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Role of trimerization in the osmoregulated 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.)

1st Editorial Decision 11 March 2011

Thank you for the submission of your research manuscript to our editorial offices. Your study has been sent to three referees, and we have so far received the reports from two of the experts who were asked to assess it (referees 1 and 3). As these referees agree on the potential interest of the findings, I would like to ask you to begin revising your manuscript according to their comments. Please note that this is a preliminary decision made in the interest of time, and that it is subject to change should the third referee offer very strong and convincing reasons for this. We will forward you the third report as soon as we have received it and we expect you to not only address the concerns of the first two referees, but also of the third reviewer once we have received them.

As I said, both referees agree on the potential interest of the findings reported in your manuscript. They do, however, also suggest potential ways of how to strengthen the message of the study. Referee 1, for example, states that the trimeric state of the T351A mutant in membranes should be tested using FF-EM in addition to showing the trimeric state in BN-PAGE. Referee 3 suggests comparing the Vmax of the individual BetP mutants to provide further support that the slower rate of betaine transport is indeed due to disruption of the oligomers. This referee also suggests analyzing mixed trimers (WT and mutants) to provide further support for the idea of conformational coupling between the different monomers.

Please note that the main referee concerns (including the ones from referee 2 once we have received them) must be addressed and that acceptance of the manuscript will depend on a positive outcome of a second round of review. I should also remind you that it is EMBO reports 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 responses included in the next, final version of the manuscript.

I will get back to you once we have received the final report from referee 2.

Kind regards

Editor
EMBO reports

REFEREE REPORTS

Referee #1:

The labs of Reinhard Kramer and Christine Ziegler have studied the BetP transport protein for many years and obtained wonderful functional and structural data on the mechanism of transport and osmotic regulation. They have now constructed monomeric variants of BetP and show convincingly that the monomers are functional in transport but no longer regulated by osmotic (ionic) stress. They demonstrate that the mutants are monomeric by using BN-PAGE, SEC, freeze-fracture EM and cross-linking studies. The FF-EM studies are particularly important as the degrees of freedom to move around in the membrane are restricted compared to the situation in the detergent-solubilized state. Thus, the tendency of a membrane protein to oligomerize is much higher in the 2D space of the membrane than in the 3D space of a solution. The authors show that the mutants are monomeric, following reconstitution into lipid vesicles. The full dissociation of the complex makes the interpretation of the transport data relatively straightforward, and the conclusions of the paper seem fully justified.
This is a beautiful piece of work and I only have few minor comments.
1. It is not very clear to what extent the MD simulations have contributed to the design of the mutants. Wouldn't one have come to the same conclusion by inspecting the crystal structure?
2. It would have been nice to show that the T351A mutant is trimeric in the membrane, using FF-EM.
3. The paper needs to be checked for typos.

Referee #3:

This is a very nice paper that studies the effect of trimerization on the function and regulation of the Betaine/Na transporter BetP. The authors use in silico alanine scanning mutagenesis to identify residues that are critical for trimerization of the protein. These mutants indeed disrupt oligomeric assembly, resulting in mostly monomeric protein. The monomers are functional and show the same Km for transport as the WT trimeric protein but lose osmo-regulation and show a ~10-fold reduced Vmax for transport.

This is an extremely interesting paper and I have no major criticisms. There are however, some issues that should be clarified.
Even though the monomer is slower than the trimer I am not convinced that the authors have shown that the reduced rate is due to the disruption of the oligomer rather than to the mutations themselves or to the loss of regulation by volume.
To address this issue the authors should determine the Vmax of the individual mutants and of the C-terminal deletion? Is any of these proteins as slow as the monomer? Or they all maintain WT-like rates?

Related to this point, I think that the author's proposal that the "conformational coupling in the catalytic cycle when BetP is trimeric" could be tested more rigorously by creating mixed trimers of WT and a transport dead (or impaired) and seeing if impairing one monomer has repercussions on

the neighboring ones.

When referring to the LeuT fold the authors should refer to the structures from the Gouaux lab that originally described the fold

Additional Correspondence 12 March 2011

We have just now received the final report on your study and I am pasting it below for your information.

In brief, referee 2 has some suggestions on how to improve the study, one of them has also been brought up by referee 3 and while referee 2 suggests toning down the conclusions, the experiments suggested by referee 3 may help to clarify the observations.

I would kindly ask you to address the concerns of this referee before submitting the revised version to our office.

