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# Structural evidence for functional lipid interactions in the betaine transporter BetP

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## 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.)

*Editor: Andrea Leibfried*

1st Editorial Decision 07 June 2013

Thank you for submitting your manuscript entitled 'Structural evidence for functional lipid interactions in the betaine transporter BetP'. I have now received the reports on your paper.

As you can see below, the two referees find your study of general interest and value your results. However, they also find that significant revisions are needed to reinforce your data and claims.

If you are able to substantially support your conclusions by the inclusion of additional data, I would like to invite you to submit a revised version of the manuscript. To support publication here, however, I would like to point out that a revised version should address all concerns of the referees.

Both referees also indicate that the manuscript needs considerable work in terms of the presentation and the writing style. I agree with this view. At the moment the manuscript is at places hard to follow and it is written very much for a specific audience. Please note that The EMBO Journal is a general interest journal and we aim to reach out to a broad audience.

Please take care to
- broaden the introduction to make the manuscript accessible to a general audience: stressing here right in the beginning the general role of lipids in regulating protein/transporter function would be helpful
- shorten the text regarding our current knowledge of BetP in the introduction

 

- extensively re-structure the results and discussion part to the full satisfaction of both referees. Please remove all unfounded speculation. It would also be helpful for the reader to separate the results section from the discussion.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

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REFEREE COMMENTS

Referee #1

The manuscript by Koshy et al. & Ziegler reports a structure of the BetP transporters at a significantly improved resolution (2.7 Å) and as a rather symmetric trimer of all inward-open protomers. Several PG lipid binding sites are identified and discussed in relation to BetP function. The nature of the PG lipids are verified by thin-layer chromatography. The sites are presented in structural detail and discussed in relation to uptake data on mutated forms and available literature. The data clearly support an interesting report and important conclusions, but the current form of the manuscripts does not convey well such messages and seems overly concerned with a systematic description rather than on key messages, and end at times also in overly speculative conclusions. Sharpening by shortening would greatly facilitate the readability and impact. Fuerthermore a stronger focus on the functional of BetP and how the observed lipid sites relate to this would seem fruitful rather than the current attempt to broaden out to stress/osmosensing transporters in general and related LeuT fold transporters.

Specific points:
1. the abstract ends in pathos, which could be downplayed a bit
2. The introduction makes some shortcuts on previous studies of lipid sites in transporters. The cited papers by Lee AG have a large focus on transporters, certainly not just regulatory lipid interactions with channels. The literature on regulatory interactions of lipids with transporters is rich, including also LeuT fold transporters and various ATPases. Also several MD studies would be relevant, e.g. Mondal et al. 2013 (http://www.ncbi.nlm.nih.gov/pubmed/23376428)
3. The trimer is described as a conformationally symmetric (first subtitle under Results) and the manuscript then moves on to describe differences between monomers (bottom page 6) - this seems unnecessarily confusing. Probably better to describe the trimer as e.g. functionally symmetric.
4. The description gets very detailed and refers repeatedly to a proprietary nomenclature on helix numbers (e.g. TM(-1), TM5' etc) - there is a dire need of an overview figure in the main text introducing these concepts in relation to the overall structure and LeuT fold, as they are referred to throughout the manuscript. The SI material is too remote and does not present it well either. Also a Ci naming of the inward-open state is presented with no sufficient explanation (page 7) - and is it at all needed?
5. The lipid content analysis (page 8-9) is very detailed, and details repeated again for the methods section (where they belong)
6. Having presented earlier the concept of annular and non-annular lipid sites it would be better to maintain this distinction throughout rather than referring to annular sites as e.g. "flexible lipids present along the periphery of the trimer" (page 9)
7. Page 10 suddenly introduces "the down-regulated BetP trimer" as the functional interpretation of the functionally symmetric trimer. This seems like an important concept that shoudl also be addressed in the introduction. A consideration of e.g. Mondal et al. would also seem appropriate on this note.
8. Pages 11-14 on the functional role of the lipid sites within the trimer are a tough read, but considerations of the effect of (or on) conformational changes associated with the BetP funtional cycle do not stand out
9. The comparison to AFM data is difficult to follow (pg. 12-13) - what is the logic here?
10. the final discussion addresses osmosensing and has a good point on the amphipathic helix h7. I would otherwise advice that a stronger BetP focus is maintained which relates the observed sites to models of the functional cycle of BetP. The comparison to AdiC for example appears less rewarding (figure 5)
11. It would seem possible to expand further on the observed sites in relation to previous BetP structures, such as by re-refinement (fig. S6 lends very strong support for an identical site once it

