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Substrate specificity and ion coupling in the Na^+/betaine symporter BetP

Camilo Perez, Caroline Koshy, Susanne Ressl, Sascha Nicklisch, Reinhard Krämer and Christine Ziegler

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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 20 December 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the exceptionally long delay in getting back to you with a decision. Unfortunately, two of the referees were not able to get back to us with their reports as quickly as initially expected.

Your manuscript has now finally been seen by three referees whose comments are shown below. You will be pleased to see that the referees are generally positive about your work and that they would support its ultimate publication in The EMBO Journal after appropriate revision. I would thus like to invite you to prepare a revised manuscript in which you need to address the referees' criticisms in an adequate manner. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer 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 your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In their manuscript the authors have investigated the substrate specificity and transport mechanism of the sodium-coupled betaine cotransporter BetP and of point mutations that were designed based on sequence comparisons with homologous transporters. In transport assays the authors were able to show that a single point mutation widens the substrate specificity of BetP towards choline and that in this mutant Na+ can be replaced by protons as cotransported ion. A crystal structure of the point mutant that was determined at 3.4 Å resolution in the presence of choline has revealed a novel conformation of the molecule. Residual electron density was assigned to choline binding in a novel position in the protein. The successful conversion of the substrate selectivity and the novel structural information was used to formulate a hypothesis on the mechanism of proton coupled transport in certain members of the family.

Overall I think that this an interesting manuscript, which is clearly a candidate for publication in The EMBO Journal. The experimental data appears to be of high quality and the observed phenotypes are sound. There are, however, some weaknesses in the presentation of the data that should be addressed by the authors to make the manuscript comprehensible for a broader audience.

Specific issues:

The electron density displayed in Figure 4b does not allow a proper judgment of its quality. The authors should provide a stereo figure. It is also not clear how the substrate was placed into the electron density and whether it was calculated in the presence or absence of the ligand. It would be helpful if the authors could display a positive difference density with phases from a model that does not contain the substrate.

Since the crystallization was carried out in comparably high (5mM) concentrations of the substrate and a bound substrate was only observed in one subunit it is possible that the observed binding mode does not represent an intermediate position in the transport cycle.

It would be interesting to see comparison of the overall conformation of the structure of the mutant with the previously determined structure of the WT-protein to illustrate why the authors distinguish between an occluded and open inward-facing conformation.

The discussed transport mechanism and its depiction in Figure 6 is somewhat confusing. I wonder whether the authors could simplify the description.

Minor:

There are weaknesses in the language throughout the text.

Referee #2 (Remarks to the Author):

The article entitled, "Substrate specificity and ion coupling in the Na2+/betaine symporter BetP" describes a new inward open conformation of BetP generated by a single point mutation (G153D), which converts the transporter into a H+ coupled choline transporter. This article presents a clear and very interesting story which further shows the robustness of the LeuT-fold. I recommend publication and would ask the authors to make some minor changes and clarifications.

In a number of places the author cite Forrest 2010 to cover many generalities of the LeuT-fold, while this is an appropriate citation it does not represent the initial observations. I have highlighted a few below:
• Almost each individual structure of LeuT transporters... (more appropriate references: Structure and function of Na(+)-symporters with inverted repeats. Abramson J, Wright EM.)
• However,...first helix of the first repeat... (Coupling substrate and ion binding to extracellular gate of a sodium-dependent aspartate transporter. Boudker O, Ryan RM, Yernool D, Shimamoto K, Gouaux E.)

It would be more appropriate to use LeuT numbering of the TM helices. This allows for direct comparisons between all the different structures. Start numbering at the first inverted repeat.

The Na2 site should be defined. Does it deviate from the other sodium dependent structures (LeuT, SGLT and Mhp1)? All other structure has trthe Na2 site between TM's 1 and 8 and it appears BetP does as well.

Figure 1B. It would be nice to highlight the Na2 site. Which carbonyl oxygens are thought to coordinate Na2? It looks like D153 is just above these carbonyls. It would be nice to have a better picture in comparison to the Na2 site.

In the results section: Thus, we conclude... conformational flexibility to TM3... The flexibility of TMs 1 and 6 has been proposed on all LeuT papers and should be cited.

Uptake experiments are really nice and compliment the structure. I was wondering (unlikely) of the authors have calculated the stoichiometry of H+ to choline? It would be interesting if it is a 1:1 or 2:1. But, this may be difficult to do and acceptance should not depend on this result.