Please do not hesitate to contact me if you have any further questions.

Kind regards

Editor
EMBO reports

REFEREE REPORTS

Referee 2:

This is a very nice paper from the Zeigler lab examining the role of trimerization in the function of the transporter BetP. The authors did a careful, molecular dynamics analysis of the intersubunit interface in the trimer, focusing on residues with a significant energetic impact on interaction energy when mutated to alanine. They then mutated these residues in the protein and found several combinations of changes that cause the protein to express and purify only in a monomeric form. The data establishing monomeric transporter are thorough and convincing with several independent methods demonstrating stable monomers in both detergent solution and in when in lipid bilayer membranes. Upon functional analysis, the authors find that the purified reconstituted monomeric transporter is active in transport (albeit with slightly reduced activity), but is not regulated by osmolarity (as opposed to the trimeric form, which is). Even when functionally examined in cells these observations hold. The authors conclude that trimerization plays a role in transport function but is essential for regulation of BetP.
The work reported here is novel, interesting, and is well performed. It will make an excellent addition to the literature and I have only minor comments, mostly on the presentation of the data.
1) I find it confusing that the authors use two different nomenclatures for their mutants, with standard usage in the text and some figures (eg. W101A/T351A) and a different system in other figures (M1 etc.). They should choose a single notation (preferably the former) and stick with it.
2) I think the discussion in paragraph 2 of the discussion is a bit too speculative and based on minimal data. The activity reduction in the monomer mutant is fairly modest (~50%) and it's hard to say whether this results from monomerization or just from one of the mutations. I would prefer to see this section toned down a bit.
3) The mutant W101A/F345A/T351A is mentioned in the text on page 7 but not shown on Figure 2. I understand that I can find it in the supplement, but since it is

featured prominently I would suggest that it be included in the gel in Figure 2B.
4) The last sentence on page 7 (extending into page 8) is a bit confusing-it might be easier to understand if it read "We determined the average diameter of the particles from their average areas (239 Particles....) by assuming that they were circular.

1st Revision - authors' response 01 April 2011

Point-by-point response to the reviewer's comments on

MS# EMBOR-2011-34809-T

"Role of trimerization in the osmoregulated betaine transporter BetP"

We are grateful to the reviewers for their constructive comments. All their suggestions have been taken into consideration and the response to each point is presented below. In the revised version of the manuscript, we have made changes in the text and figures according to their suggestions. Moreover, we added freeze-fracture electron microscopy and BN-PAGE data showing the trimeric state of one of the single-residue mutants, and provided further support that the slower rate of betaine transport is indeed due to disruption of the oligomers by comparing the Vmax values. For clarity, we copied each point from the reviewers' comments and provide our responses below (in italics).

Referee #1 (Remarks to the Author):
… This is a beautiful piece of work and I only have few minor comments.

Comment 1

It is not very clear to what extent the MD simulations have contributed to the design of the mutants. Wouldn't one have come to the same conclusion by inspecting the crystal structure?

Answer to comment 1

Given that 23 residues were found to contribute to the interface, we felt that it was worthwhile to obtain $\Delta\Delta G_{bind}$ values that were as accurate as possible, and therefore to reduce the required experimental effort as much as possible. By simulating the protein in a hydrated lipid bilayer environment, we obtain more physiologically relevant local interactions between residues at the membrane/water interface than for the detergent-solubilized crystal condition. In addition, during the simulations, the protein can sample an ensemble of conformations, which better reflects such a dynamic system at physiological temperatures. For example, several polar groups that were predicted to form hydrogen bonds in the structure in fact sampled alternate conformations when allowed to form hydrogen-bonds with the water during the simulation; this also reflects the low resolution at which the BetP structure was obtained (3.35 Å), and the corresponding uncertainty of the side-chain configurations. For all these reasons, the alanine scanning mutagenesis was more effective when calculated on snapshots from the simulations, showing, for example, the strong contribution of T351 ($\Delta\Delta G_{bind}$ = 1.1 ± 0.9 kcal/mol) which was certainly not clear from alanine scanning on the crystal structure alone ($\Delta\Delta G_{bind}$ = 0.3 ± 0.1 kcal/mol). That T351 is crucial was

subsequently demonstrated by the fact that the BetP-T351A is the only single mutant tested for which dimeric protein is observed (new Figure S4). We now explicitly mention the large number of interfacial residues in the Results section (page 4), and we have included a discussion of the expected difference between alanine scanning results in the Supplementary Text, to highlight the usefulness of the MD simulations in this context.

