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Structure of the archaeal Na^+/H^+ antiporter NhaP1 and functional role of trans-membrane helix 1

Panchali Goswami, Cristina Paulino, Dilem Hizlan, Janet Vonck, Özkan Yildiz and Werner Kühlbrandt

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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.)

1st Editorial Decision 18 May 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. I have received the final report from the three referees asked to evaluate your manuscript and I enclose their comments below.

As you will see from their reports the referees express potential interest in the structural information of NhaP1, however, they also provide mixed recommendations and it is clear that some further experimental analysis is required to make the study suitable for publication in the EMBO Journal. The referees find that the importance of the extra TM helix is currently unclear and both referee #2 and #3 would like to see the impact of removal of this helix on the function and/or structure of the antiporter. Referee #2 would also like to see the addition of a projection map of R347A mutant at pH8 to show that his residue is the important pH sensor. It is important to note that addressing the concerns of referee #2 is important for him/her to recommend publication in EMBO J. Should you be able to address the referees concerns we would be willing to consider a revised version of the manuscript.

I would like to remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your revisions included in the next, final version of the manuscript.

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

revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

With the three-dimensional map of the archaeal sodium/proton antiporter NhaP1 obtained at 7Å resolution by electron crystallography, this study provides the first structural information of a member of the cation proton antiporter 1 (CPA1) family, which also includes the pharmacologically relevant human NHE sodium/proton antiporters. NhaP1 shares a higher sequence homology with NHE1 than with NhaA, the main sodium/proton antiporter from E. coli, which is the only other structurally characterized representative of the CPA superfamily and which belongs to the CPA2 family. The pH regulation of transport activity of NhaP1 and NHE1 is similar and orthogonal to that of NhaA, with the former being active below pH 7 whereas the latter is active above pH 7.

The interpretation of the cryo-EM map and the model build on the basis of the NhaA X-ray structure describe a similar fold for the 6-helix bundle transport domain, which very likely also includes the same ion transport motif of two partially unwound helices. Differences in fold are present for the other helix-bundle at the dimer interface including an additional N-terminal transmembrane helix. Based on highly similar projection maps of the inactive NhaP1 R347A mutant and of wildtype NhaP1 in inactive conformation at pH 8, the authors suggest a central role for Arg347 in transport for NhaP1 and for human NHE transporters.

This thoroughly conducted and clearly presented study provides significant new structural information for this important family of membrane proteins and is of general interest for understanding structure and function of secondary active transporters. Yet, there are a number of questions and comments the authors should address. In particular, the argumentation concerning the functional importance of Arg347 is not fully conclusive and needs clarification.

Results and Discussion.

1. Electron crystallography. It should be stated that crystal symmetry is the same as in Vinothkumar et al. 2005 and whether unit cell dimensions changed.

2. Sequence comparison. The areas marked in cyan in figure 4 probably indicate the alpha-helical secondary structure based on the sequence alignment, which should be stated in the legend. How does it compare to the known E.coli NhaA structure? Information should be included.

3. Molecular model of NhaP1. The model of helix VI was extended compared to NhaA by 10 Å to match the density. This modification is not supported by the sequence alignment in Figure 4. How do you explain?

An additional graphical presentation with the superimposition of the NhaP1 model and the NhaA structure is recommended and could be included in figure 5.

4. Structure of inactive mutant of NhaP1. The comparison is made between projection maps of wildtype NhaP1 and inactive mutant R347, both at pH4. According to Vinothkumar et al. 2005, the authors apparently assume that NhaP1 is inactive at pH4. This should be stated at the beginning of this section. That means the first comparison is made between wild type inactive at pH4 and an inactive mutant at pH 4. What is the relevance of these differences? The helix movements deduced from difference density peaks described in the second paragraph are compared to the substrate-ion induced differences in NhaA (Appel et al. 2009). How does this fit into the context?

The 3rd paragraph starts with the remark that the mutant R347 is virtually inactive at pH6, whereas the conclusion in that paragraph is that it has residual activity. Are there any transport data to support that assumption?

The authors proposed in Vinothkumar et al. 2005 a model with two different inactive conformations for pH<5 and pH > 7. The discussion here points out that the mutant at pH 4 has the same conformation as the wild type at pH8. Is there now a new model or is the mutation driving the transporter to the pH8 inactive conformation? Furthermore, it should be considered that an alanine substitution of arginine is a substantial change in side chain size and not only in ionization capability. A secondary effect may inactivate the transporter and the pKa of R347 cannot be estimated with the current data. Thus, the unidirectional conclusion that the protonation state of R347 acts as the control switch between pH 7 and 8 appears not to be justified.
Legend Figure 8. The pH for R347 (pH4, I assume) should be included in legend for clarity.

