets can be distinctly seen. The 2D class averages are added to provide an insight into the distribution of particles (with respect to the liposomes) considered for reconstruction. We also showed that including a massive stretch of lipid dampens the resolution of our reconstruction (Figure S1A). Hence, we have used a soft solvent mask to limit the lipid density included within structure calculation while keeping the TM channel comfortably contained within the mask. The 2D class averages are added

Answer: We agree that the representation could be made more prominent. Hence, we have now altered (Figure S4) the orientation such that the heights of the cylinders can be understood.

Reviewer 2:

The signal from the lipids is depicted in several figures (figures 3, 4, 5), which appear as noisy densities (color coded orange), however the densities for the protein, which the reader would expect to see, are not shown (figures 4-D and 4-E) or hard to see (figures 5-C and 5-D). The authors should have concentrated more on depicting the protein densities.

Answer: We thank the reviewer for their helpful suggestions. In order to focus more on the protein densities, we have now updated the relevant results into a modified Figure 5.

Figure 6 shows a comparison between the crystallographic and the reported cryoEM atomic model; the observed displacements between the two structures are mostly in the 1 to 3 Å range, which is below the overall resolution of the 4.5Å cryoEM structure. At a minimum, the experimental 3D cryoEM map should be shown in the figure, so as to appreciate the quality of the data and the relevance of the reported differences.

Answer: As suggested by both the reviewers, we have added the experimental 3D cryoEM map within a modified Figure 6. Since the observed differences are less than the local resolution of the specific regions of the map, we have refrained from making any conclusive statement.

Only for figures 7-D and S4-B are shown detailed fits between the 3D cryoEM map and the derived atomic model, but the stacking of residues in figure 7-D makes it difficult to appreciate the fitting, and **in figure S4-B only a sequence of a few residues, which are not identified, are shown.**

Answer: We thank the reviewer for their valuable comment. In order to make the fitting clear, we have changed the representation style and presented it in Figure 6. To rectify our mistake in Figure S4-B, we have also included the amino acid residue stretches considered in Figure 6C. Figure 6 is attached here
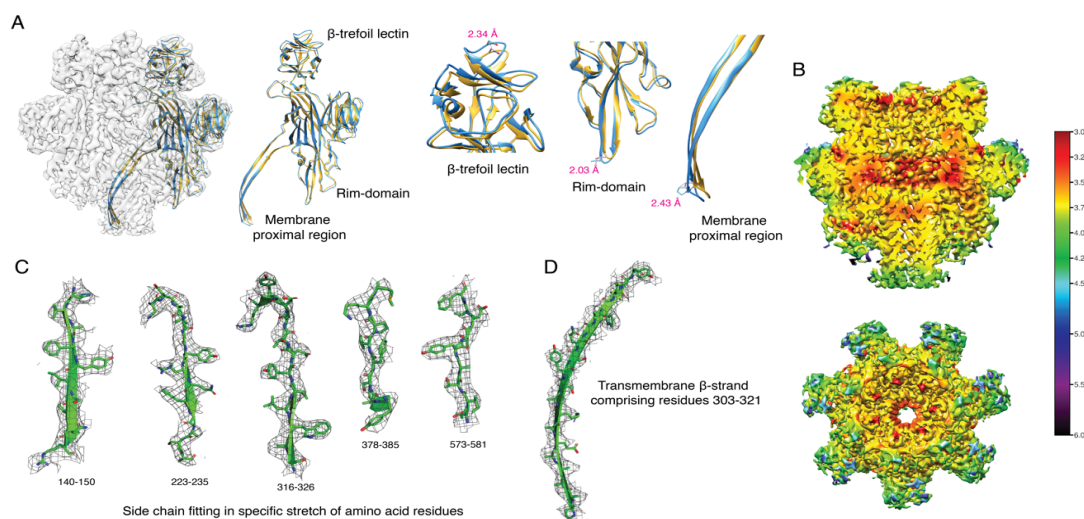
September 14, 2021

RE: JCB Manuscript #202102035R-A

Dr. Somnath Dutta
Indian Institute of Science Bangalore
Molecular Biophysics Unit
CV Raman Road
Bangalore
Bangalore, Please Select State 560012
India

Dear Dr. Dutta,

Thank you for submitting your revised manuscript entitled "Capturing the oligomeric β-barrel pore (VCC) in the lipid environment using single particle cryo-EM." We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to figures 1D, 2B, and S1A&B.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Title: In order for the paper to be accessible to a broader cell biology audience we suggest the following title: "Single particle cryo-EM reveals conformational variability of the oligomeric VCC β-barrel pore in a lipid bilayer."

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "…as previously described."

7) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate). Please be sure to provide primer sequences and enzyme restriction sites used for cloning of all plasmids.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures and 10 videos. As you are over the figure limit please combine supplemental figures 2 and 3 into a single figure. A summary of all supplemental material should appear at the end of the Materials and methods section. Please include one brief sentence per item.

11) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al…" to match our preferred style.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) A separate author contribution section is required following the Acknowledgments in all

research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

14) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

James Hurley, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The revised manuscript offers an improved structure and significantly improved visualizations. The requested changes to analysis techniques for resolution evaluation were also performed. The conclusions in the revised manuscript are now supported by the provided figures, and I have no further suggestions for improving the manuscript prior to publication.

Reviewer #2 (Comments to the Authors (Required)):

The revised version of Sengupta et al. has significantly improved. The figures now clearly show the VCC protein density compared to the lipid membrane.
The authors describe three different states of the protein embedded in the lipid membrane, which show the same general conformation except for the barrel domain which is either not visible, partially visible, or completely visible and penetrating the membrane. The structure with the whole barrel visible is the one with the highest resolution and allows a structural comparison with the previously reported structure of VCC in detergent micelles. While the comparison does not show significant differences, the structure reported by Sengupta el al. has been determined in a natural lipidic environment. Importantly, it shows that the rim regions penetrate the membrane.