Comment 2

It would have been nice to show that the T351A mutant is trimeric in the membrane, using FF-EM.

Answer to comment 2

We have included now a freeze-fracture electron microscopy (FF-EM)analysis of the BetP-T351A mutant in Figure S4. The data show that this mutant is mainly trimeric with a small population of dimers. The fact that this distribution is broader than for the wild-type, and includes dimeric protein provides strong support for our statements in the Results and the Discussion that the mutation T351A destabilizes the trimer, albeit not completely.

Comment 3

The paper needs to be checked for typos.

Answer to comment 3

The paper was thoroughly checked for typos

Referee #2:

…It will make an excellent addition to the literature and I have only minor comments, mostly on the presentation of the data.

Comment 1

I find it confusing that the authors use two different nomenclatures for their mutants, with standard usage in the text and some figures (eg. W101A/T351A) and a different system in other figures (M1 etc.). They should choose a single notation (preferably the former) and stick with it.

Answer to comment 1

The nomenclature was changed as requested.

Comment 2

I think the discussion in paragraph 2 of the discussion is a bit too speculative and based on minimal data. The activity reduction in the monomer mutant is fairly modest (~50%) and it's hard to say whether this results from monomerization or just from one of the mutations. I would prefer to see this section toned down a bit.

Answer to comment 2

We agree with the reviewer, that this paragraph was too speculative, and so we have shortened it accordingly (See page 10, paragraph 2).

Comment 3

The mutant W101A/F345A/T351A is mentioned in the text on page 7 but not shown on Figure 2. I understand that I can find it in the supplement, but since it is featured

prominently I would suggest that it be included in the gel in Figure 2B.

Answer to comment 3

Figure 2 was changed accordingly and the BN-gel of the mutant W101A/F345A/T351A is now shown.

Comment 4

The last sentence on page 7 (extending into page 8) is a bit confusing-it might be easier to understand if it read "We determined the average diameter of the particles from their average areas (239 Particles....) by assuming that they were circular.

Answer to comment 4

We have changed the sentence accordingly. However, please note that the whole paragraph on the FF-EM analysis approach has been transferred to the Supplementary text (Page 7 first paragraph) due to the strict length limits.

Referee #3 (Remarks to the Author):

…This is an extremely interesting paper and I have no major criticisms. There are however, some issues that should be clarified.

Comment 1

Even though the monomer is slower than the trimer I am not convinced that the authors have shown that the reduced rate is due to the disruption of the oligomer rather than to the mutations themselves or to the loss of regulation by volume. To address this issue the authors should determine the Vmax of the individual mutants and of the C-terminal deletion? Is any of these proteins as slow as the monomer? Or they all maintain WT-like rates?

Answer to comment 1

The Vmax of BetP WT at 800 mOsm is 138 ± 6 nmol/(min x mg cdw) (Fig. S6B) is around 6-fold faster than the monomeric BetP-W101A/T351A (23.6 ± 1.7 nmol/(min x mg cdw)), as noted by the reviewer. We have now measured the Vmax values for the trimeric single mutants W101A and F345A, showing similar values to the WT, i.e. 140 ± 8 and 132 ± 4 nmol/(min x mg cdw), respectively, indicating that the mutations themselves do not reduce the turnover rate. The other single mutant studied, T351A, showed a 3-fold lower Vmax than the other single mutants, i.e. 47 ± 4 nmol/(min x mg cdw), although not as slow as the monomeric constructs. This can be explained by the fact that T351A displays a broad distribution of size particles in the membranes of liposomes, including both trimers and dimers (Fig. S4), indicating that this point mutation partially destabilizes the trimer, probably resulting in a partial loss of coupling and consequently a lower Vmax. Thus, disruption of the oligomer interface by forming destabilized dimeric or monomeric mutants (as in T351A, W101A/T351A), reduces the turnover rate of the transporter, whereas single mutants at the interface that remain trimeric are as fast as wild-type. These results provide further support for our proposal that inter-protomer coupling is required for obtaining optimal turnover rates in BetP, and we have included them in the manuscript accordingly (Table S2, Discussion, page 9 and 10).