The authors refer to their previous structure as "occluded inward-facing" which differs from their original paper where they stated there was no clear cavity. Is this a new structure or have they modified their terminology. In light of these two structures the author should compare the differences to the recent paper on sglt where these same two conformations have been compared. In particular are the movements of TM1 the same? (The mechanism of sodium and substrate release from the binding pocket of vSGLT. Watanabe A, Choe S, Chaptal V, Rosenberg JM, Wright EM, Grabe M, Abramson J.)

Can the authors expand on their comparison with ApcT? The Lys in ApcT replaces the location of the Na2 site but I believe D153 is in quite a different position, which was surprising for me.

Referee #3 (Remarks to the Author):

The manuscript by Perez et al. describes an interesting mutagenesis study of the conserved GXGXG unwound region of TM3 from C. glutamicum BetP highlighting the importance of this region for conformational flexibility of TM3 in connection with betaine transport. Mutagenesis of the third glycine of this motif to aspartate intriguingly changed the specificity of BetP to choline in the presence of a H+ gradient while both betaine and choline are transported in the presence of a Na+ gradient. This position is also an aspartate in the closely related BetT and other choline transporters of the BCCT family. The structure of choline bound to the G153D mutant of BetP to 3.35 Å revealed a new inward facing conformation. Surprisingly, choline was shown to bind in a site suggested to be the second Na+ site in BetP. Based on the structure and modeling studies the authors present a rather speculative transport mechanism for choline.

The MS is interesting and addresses a key point of how Na+ vs. H+ driven transport is controlled and the project design is well-suited for that and shows convincing evidence of the Asp/Gly residue being a determinant .

The crystal structure highlights an additional surprise - an inward facing conformation with choline bound at the Na2 site. The structure however intersting it is, still leaves many questions open, disfavouring elaborate mechanisms to be exploited. It is recommended to put less weight on its significance - ~5 mM choline and pH 5.5 in the crystal is after all a special case, and the discussion of a possible transport mechanism therefore becomes shaky - either more structures are to be determined - e.g. soaking crystals at pH 7 and including also K+ and Mg2+ to see if the site actually makes sense in a more physiologically relevant setting - or the section on the transport mechanism should be shorter and more critical.

additional comments:

- Were mutated plasmids sequenced and confirmed?

- Was chain A or C of 2WIT used for MR? MS and supporting info say differently (not a big deal)

- Is the proton-driven transport inhibited by Na+, either side?

- Fig. 4 is in strong need of an unbiased difference map revealing choline. Even the 2Fo-Fc is not terribly convincing, and a blue density mentioned in the legend is not shown. Also the methods description should emphasize how the choline ligand and its specific modelling was validated at 3.35 Å resolution - this is not trivial.

- PROCHECK validation is obsolete, molprobity is recommended

1st Revision - authors' response 18 January 2011

Rebuttal letter to the reviewer's comments on

MS# EMBOJ-2010-76382

"Substrate specificity and ion coupling in the Na^+/betaine symporter BetP"

We are thankful to all reviewers for their constructive comments. All suggestions were taken into consideration and the pertinent response to each point brought up by the reviewers is presented below. In the revised version of the manuscript, we have included a number of new figures requested by the reviewers. We also made changes in the text according to their suggestions and added the suggested references. For the sake of clarity each point from the reviewers' comments is copied below followed by our response (in italics) and changes in the manuscript are underlined.

Referee #1 (Remarks to the Author):

In their manuscript the authors have investigated the substrate specificity and transport mechanism of the sodium-coupled betaine cotransporter BetP and of point mutations that were designed based on sequence comparisons with homologous transporters. In transport assays the authors were able to show that a single point mutation widens the substrate specificity of BetP towards choline and that in this mutant Na+ can be replaced by protons as cotransported ion. A crystal structure of the point mutant that was determined at 3.4 Å resolution in the presence of choline has revealed a novel conformation of the molecule. Residual electron density was assigned to choline binding in a novel position in the protein. The successful conversion of the substrate selectivity and the novel structural

information was used to formulate a hypothesis on the mechanism of proton coupled transport in certain members of the family.

Overall I think that this is an interesting manuscript, which is clearly a candidate for publication in The EMBO Journal. The experimental data appears to be of high quality and the observed phenotypes are sound. There are, however, some weaknesses in the presentation of the data that should be addressed by the authors to make the manuscript comprehensible for a broader audience.