 

would be modeled and the lower res structure be improved from the high res structure input). This would allow a deeper discussion of lipid sites and conformational changes in the BetP cycle.
12. figures - already mentioned a need for an overview figures to hep the reader understand the whereabouts of individual TM segments. Many figures on lipid sites, but none relating strongly to the points of lipid sites and conformational changes associated with transport.
13. Table S1 - please indicate values for CC-0.5 on data processing and include outer resolution bins for the R-factors. What were the derived tensors of the anisotropic scaling/ellipsoidal truncation?
14. Table S2 is not worth much without error ranges associated with the derived retention factors. Should be combined with the later suppl. figures on the gels where the migration measurements are indicated on the gels

Referee #2

The manuscript entitled "Structural evidence for functional lipid interactions in the betaine transporter BetP" describes a new crystal structure of the BetP membrane protein. This study is focused on the structure of lipid molecules found associated with the protein in the crystal and on the network of interactions established between the lipids and the protein.

It is well established that lipids determine the properties of membrane proteins however it is still unclear how this occurs at the molecular level. This study tries to address this important issue. Unfortunately the authors present a manuscript that describes a beautiful structure together with a large amount of unsubstantiated speculation.

1- The first issue that the authors have to address is to state what exactly are the maps presented in Figures 1,3,4 and several supplementary figures. These maps are labeled as 2Fo-Fc but what it really matters is how the phases were calculated. It is essential to know if the phases were calculated from the final model (protein +lipids) or are they calculated from models without lipids during refinement. If it is the former (protein+lipids) then the maps are useless as validation of the correct positioning of the lipid molecules and have to be replaced by simulated annealing omit maps where the lipid molecules are first removed.

2- The other issue I have with this paper is that some of its ideas are not clear. This is particularly evident in section entitled "structural and functional role of lipids within the BetP trimer". For example in page 12 at the top, the authors say " In either scenario, having a lipid-binding site directly associated with the heart of the conformation changes would be beneficial to communicate changes to the state of the membrane, an important factors in ...." .
I am not sure what this means since it implies that the protein affects the state of the membrane which does not seem to fit what is said about the properties of the transporter in the introduction.

3- In addition, this section ("structural and functional role of lipids within the BetP trimer") finishes with a paragraph discussing AFM data presented in a previous publication. Reading this other paper it is not obvious at all the need for a lipid interaction seen in the new structure for explaining the previously described AFM results. The authors need at least to present a figure in the supplemental information that substantiates their argument.

4- In page 14, 2nd paragraph, the authors describe two lipids in opposing membrane leaflets which interact in the crystal structure and that this interaction may explain long distance coupling between the two faces of the transporter. Why is this specific lipid interaction a better explanation for the functional properties of the protein than long distance coupling through the protein itself? While long distance coupling has been demonstrated experimentally in many proteins the idea that the authors raise depends on a specific interaction between two specific lipid molecules. At the moment this is pure speculation.

5- Another paragraph that is pure speculation is the argument of evolutionary link between the positively charged residues in the termini of the helices in the BetP transporter and the composition

 

of the membrane in the organism from where the protein is cloned. This sort of evolutionary link is speculation which is not substantiated with proper data and serves no purpose.

6- Section entitled "impact of lipid interactions on catalytic core" raises serious issues. The authors observe in the crystal structure the side-chain of residue Met 150 within interaction distance of the acyl chain of lipid L7. This residue is placed in a functional important region of the protein. The authors mutate the residue and observe interesting functional changes in the protein. The authors conclude that the functional changes result from the alteration in the interaction between side-chain and lipid. This conclusion is surprising, to say the least, since this experiment only demonstrates the impact of mutations in a particular residue. It does not prove that the interaction seen in the crystal structure between that residue and a specific lipid has any importance.

I am sure that the authors are aware that the energetic importance of interactions observed in 3D-structures is highly variable. Some interactions are very important and others are not. This means that seeing two residues or a residue and a lipid within interacting distance cannot be interpreted as meaning that the interaction is energetically important. This has to be demonstrated experimentally through mutant cycle analysis or other approaches. A simple experiment like the one performed is not sufficient. I understand the experimental difficulty for this particular case (residue -lipid) but this does not excuse the lack of rigor shown in the interpretation of these results.

