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Structural basis of GDP release and gating in G protein coupled Fe2+ transport

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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 17 June 2009

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office. I received the comments of three scientists that are familiar with the topic that I enclosed for further reference. They all appreciate the structural insight provided, but still raise critical issues that would need further experimental work and refinements before they would be willing to lend their full support for publication of your study. Specifically, much stronger evidence (and ample of experimental suggestions from the referees are given) would be needed to verify the existence of the proposed trimer. This would need significant further validation and should be complemented with outlining the actual interfaces. The second point relates to the role of the G5 motif (respective other parts of the structure) that might contribute to the off rate for GDP release (please refer to the comments of ref#2 on this issue). All in all, we would like to offer you the chance to carefully attend to these comments before returning a thoroughly revised version of your work. I also have to remind you that it is EMBO_J policy to allow a single round of revisions only, which means that the final decision on acceptance or rejection will depend on the content of the final version of your manuscript.

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

REFEREE REPORTS

Referee #1 (Remarks to the Author):

One point I missed; is it enough for the opening of the chanel when only one GTP is bound to one subunit of the trimer - or is coincidence of GTP binding to the other trimers required? This should be discussed more clearly.

Some minor points should be looked at.

Page 23, reference Cartron et al. Feo-transport ...
In the supplementary Methods 1 mg/ml ampicillin was used - this 10 fold more than ususal?
On the same page 6 lines from below "Cleavage protein" sounds funny for me.
Explain in the legend to Supplementary Fig. 1 the meaning of the black wavy line.

Referee #2 (Remarks to the Author):

The manuscript by Guilfoyle et al. presents crystal structures of the soluble domain of FeoB, a bacterial G protein-gated Fe2+ transporter, in the apo form and bound to mant-GMPPNP. Non-crystallographic trimers are observed in both cases. The authors attempt to demonstrate the relevance of the trimers in solution by engineering double cysteine residues at the trimer interface and showing that apparent trimers can be detected by gel filtration after treatment with an oxidizing agent. An interesting feature of the trimer is a central pore that is evidently closed in the apo structure but just large enough at narrowest point to accommodate a desolvated Fe2+ ion in the mant-GMPPNP structure. Based on comparisons between the apo and mant-GMPPNP structures, the authors propose that an additional helical domain functions as an internal effector that couples the conformation of the pore to the nucleotide state. The authors also show that a very high off rate for GDP-release is attributable in part to substitutions in the G5 motif, which adopts different conformations in the apo and mant-GMPPNP structures.

Overall, this is an interesting study that provides insight into an atypical homotrimeric G protein assembly that appears to be utilized by FeoB to gate Fe2+ transport.

Specific comments:

1. Why was the mant derivative of GMPPNP used for crystallization?

2. The experiments involving the double Cys mutants show that a trimeric state can be engineered but do not directly address the oligomeric state of the wild type protein in solution. Is there any evidence that the wild type hydrophilic domain of FeoB can assemble into trimers in solution?

3. Mutations in the G5 motif reduce the high off rate for GDP release. However, the off rate remains very high compared to canonical small GTPases and alpha subunits of heterotrimeric G proteins, indicating that other differences must also be important. This should be discussed.

4. p13, the discussion regarding hypothetical G5 conformational changes in Galpha subunits is highly speculative and should be eliminated.

Referee #3 (Remarks to the Author):

This manuscript describes the crystal structure of the extracellular domain of FoeB, a prokaryotic membrane protein. The extracellular domain includes a G-protein that is coupled to iron transport. This is interesting work, and the comparison of the apo vs. GTP bound form of this very uncharacteristic G-protein constitute an important addition to the G-protein literature. Also, from the look of it, the crystal structure determination (with only 2.9 Angstrom resolution in house heavy atom data for initial phasing) seems as though it must have been quite a challenge. The structure is well-refined, and the authors do a good job of putting this structure into a broader context so that it will be of interest to a wide audience. I believe the subject matter is appropriate for publication in EMBO but a variety of issues need to be addressed before this manuscript is accepted for publication. These are as follows:

The structure determinations utilized a methyl-anthraniloyl modified non-hydrolyzable GTP analog, referred to in the paper as mGTP. The mant moiety is fairly large, and it can easily make interactions with the protein. It can also disrupt the conformation of the nucleotide observed in the crystal structure. It is not clear why the fluorescent analog was used for the crystallization. Moreover, the mant group is not shown in any of the figures. If it was disordered, the authors should say so. They should also make it clear whether this modification is required for crystallization and whether the mant group enhances or detracts from binding affinity relative to the natural substrate.