Specific issues:
1. The electron density displayed in Figure 4b does not allow a proper judgment of its quality. The authors should provide a stereo figure. It is also not clear how the substrate was placed into the electron density and whether it was calculated in the presence or absence of the ligand. It would be helpful if the authors could display a positive difference density with phases from a model that does not contain the substrate.

Answer to comment 1:
As suggested by the reviewer we now provide a stereo figure, new Figure 5A, from the binding site together with the 2Fo-Fc electron density map to confirm the choline electron density. Furthermore we provide another stereo figure, new Figure 5B, that shows the 2Fo-Fc and Fo-Fc difference map of the binding site of a model in an early refinement round in which choline was not positioned yet. This additional figure shows a clear positive difference density in the position in which we locate the choline molecule in a subsequent refinement round. Overall these figures support the correct positioning of choline.

2. Since the crystallization was carried out in comparably high (5mM) concentrations of the substrate and a bound substrate was only observed in one subunit it is possible that the observed binding mode does not represent an intermediate position in the transport cycle.

Answer to comment 2:
With a protein concentration of 10-15 mg/ml (~ 0.2 mM BetP) in the crystallization drop we deal with a 10-30 fold excess of substrate, which we consider still as moderate. Most likely, the occupancy of only one protomer with substrate is an effect of the asymmetric distribution of conformations in the trimer due to an asymmetry of C-terminal interactions and not an effect of unspecific binding. Differences in substrate occupancy caused by conformational asymmetry were present already in our first betaine bound structure (Ressl et al., 2009), although hidden due to strict imposition of a three-fold non-crystallographic symmetry, which was essential to solve the phase problem. A comparable asymmetry was also observed in electron microscopy data (Tsai et al., accepted in JMB).
We have added the following sentence for clarification on page 7: The structure of BetPΔN29G153D (PDB entry 3PO3) was solved to 3.35 Å (Table II) without imposition of a

threefold non-crystallographic symmetry to account for the conformational asymmetry of individual protomers within the trimer (Tsai et al, 2010). A choline molecule was observed in a central binding site in one of the three protomers within the trimer (light blue protomer in Fig. 4A) that is fully accessible from the cytoplasm (Fig. 4B).

Moreover, the proposed coordination fits very well with mutagenesis data. An exchange of Trp377 that mediates binding of choline and betaine against leucine (Ressl et al. 2009) abolished transport and support therefore the substrate coordination proposed in our manuscript. We clarified this point in the results on page 8 : Trp377 is a crucial residue in substrate transport in BetP and replacement against leucine abolished betaine transport (Ressl et al, 2009).

3. It would be interesting to see comparison of the overall conformation of the structure of the mutant with the previously determined structure of the WT-protein to illustrate why the authors distinguish between an occluded and open inward-facing conformation.

Answer to comment 3:
The reviewer raised an important point here since two different conformational states are discussed. In a new Figure 4B we now provide a comparison of the binding site accessibility in the previous structure of BetP (PDB entry 2WIT) and the new structure as well as a comparison of the main chains of both structures in a new Figure 4C. We have included a short section on the conformational changes on page 8: This open inward-facing state constitutes a new conformation of BetP in comparison to the betaine-bound structure of BetP (PDB entry 2WIT) (Fig. 4B) reported previously as occluded state (Ressl et al, 2009). In the light of the open inward-facing state observed for BetP-G153D, hereafter, we will refer to the betaine-bound structure (PDB entry 2WIT) as occluded inward-facing conformation. Superimposition of both structures (Fig. 4C) supports a rigid-body movement of the bundle domain (first two helices of each repeat) relative to the scaffold of adjacent helices (helix 3 and 4 of each repeat) that is comparable to the conformational changes described very recently for vSGLT (Table SI) (Watanabe et al, 2010). The intracellular halves of TM3 and TM8 are displaced by 6° and 5°, respectively (Fig. 4C). Side chain displacements of residues Ala144, Met144, Ile302, Gln303, Phe380, Phe384, Ile388 and Ser471 facilitate the accessibility of the binding site from the cytoplasm (Fig. 4C and inset).

4. The discussed transport mechanism and its depiction in Figure 6 is somewhat confusing. I wonder whether the authors could simplify the description.