On the other hand, the unregulated C-terminal deletion mutant (BetP-ΔC) is also significantly slower than WT BetP, with a Vmax of 12 ± 3 nmol/(min x mg cdw). If the only role of the C-terminal tail were suppressive interactions with the loops of a neighboring protomer, we would expect the deletion mutant instead to be faster in turnover. However, analysis of single-point mutations within the C-terminal tail (Schiller D, Ott V, Krämer R, Morbach S. Influence of membrane composition on osmosensing by the betaine carrier BetP from Corynebacterium glutamicum. J Biol Chem. 2006 281(12):7737-46) indicates that such interpretations are oversimplified, and point to important consequences for the conformation of another pore-lining helix, TM12 (connected to the C-terminal tail), which could also lead to differences in turnover rates. Thus, we believe it makes sense to reserve discussion of the Vmax of BetP-ΔC for future work, in which the role of the C-terminal tail can be examined more thoroughly.

Comment 2

Related to this point, I think that the author's proposal that the "conformational coupling in the catalytic cycle when BetP is trimeric" could be tested more rigorously by creating mixed trimers of WT and a transport dead (or impaired) and seeing if impairing one monomer has repercussions on the neighboring ones.

Answer to comment 2

We fully agree with the suggestion of this reviewer. The strategy to create artificial heterotrimers of BetP and to test them for negative dominance would be an extremely helpful approach. We are on the way to doing that - however, it turned out to be significantly more difficult than expected. The reason for that is mainly due to the fact that fusing different tags to the termini of BetP, which themselves harbor regulatory domains on both sides, has a serious impact on expression as well as function. We expect that this approach will keep us busy until end of this year at least.

Comment 3

When referring to the LeuT fold the authors should refer to the structures from the Gouaux lab that originally described the fold.

Answer to comment 3

This has been changed accordingly (Page 2, first paragraph in Introduction).

2nd Editorial Decision 28 April 2011

Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed report from the referee that was asked to assess it. I am happy to tell you that this referee supports publication of your revised manuscript in EMBO reports. S/he only has one more, rather minor, suggestion, which is to provide the quantification of dimer appearance in the FF-EM experiment (see below).

I would kindly ask you to address this before we proceed with the official acceptance of your study and I look forward to seeing the final version of your manuscript as soon as it is ready.

Please do not hesitate to contact me if you have any further questions.

Best wishes

Editor
EMBO Reports

Referee #3:

The authors have satisfactorily addressed all of my concerns.
I think that the added data on the transport rates and FFEM of the mutants considerably strengthens and validates their conclusions.
My last residual minor concern is that the authors argue that the single T351A mutant partially disrupts trimerization of BetP, however already in the distribution of the WT FFEM particles (Fig. 3B) there is a small peak at what presumably is the dimer position (~6 nm diameter). Would it be possible to quantify the increase in the relative weight of the dimeric peak induced by the T351A mutation, for example by fitting the distribution to the sum of two Gaussians and then comparing the increased weight of the dimeric component in the mutant? The increased frequency is visible by eye (it goes from ~10% to ~20-25%) so its quantification should be feasible.

2nd Revision - authors' response 04 May 2011

Response to the reviewer # 3 comment on MS# EMBOR-2011-34809-V2

"Role of trimerization in the osmoregulated betaine transporter BetP"

Referee #3 comment:

The authors have satisfactorily addressed all of my concerns.
I think that the added data on the transport rates and FFEM of the mutants considerably strengthens and validates their conclusions.
My last residual minor concern is that the authors argue that the single T351A mutant partially disrupts trimerization of BetP, however already in the distribution of the WT FFEM particles (Fig. 3B) there is a small peak at what presumably is the dimer position (~6 nm diameter). Would it be possible to quantify the increase in the relative weight of the dimeric peak induced by the T351A mutation, for example by fitting the distribution to the sum of two Gaussians and then comparing the increased weight of the dimeric component in the mutant? The increased frequency is visible by eye (it goes from ~10% to ~20-25%) so its quantification should be feasible.

Answer to comment

We agree with reviewer 3 on the quantification of the dimeric component. In the revised version of the manuscript, we added a cumulative percentage plot in Figure S4C to quantify the increase in the relative weight of the dimeric peak induced by the mutation T351A.
The plot shows the percentage of picked particles as a function of particle size and demonstrates that the dimeric form of BetP-T351A constitutes around 18% of the total counted particles. We added and commented this value in the first lines in page 8.

3rd Editorial Decision 06 May 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

Yours sincerely,

Editor
EMBO Reports