Referee #2 (Remarks to the Author):

The manuscript by Goswami et al. describes a density map of the sodium/proton antiporter NhaP1 at a resolution of 7 Å in-plane and 15 Å perpendicular to the membrane obtained by electron crystallography of two-dimensional crystals. The map is interpreted based on sequence homology and by manually fitting the atomic coordinates of the X-ray structure of NhaA into the density map. Comparison of the NhaP1 model with the NhaA structure demonstrates a common fold shared by members of the CPA superfamily, containing a structurally conserved six-helix bundle that is thought to contain the ion binding and translocation sites. The dimerization interface is less well conserved and in the case of NhaP1 comprises an additional helix that is not present in NhaA. This helix is discussed, but its physiological relevance is unclear. The authors also calculated a 7Å projection map of the inactive R347A mutant, which they compared with projection maps of the wild-type protein at pH 4 (active form) and at pH 8 (inactive form). Based on the similarity of the projection maps of the inactive mutant and the pH-inactivated wild-type protein, the authors suggest that residue R347 may be the pH sensor. Sequence alignment further shows that there is no equivalent residue in NhaA, leading the authors to conclude that the presence/absence of this residue in NhaP1/NhaA may be responsible for the inverse pH activation profiles of the two distantly related sodium/proton antiporters. While the work is solid, additional experiments would substantially strengthen the paper and raise it to the level expected from a publication in EMBO Journal.

Major points

1) The authors spend several paragraphs describing the additional helix present in NhaP1 compared to NhaA, but it is not even clear whether this helix is at all physiologically relevant or just an artifact of heterologous expression of NhaP1 in E. coli. The authors also state that among NhaP1 homologs this helix acts as a signal sequence that can be cleaved or maintained as part of the mature protein. The authors continue to describe how this helix may or may not be relevant in eukaryotic CPAs. This argument is difficult to follow and does not add much to the paper. To address the physiological relevance of the additional helix, the authors could simply express a construct in which the helix is missing and analyze the effects of the truncation. Alternatively, most of the discussion concerning the additional helix could simply be deleted, since the manuscipt is very wordy anyway.

2) The conclusion that R347 is the pH sensor is based on comparison of a 7 Å projection map of the R347A mutant at pH 4 with those of wild-type NhaP1 at pH 4 and 8. This argument appears sound, but would be substantially strengthened by the addition of a projection map of the R347A mutant at pH 8, which would demonstrate that this mutation renders the antiporter insensitive to changes in pH. A three-dimensional map of this mutant would be most helpful, particularly as the authors state that these crystals were of a higher quality than those made with wild-type NhaP1.

Minor points

1) The manuscript lacks page numbers, which makes it difficult to refer to specific sentences.

2) I am not sure whether "orthogonal" is the right word to describe the activity profiles of NhaA and NhaP1. Would "inverse" not be more appropriate?

3) Figure 1 is not essential for the main text and should be moved to supplementary material. The supplementary material should also show IQ plots of representative images recorded at tilt angles of 0 and 45 degree.

4) The authors state that the z-resolution of the structure is limited by the missing cone of electron crystallography. This statement is misleading. The severe anisotropy in resolution in the presented density map is not a general feature of electron crystallography but is due to the lack of highly tilted images in this particular data set (only images up to a tilt angle of 45 degree were included). The lack of highly tilted images is not a problem in this case, but the authors should describe the issue more accurately.

5) Did the authors indeed mean to say that "subsequent studies showed that it (the N-terminus) is cleaved of in vitro (Miyazaki et al, 2001)". It appears that "in vivo" would make more sense in this context.

6) The authors state that "the unwound region of helix IV in NhaA extend from A136 to A134". This must be incorrect.

7) The authors describe in the text the ATDI/ATDP sequence (4 residues) in in the unwound region of helix IV, but only box 3 residues in Figure 4. Similarly, the text describes the GIGFT/PRGVV sequence (5 residues) in in the unwound region of helix XI, but only box 4 residues in Figure 4.