Answer to comment 4:
We thank the reviewer for this useful suggestion. We simplify the description on page 10 and 11 of the mechanism to make it more comprehensible. However, it is a rather complex mechanism we describe here, so we could not shorten this part excessively. However, we re-phrased the whole section on page 10 and 11 so that it is hopefully more comprehensive to a broader audience.

Minor:

5. There are weaknesses in the language throughout the text.

Answer to comment 5:

A native English speaking person has now revised our manuscript.

Referee #2 (Remarks to the Author):

The article entitled, "Substrate specificity and ion coupling in the Na2+/betaine symporter BetP" describes a new inward open conformation of BetP generated by a single point mutation (G153D), which converts the transporter into a H+ coupled choline transporter. This article presents a clear and very interesting story which further shows the robustness of the LeuT-fold. I recommend publication and would ask the authors to make some minor changes and clarifications.

1. In a number of places the authors cite Forrest 2010 to cover many generalities of the LeuT-fold, while this is an appropriate citation it does not represent the initial observations. I have highlighted a few below:

• Almost each individual structure of LeuT transporters... (more appropriate references: Structure and function of Na(+)-symporters with inverted repeats. Abramson J, Wright EM.)

• However,...first helix of the first repeat... (Coupling substrate and ion binding to extracellular gate of a sodium-dependent aspartate transporter. Boudker O, Ryan RM, Yernool D, Shimamoto K, Gouaux E.)

Answer to comment 1:

We thank the reviewer for this important concern. We made the changes in the references accordingly. References (Abramson et al, 2009 and Boudker et al, 2007) are now included.

2. It would be more appropriate to use LeuT numbering of the TM helices. This allows for direct comparisons between all the different structures. Start numbering at the first inverted repeat.

Answer to comment 2:

We changed the TM numbering in the figure of the proposed mechanism, new Figure 7, for better comparison with other LeuT-fold transporters.

3. The Na2 site should be defined. Does it deviate from the other sodium dependent structures (LeuT, SGLT and Mhp1)? All other structure has the Na2 site between TM's 1 and 8 and it appears BetP does as well.

Answer to comment 3:
Yes, in BetP the location of the Na2 site is the same as the one observed for LeuT, SGLT and Mhp1. A figure for clarification is provided in the supplementary material (Fig. S2B) and the conservation of the binding site is added to the manuscript on page 9: The Na2-sodium is situated between the 4TM-helix bundle formed by the two first helices of each repeat (helix 1 and 6 in Fig. 7A) and the hash domain comprising the 3rd and 4th helix of each repeat (helix 3 and 8 in Fig. 7A).

4. Figure 1B. It would be nice to highlight the Na2 site. Which carbonyl oxygens are thought to coordinate Na2? It looks like D153 is just above these carbonyls. It would be nice to have a better picture in comparison to the Na2 site.

Answer to comment 4:
We agree with the reviewer. Therefore, we now compare the location of the Na2 site with the position of choline coordinated by the D153 residue in Figure S2.

5. In the results section: Thus, we conclude... conformational flexibility to TM3... The flexibility of TMs 1 and 6 has been proposed on all LeuT papers and should be cited.

Answer to comment 5:
We have added appropriate citations on page 5: A similar conformational flexibility in the midsection of the first helix of the first repeat has been described previously for LeuT (Yamashita et al, 2005; Shi et al, 2008).

6. Uptake experiments are really nice and compliment the structure. I was wondering (unlikely) of the authors have calculated the stoichiometry of H+ to choline? It would be interesting if it is a 1:1 or 2:1. But, this may be difficult to do and acceptance should not depend on this result.

Answer to comment 6:
We thought about this experiment too, but this is not a trivial experiment and so far we could not provide data concerning stoichiometry.

7. The authors refer to their previous structure as "occluded inward-facing" which differs from their original paper where they stated there was no clear cavity. Is this a new structure or have they modified their terminology. In light of these two structures the author should compare the differences to the recent paper on sglt where these same two conformations have been compared. In particular are the movements of TM1 the same? (The mechanism of sodium and substrate release from the binding pocket of vSGLT. Watanabe A, Choe S, Chaptal V, Rosenberg JM, Wright EM, Grabe M, Abramson J.)

Answer to comment 7:

Yes, the terminology to describe the conformational state of the published betaine-bound BetP structure (PDB entry 2WIT) was modified based on the new structure of BetP G153D. This information is now included in the results on page 8: This open inward-facing state constitutes a new conformation of BetP in comparison to the betaine-bound structure of BetP (PDB entry 2WIT) (Fig. 4B) reported previously as occluded state (Ressl et al, 2009). However, in the light of the open inward-facing state observed for BetP-G153D, hereafter we will refer to the betaine-bound structure as occluded inward-facing conformation.