1st Revision - authors' response 14 July 2013

We wish to thank the reviewers for a careful evaluation and the insightful comments that helped us to improve our manuscript. In general, we have shortened the text excluding all aspects of speculation. The abstract and introduction have been re-written to provide a general description of lipids in membrane protein regulation and keeping only the essentials of previous BetP knowledge. We re-organized the main text to separate the results and discussion sections for an easier read. Moreover, we provide a point-by-point response to the specific points raised by the reviewers.

**Referee #1**

*1. the abstract ends in pathos, which could be downplayed a bit*

We have changed the last sentence in the abstract so that it reflects the relevance of lipids for BetP: "The lipid-protein interactions observed here in structural detail in BetP provide molecular insights into the role of lipids in osmoregulated secondary transport."

*2. The introduction makes some shortcuts on previous studies of lipid sites in transporters. The cited papers by Lee AG have a large focus on transporters, certainly not just regulatory lipid interactions with channels. The literature on regulatory interactions of lipids with transporters is rich, including also LeuT fold transporters and various ATPases. Also several MD studies would be relevant, e.g. Mondal et al. 2013 (http://www.ncbi.nlm.nih.gov/pubmed/23376428)*

This section has been re-written to include a broader description of lipid interactions in membrane proteins (channels and transporters) with recent studies on LeuT also cited: Pg 3 "Transmembrane proteins need to function…secondary transporters".

 

*3. The trimer is described as a conformationally symmetric (first subtitle under Results) and the manuscript then moves on to describe differences between monomers (bottom page 6) - this seems unnecessarily confusing. Probably better to describe the trimer as e.g. functionally symmetric.*

We would rather prefer not to use functionally symmetric as to date we do not know how the different orientations of the osmosensing C-terminal domain in BetP affect the functional state (activated or inactivated). But we see the point of the referee and avoided the term "conformationally symmetric". Instead we describe the trimer as either "trimer with all three protomers adopting an inward facing state" or as "transporter state-symmetric" through the paper.

*4. The description gets very detailed and refers repeatedly to a proprietary nomenclature on helix numbers (e.g. TM(-1), TM5' etc) - there is a dire need of an overview figure in the main text introducing these concepts in relation to the overall structure and LeuT fold, as they are referred to throughout the manuscript. The SI material is too remote and does not present it well either. Also a Ci naming of the inward-open state is presented with no sufficient explanation (page 7) - and is it at all needed?*

A relevant figure denoting the nomenclature of TM helices has been included as Figure S1c in the supplementary. We have changed the description to introduce the $C_i$ naming convention at the beginning of the results section titled *Crystal structure of a functionally symmetric BetP trimer.* The $C_i$ state has been named so in keeping with the convention introduced in Perez et al 2012 for the different states in the transport cycle.

*5. The lipid content analysis (page 8-9) is very detailed, and details repeated again for the methods section (where they belong)*

This section has been shortened with details included only in the supplementary.

*6. Having presented earlier the concept of annular and non-annular lipid sites it would be better to maintain this distinction throughout rather than referring to annular sites as e.g. "flexible lipids present along the periphery of the trimer" (page 9)*

All references to flexible lipids have been changed to 'annular lipids'.

*7. Page 10 suddenly introduces "the down-regulated BetP trimer" as the functional interpretation of the functionally symmetric trimer. This seems like an important concept that shoudl also be addressed in the introduction. A consideration of e.g. Mondal et al. would also seem appropriate on this note.*

 

We agree with the referee here, the comparison with Mondal et al is very appropriate. Therefore, we have re-written this paragraph and discuss now the effect of lipids on structural elements involved in conformational changes both in BetP and LeuT (Mondal et al, 2013): " In the light of transport regulation in BetP, specific lipid interactions with structural elements involved in the alternating access mechanism might represent an elegant way to populate distinct transporter states contributing to in/activation. A similar concept was proposed previously for LeuT (Mondal et al, 2013). MD simulations revealed that in LeuT lipid-protein interactions are different in the functionally distinct conformations (outward-open, occluded, inward-open) and that the differences are mainly connected to structural elements (e.g., TM1a), which play key roles in transport."