8) The authors state that the 2D crystals formed by the R347A mutant were better ordered, which was confirmed by the high quality of electron crystallographic data collected from the mutant crystals. No data are presented that demonstrate that the electron crystallographic data collected from the mutant crystals were indeed better than those collected from the wild-type crystal.

9) The difference map between the R347A mutant and wt incubated at pH 8 shows only weak difference peaks, and the authors state that the two proteins have similar but not identical structures. Are the observed differences statistically meaningful or do they just represent noise?

Referee #3 (Remarks to the Author):

This paper describes 2D electron diffraction study at 7 A resolution of cation/proton antiporter protein NhaP1. Thank to the 3.45A-A resolution crystal structure of NhaA, but belonging to different subfamily, 12 transmembrane helices can be fitted into the electron density well, which enables various discussion. One interesting point is the two unwound TM helices harboring specific stretched peptide motifs, which present Asp in NhaA and Arg347 in NhaP1, that might be involved in ion translocation. Second, in NhaA, buried TM helix V harbors 2 Asp, while NhaP1 has one, which may be related to antitransport of two proton/Na or one proton/Na. Third, inverted TM helix repeat orientation highlights that NhaP1 has an extra Np-terminal TM helix that might be important for molecular dimerization, as compared to NhaA, while whether it is uncleaved signal sequence or not remains elusive. Finally, projection difference map reflecting TM helix movement upon ion translocation (active versus inactive) is intriguing.

All together, the structure determination, although resolution is low, is solid and the discussion is interesting to wide audience, and the work should be published in EMBO J., but after some revision.

1. This reviewer still concerns about the extra N-terminal TM helix. If the authors genetically posttranslationally removed the helix by introduction of specific protease site, how the structure as well as the function are changed ?

2. Illustration has some problem. The explanation of the unwound TM helices is not easy to be understood. In Figure 5, the author named the number of TM helices, but the author should also name TM helices in Figure 3 to clarify the discussion.

3. Also the discussion about projection difference map is not easy to be understood without referring to any Figure. In Figure 8, putting the TM helix names into the map may allow the readers to understand better the discussion.

4. In line 3 of the first paragraph of Results and Discussion section, "Both the size and the order of the crystals improved" may be "Both the size and the order of the crystals were improved".

Additional correspondence 27 January 2010

I am writing to discuss our long overdue revised manuscript # EMBOJ-2009-71145. There we are facing a bit of a dilemma. Having overcome an endless train of difficulties (electron microscope broken, crystals running out, new preparation necessary etc), we finally obtained the missing projection maps requested by the referee, but they did not make sense. It turned out that we had been sent a mislabelled plasmid by our colleagues in Hannover, and that took a while to sort out. We have now re-cloned the construct ourselves, but still the projection maps do not tell a straight story. It is possible that the His/STREP tags, which are different from our original construct, cause the problem. In short, we still need to sort this out, and it does not look as easy as I thought.

We have also conducted functional studies with various constructs, including in which the first helix has been removed, but the results are not yet conclusive.

The question is, where do we go from here? Would you consider a revised version without these two extra experiments? It is still an important piece of work, the structure is unique, and we would like to see it published soon.

Or should we retract the manuscript and start again once we have sorted out the problems I mentioned above? I have no idea how long this would take.

Additional correspondence 01 February 2010

Thank you for your letter, I am sorry to hear of all the difficulties that you have experienced with attempting to address the referees concerns. I have read through the manuscript once more and also the referee's comments and my letter. While I appreciate that you have put in a lot of effort to try and address the major issues I do not feel that we can proceed without the two experiments being incorporated into the manuscript. The reason for this is that these were both the major issues raised by the referees, referee #2 wanted the role of R347A as a pH sensor to be validated by acquiring the maps of the mutant at pH8, the mechanism of activating the transporter was also touched upon by referee #1. In addition, both referee #3 and #2 stated that they needed the functional relevance of the new TM helix 1 to be tested. Given that you have unable to address these concerns, even though this is due to unforeseen consequences, and that these were the important issues that were highlighted in my original decision letter as being required to address, it is not currently possible to proceed.

If you are able to resolve these issues in the future we could consider the study again as a new submission. While I would try to get the same referees to review the manuscript there is no guarantee that they would be available at that time and therefore, this may result in new referees being assigned. It should be noted that as a new submission the novelty of the study will be once more assessed.