In a new Figure 4B we now provide a comparison of the binding site accessibility in the betaine-bound structure of BetP (PDB entry 2WIT) and the choline-bound structure (PDB entry 3PO3). Furthermore we provide now a full comparison of the two overall structures, new Figure 4C. A slight rigid body movement of the bundle relative to the hash domain is observed, in particular of the intracellular half of TM3 and TM8 which are displaced by around 6° and 5°, respectively. Movement of side chains of residues located in TM3, TM8 and TM7 render the binding site more accessible (Fig. 4C inset).

An RMSD analysis of the superposition of the structures of BetP-G153D and BetP WT (PDB entry 2WIT) (Table SI) shows that RMSD values are similar to the ones observed in the recent paper on vSGLT (Watanabe et al 2010), in which the inward-facing open and inward-facing occluded conformations of vSGLT are compared.

A short section on the comparison has been added on page 8: " Superimposition of both structures (Fig. 4C) supports a rigid-body movement of the bundle domain (first two helices of each repeat) relative to the hash-sign like scaffold of adjacent helices (helix 3 and 4 of each repeat) that is comparable to the conformational changes described very recently for vSGLT (Table SI) (Watanabe et al, 2010). The intracellular halves of TM3 and TM8 are displaced by 6° and 5°, respectively (Fig. 4C). Side chain displacements of residues Ala144, Met144, Ile302, Gln303, Phe380, Phe384, Ile388 and Ser471 facilitate the accessibility of the binding site from the cytoplasm (Fig. 4C and inset).

8. Can the authors expand on their comparison with ApcT? The Lys in ApcT replaces the location of the Na2 site but I believe D153 is in quite a different position, which was surprising for me.

Answer to comment 8:

In ApcT, Lys158 residue is located in the Na2 site and it is suggested that this residue plays a similar role than sodium when undergoes protonation-deprotonation. In our case we propose that either sodium or choline can go trough this position and that D153 is required for coordination of choline. Therefore, aspartate (BetP) and lysine (ApcT) are involved in different ways. We included the following sentence for clarification in the discussion (Page 11): Obviously, protonation of Asp153 has a different role in the transport cycle of BetP compared to Lys158 in ApcT, where protonation of this residue renders the central binding site accessible for the substrate (Shaffer et al, 2009).

Referee #3 (Remarks to the Author):

The manuscript by Perez et al. describes an interesting mutagenesis study of the conserved GXGXG unwound region of TM3 from C. glutamicum BetP highlighting the importance of this region for conformational flexibility of TM3 in connection with betaine transport. Mutagenesis of the third glycine of this motif to aspartate intriguingly changed the specificity of BetP to choline in the presence of a H+ gradient while both betaine and choline are transported in the presence of a Na+ gradient. This position is also an aspartate in the closely related BetT and other choline transporters of the BCCT family. The structure of choline bound to the G153D mutant of BetP to 3.35 Å revealed a new inward facing conformation. Surprisingly, choline was shown to bind in a site suggested to be the second Na+ site in BetP. Based on the structure and modeling studies the authors present a rather speculative transport mechanism for choline.
The MS is interesting and addresses a key point of how Na+ vs. H+ driven transport is controlled and the project design is well-suited for that and shows convincing evidence of the Asp/Gly residue being a determinant

The crystal structure highlights an additional surprise - an inward facing conformation with choline bound at the Na2 site. The structure however interesting it is, still leaves many questions open, disfavouring elaborate mechanisms to be exploited.

1. It is recommended to put less weight on its significance - ~5 mM choline and pH 5.5 in the crystal is after all a special case, and the discussion of a possible transport mechanism therefore becomes shaky - either more structures are to be determined - e.g. soaking crystals at pH 7 and including also K+ and Mg2+ to see if the site actually makes sense in a more physiologically relevant setting - or the section on the transport mechanism should be shorter and more critical.

Answer to comment 1:
We thank the reviewer for this useful suggestion. We have now modified the transport mechanism section to make it shorter and more critical and less speculative. See also our answer to comment 4 of reviewer#1.

2. additional comments:
- Were mutated plasmids sequenced and confirmed?