*8. Pages 11-14 on the functional role of the lipid sites within the trimer are a tough read, but considerations of the effect of (or on) conformational changes associated with the BetP funtional cycle do not stand out*

This is an important point and we have restructured the information on Pages 11-14 into the results section, which only describe the architecture of the observed sites and the discussion where possible effects of lipid binding on the functional cycle have been considered.

*9. The comparison to AFM data is difficult to follow (pg. 12-13) - what is the logic here?*

We understand that introducing the AFM results drifts away from focus and have excluded them from the manuscript to keep with the flow.

*10. the final discussion addresses osmosensing and has a good point on the amphipathic helix h7. I would otherwise advice that a stronger BetP focus is maintained which relates the observed sites to models of the functional cycle of BetP. The comparison to AdiC for example appears less rewarding (figure 5)*

Generalizing to the LeuT-fold and references to AdiC have been deleted.

*11. It would seem possible to expand further on the observed sites in relation to previous BetP structures, such as by re-refinement (fig. S6 lends very strong support for an identical site once it would be modeled and the lower res structure be improved from the high res structure input). This would allow a deeper discussion of lipid sites and conformational changes in the BetP cycle.*

A re-refinement of the lower resolution 3.1Å (PDB 4AIN) and 3.25Å (PDB 4DOJ) structures indeed brought up density for the central lipid L4 and is shown in Figure S4. This section has been re-written on pg 15 "since spots of lipid density….crystallization conditions themselves".

*12. figures - already mentioned a need for an overview figures to help the reader understand the*

 

*whereabouts of individual TM segments. Many figures on lipid sites, but none relating strongly to the points of lipid sites and conformational changes associated with transport.*

The figures have been redone to show the effect of lipids on conformational changes (Figure 1b, 2, 3, 4). A new color scheme has been introduced in the figures, where only those helices providing lipid coordination residues and which are also involved in conformational changes are highlighted and colored, following the convention used to describe elements involved in the conformational cycle in Figure 1b.

*13. Table S1 - please indicate values for CC-0.5 on data processing and include outer resolution bins for the R-factors. What were the derived tensors of the anisotropic scaling/ellipsoidal truncation?*

We thank the referee for this valuable suggestion to include CC-0.5 numbers for data processing. These values, added into Table S1, emphasize the good quality of data used during refinement. Since we now report a measure for the strength/quality of the data used for structure determination, which also justifies the cut-off employed, a comparison of statistics using lower resolution cut-offs and anisotropy correction (former Table S1, former figure S13) is redundant. Neither the lower resolution cut-offs nor the anisotropy corrected data were used in the final refinement and so, in order to avoid misleading the reader, we have deleted this section from consideration in the revised Supplementary section. As suggested, we have also included outer resolution bins used for the reported R-factors.

*14. Table S2 is not worth much without error ranges associated with the derived retention factors. Should be combined with the later suppl. figures on the gels where the migration measurements are indicated on the gels*

Error ranges for the relative retention factors calculated have been combined with Figures S6a and S6b.

**Referee #2**

*1- The first issue that the authors have to address is to state what exactly are the maps presented in Figures 1,3,4 and several supplementary figures. These maps are labeled as 2Fo-Fc but what it really matters is how the phases were calculated. It is essential to know if the phases were calculated from the final model (protein +lipids) or are they calculated from models without lipids during refinement. If it is the former (protein+lipids) then the maps are useless as validation of the correct positioning of the lipid molecules and have to be replaced by simulated annealing omit maps where the lipid molecules are first removed.*

 

We understand the concern of model bias especially at a moderate resolution and have been careful to avoid this issue. The phases for the described maps come from molecular replacement and refinement with **protein** only. During the iterative refinement steps many clear tubular densities came up which we began systematically testing with different ligands, including detergents from the crystallization. None of these were able to satisfy the densities as best as lipid moieties, which then iteratively led to improved phases and better clarity at the binding sites. The final 2Fo-Fc maps shown in the figures arise after several iterative refinement steps during which the entire protein was first modeled in. The ligands were then placed only **after** clear density was visible. 2Fo-Fc maps **prior** to ligand placement during the iterative refinements have been shown in supplementary Figure S4. In order to better appreciate this density we have provided a zoom of the 2Fo-Fc map from refinement rounds prior to placement of the central lipid L4, which has been consistently observed in different structures of BetP. From all the observed densities, care was taken to only build those that could be identified best. Our confidence in the type of lipid head group was reiterated by the TLC results. To clarify this point as suggested, we have included a line describing the 2Fo-Fc maps in legends for Figures 2, 3 and 4 and now call the maps 'final 2Fo-Fc'.