I am sorry that I can not be more positive at this time,

Yours sincerely,

Editor

The EMBO Journal

Resubmission 28 July 2010

You may remember an earlier version of this manuscript (EMBOJ-2009-71145), which went through two rounds of revision last year before we finally decided that we needed more time to sort things out. This we have now done and accordingly are re-sending the manuscript to you as a new submission, as suggested in your mail of 1 February 2010.

The main result of the manuscript is the 7Å structure of the sodium/proton antiporter NhaP1 from *Methanococcus*, which has both interesting similarities and differences to its distant relative, NhaA from *E. coli*, the only other such antiporter with known structure. The NhaP1 structure is just as important and interesting now as it was a year ago, and we are fairly certain that no other group has made progress in this direction. NhaP1 is a much closer homologue than NhaA to the human sodium/proton antiporter NHE1, which has many important functions in health and disease. This makes the NhaP1 structure all the more interesting.

We found that, compared to NhaA, NhP1 has an extra trans-membrane helix. This helix appears to be an uncleaved signal sequence that is also present in NHE1. Since last year we have made several new constructs with and without this helix to see whether or not it is part of the mature protein, or merely left over from incorrect processing in the expression host. To our surprise we discovered that the helix is essential for function, as constructs without it are inactive, even though they are fully folded and able to form dimers, like the wt. The most likely explanation is that this helix, as an uncleaved signal sequence, determines the orientation of the protein in the membrane, and that the orientation has to be correct for the antiporter to function. Apart from the 7Å structure itself, this new and unexpected finding is now the main focus of our manuscript.

In our first submission, we had addressed an inactive NhaP1 mutant (R347A), as the constructs for studying the importance of the signal sequence were not yet ready. In the meantime we have done many more experiments to characterize this inactive mutant and to compare its structure to wt, which turns out to be another interesting story that is however considerably more complex than originally thought, and would go well beyond the scope of the present manuscript. We have therefore decided to publish the mutant studies separately.

We hope you find our new manuscript interesting.

2nd Editorial Decision 31 August 2010

Thank you for submitting your revised version of the manuscript for consideration at The EMBO Journal. All three original referees were able to review the manuscript once more and recommend publication pending some minor revisions. These include determining the orientation of helix 1, I leave the organisation of the figures to your discretion. Should you be able to adress the remaining concerns we would be happy to publish the manuscript in The EMBO Journal.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.
When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

With the three-dimensional map of the archaeal sodium/proton antiporter NhaP1 obtained at 7-Å resolution by electron crystallography, this study provides the first structural information of a member of the cation proton antiporter 1 (CPA1) family, which also includes the pharmacologically relevant human NHE sodium/proton antiporters. NhaP1 shares a higher sequence homology with NHE1 than with NhaA, the main sodium/proton antiporter from E. coli, which is the only other structurally characterized representative of the CPA superfamily and which belongs to the CPA2 family.

The interpretation of the cryo-EM map and the model build on the basis of the NhaA X-ray structure describe a similar fold for the 6-helix bundle transport domain, which very likely also includes the same ion transport motif of two partially unwound helices. Differences in fold are present for the other helix-bundle at the dimer interface including the additional N-terminal transmembrane helix 1. The authors now focus on the functional analysis of helix 1, a potential signal sequence. Two N-terminal deletion mutants were constructed and the truncation of helix 1 specifically resulted in loss of transport activity in everted vesicles of sodium-proton antiporter deficient E. coli cells without major effect on protein stability, alpha helix content, or dimer formation. These results open a highly interesting discussion about the role of helix 1. Yet, the question asked in the manuscript, whether helix 1 is an integral part of the antiporter or whether the presence of helix 1 in the recombinant protein is a lack of processing in E. coli is not answered. The authors rightly pointed out, that deletion of the signal peptide might lead to opposite orientation of the transporter in the membrane thus affecting its activity.

The thoroughly conducted and clearly presented study provides significant new structural information for this important family of membrane proteins and is of general interest for understanding structure and function of secondary active transporters. Publication in EMBO J is recommended after revision, which mainly is the request to demonstrate the orientation of the helix 1 deletion constructs in the membrane to permit valid interpretation of the loss of function and strengthen the biological implications.

Major point

Orientation of deletion mutants should be addressed. Accessibility of His-tag or myc-tag of wild-type and truncation mutants can be probed with antibodies in everted vesicles (e.g. Zuber et al. BBA 1709, 2005 or Rothman et al J Biol Chem 271, 1996).