Answer to comment 2:
Yes, all the plasmids used in this project were sequenced and the specific mutation confirmed. We clarify this now in the supplementary material (site-directed mutagenesis section).

3. Was chain A or C of 2WIT used for MR? MS and supporting info say differently (not a big deal)

Answer to comment 3:

We thank the reviewer for detecting this mistake in the supplementary material. Indeed chain C of BetP (PDB entry 2WIT) was used for MR. We corrected this in the supplementary material (Crystallization and structure determination section).

4. Is the proton-driven transport inhibited by Na+, either side?

Answer to comment 4:
Yes, there is definitely an inhibiting effect of sodium on the H+ coupled transport, which becomes obvious by the reduced affinity for choline in the presence of sodium. We mention this point now in the manuscript. Page 11: The observed lower affinity for choline in the presence of sodium would be caused by competition between the positive charges of choline and sodium for the Na2 binding site suggesting that Na^+ has an inhibitory effect during H^+-coupled transport.

5. Fig. 4 is in strong need of an unbiased difference map revealing choline. Even the 2Fo-Fc is not terribly convincing, and a blue density mentioned in the legend is not shown.

Answer to comment 5:
We agree with the reviewer that an unbiased difference map is needed (see also our answer to comment #1 of reviewer 1). We now provide a stereo figure showing the 2Fo-Fc and Fo-Fc difference map of the binding site of a model in an early refinement round in which choline was not positioned yet (Fig. 5B). This additional figure shows a clear positive difference density in the position in which we locate the choline molecule in a subsequent refinement round. Furthermore we now provide a stereo figure of the binding site and the 2Fo-Fc electron density map (Fig. 5A). This figure allows a better judgment of the choline electron density.

6. Also the methods description should emphasize how the choline ligand and its specific modelling was validated at 3.35 Å resolution this is not trivial.
PROCHECK validation is obsolete, molprobity is recommended

Answer to comment 6:
We thank the reviewer for this useful comment. We now provide in the section of modeling studies in the supplementary methods an additional validation with MolProbity (Chen et al, 2010 and Davis et al 2007). For the BetT model we found that over 92% residues were in the Ramachandran favored regions while 1.15% were outliers and 2.1% with poor rotamers. These deviations were examined and were found to mainly be in loop regions or in low electron density regions in the template. They were thus left unaltered.
For the model with choline additional MolProbity validation of the optimized model reported 94.81% residues in Ramachandran favoured regions with a similar 1.15% outlier and 3.5% poor rotamer residue distribution. These loop region residues were also left unaltered due to insufficient information from the template.

References

Chen VB, Arendall WB 3rd, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, Murray LW, Richardson JS, Richardson DC (2009) MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr D Biol Crystallogr **66**(Pt 1):12-21

Davis IW, Leaver-Fay A, Chen VB, Block JN, Kapral GJ, Wang X, Murray LW, Arendall WB 3rd, Snoeyink J, Richardson JS, Richardson DC (2007) MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. Nucleic Acids Res **35**:W375-83

Ressl S, Terwisscha van Scheltinga A, Vonrhein C, Ott V, Ziegler C (2009) Molecular basis of transport and regulation in the Na^+/betaine symporter BetP. Nature **458**: 47-53 Watanabe A, Choe S, Chaptal V, Rosenberg JM, Wright EM, Grabe M, Abramson J (2010) The mechanism of sodium and substrate release from the binding pocket of vSGLT. Nature **468**(7326): 988-91

2nd Editorial Decision 26 January 2011

Thank you for sending us your revised manuscript. Our original referee 1 has now seen it again, and you will be pleased to learn that in his/her view you have addressed all criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Before this will happen, there are a number of editorial issues that need further attention. I would like to ask you to include the statistical details for figure 3B into the figure legend and for table I into the table legend. Furthermore, I would like to ask you not to include the supplementary figures into the merged manuscript file, but to generate a combined supplementary material file that includes the supplementary text and the labelled supplementary figures.

Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have responded to the reviewers comments in a satisfying way. The revised manuscript has significantly improved and I thus think that the manuscript is now acceptable for publication

2nd Revision - authors' response 28 January 2011

Please find attached the revised version of our manuscript where we have included the statistical details for figure 3B into the figure legend and for table I into the table legend. Furthermore, we

merged the supplementary figures into a combined supplementary material file that includes the supplementary text and the labelled supplementary figures.