*2- The other issue I have with this paper is that some of its ideas are not clear. This is particularly evident in section entitled "structural and functional role of lipids within the BetP trimer". For example in page 12 at the top, the authors say " In either scenario, having a lipid-binding site directly associated with the heart of the conformation changes would be beneficial to communicate changes to the state of the membrane, an important factors in ...." .*
*I am not sure what this means since it implies that the protein affects the state of the membrane which does not seem to fit what is said about the properties of the transporter in the introduction.*

One of the major consequences of osmotic stress in *C. glutamicum* is a change in the state of its membrane, which is one of the signals sensed by BetP. To clarify this aspect we have re-written this section as: "To date it is not known if this regulatory restriction from the membrane results in up- or down-regulation of the transport activity, which is attributed to the fact that mutations in the ionic network of loop 2 are not tolerated. However, having key components in the functional conformational changes directly accessible by the membrane may prove to be beneficial to communicate changes in the state of the membrane due to osmotic stress, an important factor in osmoregulation (Wood, 1999), through the core of the protein."

*3- In addition, this section ("structural and functional role of lipids within the BetP trimer") finishes with a paragraph discussing AFM data presented in a previous publication. Reading this other paper it is not obvious at all the need for a lipid interaction seen in the new structure for explaining the previously described AFM results. The authors need at least to present a figure in the supplemental information that substantiates their argument.*

 

See answer to point 9, referee 1.

*4- In page 14, 2nd paragraph, the authors describe two lipids in opposing membrane leaflets which interact in the crystal structure and that this interaction may explain long distance coupling between the two faces of the transporter. Why is this specific lipid interaction a better explanation for the functional properties of the protein than long distance coupling through the protein itself? While long distance coupling has been demonstrated experimentally in many proteins the idea that the authors raise depends on a specific interaction between two specific lipid molecules. At the moment this is pure speculation.*

We acknowledge long-distance coupling through the protein as critical to the function of BetP and have deleted this paragraph in order to avoid speculation. In keeping with this we have also deleted comments on the regulatory networks on either side of the transporter being affected by these lipid interactions.

*5- Another paragraph that is pure speculation is the argument of evolutionary link between the positively charged residues in the termini of the helices in the BetP transporter and the composition of the membrane in the organism from where the protein is cloned. This sort of evolutionary link is speculation which is not substantiated with proper data and serves no purpose.*

This section has been deleted.

*6- Section entitled "impact of lipid interactions on catalytic core" raises serious issues. The authors observe in the crystal structure the side-chain of residue Met 150 within interaction distance of the acyl chain of lipid L7. This residue is placed in a functional important region of the protein. The authors mutate the residue and observe interesting functional changes in the protein. The authors conclude that the functional changes result from the alteration in the interaction between side-chain and lipid. This conclusion is surprising, to say the least, since this experiment only demonstrates the impact of mutations in a particular residue. It does not prove that the interaction seen in the crystal structure between that residue and a specific lipid has any importance.*

We agree with this concern raised by the reviewer, which is in fact a very insightful and important comment. Given the nature of the interaction (residue-lipid), it is non-trivial to discriminate between functional changes due to mutating a specific residue and changes due to lipid interactions that may be consequently altered. Therefore, we now also included a comparison of Km and Vmax values for the two key mutations at position 150, Met against isoleucine and phenylalanine, respectively, in a new Table S2. While Km and Vmax values for Met150Ile suggest that only regulation and not substrate binding is altered by this mutation, Met150Phe shows a dramatic increase in both Km and Vmax values, while the osmoprofile is not significantly changed. We also included a comparison of

 

osmoprofiles for other glycine to alanine mutations in the modified Figure 4c, which further helped to distinguish between the effect of mutagenesis on transport and regulation. Transport is strongly affected by mutations that decrease the flexibility of the stretch in a manner independent of osmotic stress, as it is observed for Met150Phe and Gly153Ala. Mutations like Met150Ile affect the flexibility apparently in a manner dependent on osmotic stress. One possible reason is the altered interaction with L7. Based on the newly added data, we conclude now in the manuscript that certain architectures of the stretch might be maintained by the interaction with the L7 lipid and the strength of this interaction affects the regulatory properties of BetP. However, we tuned down the speculation on a regulatory interaction of L7. This section has been rewritten under “Impact of lipid interactions on the catalytic core” on page 13.