Minor point.
N-terminus of deletion mutants should be marked in Figure 4

Referee #2 (Remarks to the Author):

The present revised manuscript fully satisfies my previous concerns and now should be published in EMBO J.

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The manuscript by Goswami and coworkers describes an electron crystallographic 3D density map of the archaeal sodium/proton antiportor NhaP1 at a resolution of 7 Å in-plane and 15 Å perpendicular to the membrane. The map is interpreted based on sequence homology and fitting of a homology model (based on the crystal structure of NhaA) into the density map. Comparison of the NhaP1 model with the NhaA structure indicates that members of the CPA superfamily have a common fold. A well-conserved six-helix bundle that is thought to contain ion binding and translocation sites was identified as well as a less well-conserved dimerization interface. In the

NhaA structure, the dimerization interface is mediated by b-sheets, while a series of tight helix-helix interactions were found in the NhaP1 model. In addition, the NhaP1 model contains a 13th transmembrane helix that the authors assign as helix 1 based on sequence alignments. Functional assays using two mutants lacking helix 1 showed that this additional helix is essential for function of NhaP1. The authors hypothesize that helix 1 may determine the orientation of the transporter in the membrane and the orientation matters for its activity.

This is an interesting and well-written manuscript. The experiments are well done and the interpretations are reasonable. The structure of NhaP1 is informative, and although it would be an even more interesting study if the authors could have determined the function of additional helix 1, the finding that it is essential for function is unexpected and novel. Before publication, the authors should address a few minor points:

- The manuscript in its current form has 9 figures, which appears excessive. Figures 1 and 9 should be moved into Supplementary Materials and Figures 3 and 5 or 5 and 6 could easily be combined.

- The numbers of the helices in Figure 5 are impossible to read.

- The authors should show as Supplementary Materials an image of the new, improved 2D crystals compared to the previous 2D crystals. It would also be informative for the reader if the authors would show IQ plots of crystals recorded at 0- and 45-degree tilt.

- Page 4, second-to-last line has a typo, it should be "... present in several ..."

- Page 5: The very first paragraph of Results and Discussion does not seem to make sense. The authors suggest that the low cmc of DDM may help in obtaining better crystals. However, DDM was presumably present in even higher quantities in the previous crystallizations because the protein was actually purified and kept in DDM.

- Page 5: The authors state that "In most cases, the two lattices yielded two sets of projection data per image." It is unclear whether the authors included data from both lattices or only the better ordered lattice from each crystal in the merged dataset.

- Page 8: The authors state that the "unwound region of NhaA helix IV extends from A136 to I134 and has the sequence ATDI." The numbering of the residues must be incorrect.

- Page 9: How was the fit between the helices and the corresponding density assessed?

1st Revision - authors' response 25 October 2010

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With the three-dimensional map of the archaeal sodium/proton antiporter NhaP1 obtained at 7 Å resolution by electron crystallography, this study provides the first structural information of a member of the cation proton antiporter 1 (CPA1) family, which also includes the pharmacologically relevant human NHE sodium/proton antiporters. NhaP1 shares a higher sequence homology with NHE1 than with NhaA, the main sodium/proton antiporter from E. coli, which is the only other structurally characterized representative of the CPA superfamily and which belongs to the CPA2 family.

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major effect on protein stability, alpha helix content, or dimer formation. These results open a highly interesting discussion about the role of helix 1. Yet, the question asked in the manuscript, whether helix 1 is an integral part of the antiporter or whether the presence of helix 1 in the recombinant protein is a lack of processing in E. coli is not answered. The authors rightly pointed out, that deletion of the signal peptide might lead to opposite orientation of the transporter in the membrane thus affecting its activity.

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Orientation of deletion mutants should be addressed. Accessibility of His-tag or myc-tag of wild-type and truncation mutants can be probed with antibodies in everted vesicles (e.g. Zuber et al. BBA 1709, 2005 or Rothman et al J Biol Chem 271, 1996).

In response to the referee's suggestion, we determined the activity of both wt and the NhaP1Δ22 mutant reconstituted into proteoliposomes to investigate the orientation-dependent activity in the membrane. From the $p22_12_1$ symmetry of the 2D crystals we know that NhaP1 inserts into the membrane in both orientations. If the loss of activity of the truncation mutant were due to its wrong orientation in the membrane, the antiporter should be active in proteoliposomes, since insertion in either direction is equally likely. These experiments show clearly that the first helix of NhaP is required for activity. Thus, the lack of activity in case of the truncated protein is not related to the orientation of the protein in the membrane. These new results are included in the revised manuscript on p12-13.

Minor point.
N-terminus of deletion mutants should be marked in Figure 4

The N-termini of both deletion mutants are now highlighted in the sequence alignment (Figure 3 in the revised manuscript)

Referee #2 (Remarks to the Author):

The present revised manuscript fully satisfies my previous concerns and now should be published in EMBO J.

The authors thank the reviewer for his/her comments on the initial manuscript.

Referee #3 (Remarks to the Author):

The manuscript by Goswami and coworkers describes an electron crystallographic 3D density map of the archaeal sodium/proton antiportor NhaP1 at a resolution of 7 Å in-plane and 15 Å perpendicular to the membrane. The map is interpreted based on sequence homology and fitting of a homology model (based on the crystal structure of NhaA) into the density map. Comparison of the NhaP1 model with the NhaA structure indicates that members of the CPA superfamily have a common fold. A well-conserved six-helix bundle that is thought to contain ion binding and translocation sites was identified as well as a less well-conserved dimerization interface. In the NhaA structure, the dimerization interface is mediated by b-sheets, while a series of tight helix-helix interactions were found in the NhaP1 model. In addition, the NhaP1 model contains a 13th transmembrane helix that the authors assign as helix 1 based on sequence alignments. Functional assays using two mutants lacking helix 1 showed that this additional helix is essential for function of NhaP1. The authors hypothesize that helix 1 may determine the orientation of the transporter in the

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This is an interesting and well-written manuscript. The experiments are well done and the interpretations are reasonable. The structure of NhaP1 is informative, and although it would be an even more interesting study if the authors could have determined the function of additional helix 1, the finding that it is essential for function is unexpected and novel. Before publication, the authors should address a few minor points:

- The manuscript in its current form has 9 figures, which appears excessive. Figures 1 and 9 should be moved into Supplementary Materials and Figures 3 and 5 or 5 and 6 could easily be combined.

In the revised manuscript the Figures 1 and 9 are moved into the supplementary material. The other figures were renumbered accordingly.

- The numbers of the helices in Figure 5 are impossible to read.

For better readability we increased the font size by 25% and changed their positions in the figure.

- The authors should show as Supplementary Materials an image of the new, improved 2D crystals compared to the previous 2D crystals.

An image of the new, improved 2D crystals of NhaP1 is compared now in the Supplementary Figure 1 with the previous crystals.

It would also be informative for the reader if the authors would show IQ plots of crystals recorded at 0- and 45-degree tilt.

The IQ plots of crystals recorded at 0°, 20°, 30°, and 45° is included now in the supplementary materials.

- Page 4, second-to-last line has a typo, it should be "... present in several ..."

corrected

- Page 5: The very first paragraph of Results and Discussion does not seem to make sense. The authors suggest that the low cmc of DDM may help in obtaining better crystals. However, DDM was presumably present in even higher quantities in the previous crystallizations because the protein was actually purified and kept in DDM.

In the previous crystallizations DDM was present at a concentration of 0.05% in the protein solution and the lipids were solubilized in 1% DM. The better crystals we obtained from protein solubilized in 1% OG and lipids solubilized in 1% DDM. After mixing of protein and lipids, the concentration of DDM in the latter case would be higher than in the previous crystallization, so that the statement would make sense.

- Page 5: The authors state that "In most cases, the two lattices yielded two sets of projection data per image." It is unclear whether the authors included data from both lattices or only the better ordered lattice from each crystal in the merged dataset.

As stated in the manuscript in most cases both lattices were included in calculation. Thus poor second lattices were excluded from the data set.

- Page 8: The authors state that the "unwound region of NhaA helix IV extends from A136 to I134 and has the sequence ATDI." The numbering of the residues must be incorrect.

corrected: the right residue number is A131 to I134.

- *Page 9: How was the fit between the helices and the corresponding density assessed?*

The fitting and assessment was done manually using coot (see page 9 and Material&Methods).

3rd Editorial Decision 08 November 2010

Thank you for submitting the revised version of of your NhaP1 manuscript. It has now been seen by one of the original referees who finds that you have addressed all their concerns. Therefore I am happy to accept the manuscript for publication in The EMBO Journal. You will receive the official acceptance letter in the next day or so.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The revised manuscript is recommended for publication in EMBO J.