2nd Editorial Decision 18 August 2013

Thank you for submitting the revised version of your manuscript entitled 'Structural evidence for functional lipid interactions in the betaine transporter BetP'. I have now received the reports on your paper, which you can find below.

Both referees appreciate the introduced changes and support publication here. However, before formal acceptance a number of issues have to be resolved.

Referee #1: Please address his remaining concern (1), and, if you wish, you can also include the points (2) and (3) suggested by this referee in your discussion.

Referee #2 thinks that the discussion part is too speculative and should be trimmed down.
To address this referees' criticism, I suggest the following:

- Second paragraph of the report: please refer to other possibilities (e.g. changed interactions within the protein) that could change the transport activity of the Met150 mutant.
- Third and fourth paragraph of the report: You could change the headings into "Possible impact of lipid interactions on catalytic core" and "Possible role of lipids in the trimer assembly of BetP".
- I agree with referee #2 that it would be helpful to trim down the discussion a bit.
- Please also revise the text of the discussion once more to make sure that all speculation is clearly visible as such.

I appreciate the changes that you have done for the introduction, which reads really well, and I also appreciate the separation of the results and discussion parts.

Please do not hesitate to contact me in case of further questions.
I am looking forward to reading your revised manuscript!

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REFEREE COMMENTS

Referee #1

The authors have responded well to the points raised by the reviewers, and the revised version reads far better. Only few points spring to mind

1. the following statement is being made, but is difficult to follow:
"Since we observe that lipid binding is typically pronounced in the inward-facing conformation, it follows that at least one of the three monomers in the trimer would always be assembled first in the inward-facing state. This provides a seeding point for forming asymmetric functional trimers, as seen in structures reported previously (Perez et al, 2012)." - how can they conclude that?

 

2. The lipid bundle brings to mind the pentameric ligand-gated ion channel:
http://www.ncbi.nlm.nih.gov/pubmed/23403925 - anything to compare to here?

3. There are also studies of overall loose and flexible membrane interactions of membrane transporters
http://www.ncbi.nlm.nih.gov/pubmed/21556058 - anything to compare to here?

Referee #2

The authors have simplified greatly the results section of the manuscript. Unfortunately, they have transferred a lot of the speculation into the discussion section, which is ~7.5 pages long while the results section is much shorter, ~5.5 pages long. I agree that it is perfectly acceptable to speculate and to expose possible explanations for the results obtained but there is a certain threshold that cannot be crossed. For example, in the second section of the discussion "Lipids associated with helices involved in conformational changes" the authors describe that some of the lipids uncovered in this structure are associated with functionally important regions of the protein. In their last sentence the authors speculate that "having key components in the functional conformational changes directly accessible to the membrane may prove beneficial to communicate changes in the state of the membrane...." This, I would argue, is acceptable speculation since it does not extend itself too much and is clearly put forward as an hypothesis that agrees with the experimental data.

However in the following section "Impact of lipid interactions on catalytic core" the authors no longer follow this approach. In this section the authors discuss an interaction seen between the tip of the acyl chain of a lipid and a residue in an important part of the protein, Met150. In my original review I had already alerted the authors to serious problems with their over-interpretation of this data. The authors have merely moved the section, including some experimental data, from the results section to the discussion. This data shows that mutations in the catalytic core affect the properties of the transporter and that mutation of Met150 to Ile shifts the osmoprofile of the mutant protein and alters Vmax. The authors speculate that this mutation favors a stronger binding with the lipid. The problem is that there is nothing in the data that supports that the lipid interaction observed is in anyway the underlying cause of the altered functional properties. Any of the interactions established between the same residue side-chain and the neighboring residues could as well be the cause of the changes detected. For example, in the structure of the BetP with choline (PDB code: 3P03) I detected possible interactions of Met150 with 3, 2 and 5 residues depending on which subunit I looked. Why is it then that the alteration of the interaction between the residue and the lipid is put forward as the cause of the altered functional properties, while the interactions within the protein are ignored?
As I have stated in my original review, the authors have not provided any data to demonstrate the functional importance of the specific interaction observed in the structure. To make it worse the authors continue their speculation by writing "Consequently, the enhanced dwell time of lipid interaction with the core due to the mutation may have altered the energy barrier for transitioning among conformational states resulting in slower transport." The authors have no data to support any speculation about "dwell time of the interaction" or "energy barrier". They provide Km and Vmax values which, has they are well aware, are both complicated functions of the many different equilibrium and kinetic parameters involved in the transport reaction and although useful in a general fashion, cannot be used to discuss detailed changes in the mechanism of an enzymatic reaction. Therefore I argue that this whole section is inappropriate speculation. Importantly, the title of the section "Impact of lipid interactions on catalytic core" is also inappropriate since they have not demonstrated at all the impact of the lipid interaction on the catalytic core, they have only observed a structural interaction of a lipid with the catalytic core.

Another section in the discussion where speculation goes beyond any of the data is the section entitled "Role of lipids in the trimer assembly of BetP". Unlike what the title may indicate the authors provide no data that supports a role of the lipids in the trimeric assembly. What they show is that in their new structure there are lipids bound within the central hole formed by the trimeric assembly. They speculate that like in other cases reported in the literature these lipids may have a role in trimer assembly. This is an acceptable level of speculation. However, the authors then launch

 

themselves about the possible stabilization of the monomeric structure and that the lipids may actually stabilize one of the conformational states of the monomer during assembly and that this would be a seeding point for assembly of the trimer. The authors have no data to support this level of speculation. They do not know if the monomers bind the lipids in the same fashion as the trimer and they do not seem to know anything about the folding and assembly pathway of BetP since there is no data or discussion about this issue. I also feel that the title of this section is misleading since in reality the authors offer no proof for a role of lipids in the assembly of the trimer.

Additionally, in the discussion section "Lipid association affects conformational state in BetP" the authors refrain from over-speculation in the text but the title of the section is misleading since they offer no proof for lipids affecting the conformational state of BetP.

Overall, I feel that the discussion section has to be trimmed down and the speculation has to be brought down to an acceptable level.

2nd Revision - authors' response 28 August 2013

Thank you for a positive evaluation of our revised manuscript. We have addressed the remaining concerns of the reviewers and included your suggestions in this version of the text. In general, we have changed the sub titles for those sections that include speculative interpretations to reflect the same. We have also shorten the discussion as proposed (see end of rebuttal letter). We further wish to provide a point-by-point response to the specific points raised by the reviewers.

**Referee #1**

*1. the following statement is being made, but is difficult to follow:*
*"Since we observe that lipid binding is typically pronounced in the inward-facing conformation, it follows that at least one of the three monomers in the trimer would always be assembled first in the inward-facing state. This provides a seeding point for forming asymmetric functional trimers, as seen in structures reported previously (Perez et al, 2012)." - how can they conclude that?*

We have changed the sentence to make it clearer: “We observe that lipid binding is typically pronounced in the inward-facing conformation. Therefore, the asymmetric lipid binding if originating already during assembly may provide a seeding point for forming asymmetric functional trimers, as seen in structures reported previously (Perez et al, 2012).”

*2. The lipid bundle brings to mind the pentameric ligand-gated ion channel:*
*http://www.ncbi.nlm.nih.gov/pubmed/23403925 - anything to compare to here?*

Since we could not draw any direct parallels to the lipid-filled central core in BetP, we have refrained from making a comparison with pLGIC.

*3. There are also studies of overall loose and flexible membrane interactions of membrane transporters*

*http://www.ncbi.nlm.nih.gov/pubmed/21556058 - anything to compare to here?*

This citation is useful to fortify the argument of the membrane directly influencing conformational states and we thank the referee for pointing it out. We have included it on page 16: "That the membrane can play a key role in aiding smooth conformational state transitions was also proposed for sarco (endo) plasmic reticulum $Ca^{2+}$-ATPases, when small adaptations in protein side chains and helix tilts were observed in response to local membrane deformations (Sonntag et al, 2011)".

**Referee #2**

*1- However in the following section "Impact of lipid interactions on catalytic core" the authors no longer follow this approach. In this section the authors discuss an interaction seen between the tip of the acyl chain of a lipid and a residue in an important part of the protein, Met150. In my original review I had already alerted the authors to serious problems with their over-interpretation of this data. The authors have merely moved the section, including some experimental data, from the results section to the discussion. This data shows that mutations in the catalytic core affect the properties of the transporter and that mutation of Met150 to Ile shifts the osmoprofile of the mutant protein and alters Vmax. The authors speculate that this mutation favors a stronger binding with the lipid. The problem is that there is nothing in the data that supports that the lipid interaction observed is in anyway the underlying cause of the altered functional properties. Any of the interactions established between the same residue side-chain and the neighboring residues could as well be the cause of the changes detected. For example, in the structure of the BetP with choline (PDB code: 3P03) I detected possible interactions of Met150 with 3, 2 and 5 residues depending on which subunit I looked. Why is it then that the alteration of the interaction between the residue and the lipid is put forward as the cause of the altered functional properties, while the interactions within the protein are ignored?*

This is an important concern raised by the reviewer. We agree with the fact that our data does not exclude differences in interaction with protein residues in the introduced mutants that could additionally lead to these effects. We have included this in our revised text on page 14: "While these mutations could possibly alter local protein-protein interactions around the unwound stretch, they could also directly affect the observed local protein-lipid interaction. Due to its length and hydrophobic nature isoleucine may complement the hydrophobic cleft for lipid binding and favor a stronger interaction with the acyl chain than methionine or a bulky phenylalanine. We therefore presume that the distinct architecture provided by the glycines and methionine, needs to be maintained for the plasticity required in stretch movements during the transport cycle and suggest that the lipid-protein interaction of L7 with the catalytic core has an impact on regulation in BetP".

 

We have also changed the title of this sub-section in "*Structural interaction of annular lipid with the catalytic core*".

*2. As I have stated in my original review, the authors have not provided any data to demonstrate the functional importance of the specific interaction observed in the structure. To make it worse the authors continue their speculation by writing "Consequently, the enhanced dwell time of lipid interaction with the core due to the mutation may have altered the energy barrier for transitioning among conformational states resulting in slower transport." The authors have no data to support any speculation about "dwell time of the interaction" or "energy barrier". They provide Km and Vmax values which, has they are well aware, are both complicated functions of the many different equilibrium and kinetic parameters involved in the transport reaction and although useful in a general fashion, cannot be used to discuss detailed changes in the mechanism of an enzymatic reaction. Therefore I argue that this whole section is inappropriate speculation. Importantly, the title of the section "Impact*
*of lipid interactions on catalytic core" is also inappropriate since they have not demonstrated at all the impact of the lipid interaction on the catalytic core, they have only observed a structural interaction of a lipid with the catalytic core.*

Keeping in mind the complexity of interpreting the kinetic data, we have excluded the section about energy barrier and dwell time from the discussion.

*3. Another section in the discussion where speculation goes beyond any of the data is the section entitled "Role of lipids in the trimer assembly of BetP". Unlike what the title may indicate the authors provide no data that supports a role of the lipids in the trimeric assembly. What they show is that in their new structure there are lipids bound within the central hole formed by the trimeric assembly. They speculate that like in other cases reported in the literature these lipids may have a role in trimer assembly. This is an acceptable level of speculation. However, the authors then launch themselves about the possible stabilization of the monomeric structure and that the lipids may actually stabilize one of the conformational states of the monomer during assembly and that this would be a seeding point for assembly of the trimer. The authors have no data to support this level of speculation. They do not know if the monomers bind the lipids in the same fashion as the trimer and they do not seem to know anything about the folding and assembly pathway of BetP since there is no data or discussion about this issue. I also feel that the title of this section is misleading since in reality the authors offer no proof for a role of lipids in the assembly of the trimer.*

See answer to Referee 1 point 1. In addition we have changed the title of this section to: "Putative role of lipids in trimer assembly of BetP".

*4. Additionally, in the discussion section "Lipid association affects conformational state in BetP" the*

*authors refrain from over-speculation in the text but the title of the section is misleading since they offer no proof for lipids affecting the conformational state of BetP.*

The title of this sub-section has been changed to: "Lipid association may affect conformational states in BetP"

We have deleted a part of the discussion on 2D crystals, which we felt was not helping to understand the lipid-interactions observed in 3D crystals (page 18: "2D crystals are in a more native environment compared to the mixed detergent and lipid surroundings of a 3D crystal and can therefore be direct indicators of membrane-related events. BetP shows larger inter-chain distances in the 3D fitting data from 2D maps when compared to the X-ray structures. This was attributed to differences in the tilt angle of each chain with respect to the membrane plane in the 2D crystal data(Tsai et al, 2011), indirectly suggesting a different membrane curvature.").

 