One of the major findings is that FoeB forms a trimeric complex. As I understand it, to demonstrate that the complex is formed in solution, the authors introduced cys residues at two positions such that they can form a disulfide bond between the monomers upon formation of the trimer observed in their crystals. They then incubated the protein for 3 days and then ran a gel filtration column. After the gel filtration, they took the fractions and introduced Cu phenantroline to generate disulfide bonds before running a non-reducing SDS PAGE gel. Given this sequence, it is possible that disulfide bonds were formed before the gel filtration run. For the data to be convincing, the authors should show that the wild-type protein elutes at the same place as the cys mutant. Alternatively other techniques such as AUC or SAXS could be used (on the wild-type protein) to demonstrate trimer formation in solution.

Along the same lines, it would be nice to see the electrostatics of the face of the trimer that presumably interacts with the membrane. If the trimer model is correct, one might expect that this would be positively charged or at least neutral. Also, the authors should indicate on the sequence alignment in the supplementary information which residues mediate the trimer interface. Conservation here would also bolster their case for physiological relevance of the trimer.

A 2Fo-Fc electron density map showing the nucleotide is provided. Even at 2.7 Angstroms, the electron density is not of particularly good quality given that the the model was present for phasing when the map was calculated. A simulated annealing omit map would be much more convincing. Also, the authors should show all atoms including waters, the Mg2+ ion and and main chain atoms in the revised figure. As noted above, the mant group should also be shown if it is part of the model.

Finally, the section on the fluorescence measurements should be expanded. As is, the authors present only association rate constants for the mant-GTP analog and only dissociation rate constants for the mant-GDP analog. It is unclear why the second set of experiments (dissociation of mant-GTP and association of mant-GDP) were not presented. Furthermore, while the experiments do support the involvement of S150 and R154 in binding, it is not clear that these mutants substantiate the claim that the G5 motif plays any special role in nucleotide release.

1st Revision - authors' response 26 June 2009

We are submitting this revised manuscript (EMBOJ-2009-71432) for your consideration. We have modified the text to address the reviewer's comments, as detailed below. Most importantly, we have added experimental data that further support the oligomeric configuration of $FeoB^{1-270}$. We hope the changes will be sufficient to address the referee's queries.

Response to Referees

Referee #1

Is it enough for the opening of the channel when only one subunit is bound to GTP?

This is a very interesting question, which will be thoroughly investigated in future studies. From the structures at hand, we believe that all molecules in the trimer needs to be bound to GTP for the channel to be open, and that there could be some allosteric communication/regulation between the molecules. However, at this point, this is pure speculation, which is why we have chosen not to include this in the Discussion.

Minor points:

1. *Page 23, reference Carton et al:*
   The typographical error in the reference has been corrected.

2. *In the supplementary Methods 1 mg/ml ampicillin was used:*
   1 mg/mL ampicillin has been corrected to 100 μg/mL.

3. *"Cleavage protein" on page 6:*
   This has been corrected to 'Cleaved protein'

4. *Meaning of the black wavy line in Supplementary Fig. 1:*
   In response to the referees comment, we opted to remove the black wavy line in Supplementary Fig S1.

Referee #2

Response to Specific comments:

1. *Why was the mant derivative of GMPPNP used for crystallization?(referee #2 and #3)*

   We co-crystallized $FeoB^{1-270}$ with both GMPPNP and its mant derivative. The mant GMPPNP was the analogue that provided the highest resolution data. Indeed, we found the mant group to be in close contact to a crystallographically related molecule, and could thus act to stabilize crystal contacts. When compared to structures of other GTP or GMPPNP complexed GTPases, the position and coordination pattern of the GTP moiety in our structure corresponds exactly to that of the other structures (data not shown). From this it was concluded that the mant group did not interfere with the coordination of the nucleotide. The mant GMPPNP $FeoB^{1-270}$ complex was therefore used in refinement, modeling, and for figures in the manuscript. To clarify this to the reader, we have amended the Materials and Methods section (under 'Crystallization and X-ray Data collection') to include this information.

2. *Is there any evidence that the wild type hydrophilic domain of FeoB can assemble into trimers in solution?*

   We appreciate the referees question in regards to the oligomeric configuration of FeoB. Oligomeric membrane proteins generally have the largest protein-protein interface in the membrane domain. Sub-cloning the soluble domain of some proteins, particularly membrane proteins, renders it therefore cumbersome to isolate the oligomeric protein. The $FeoB^{1-270}$ molecule is in solution as dynamic mixture of monomer/trimer, favoring the monomer state when, for example, run on gel filtration. To stabilize the trimer in solution, we generated a double Cys mutant designed (by looking at the structure) to 'lock' the trimer to be able to clearly identify the molecular weight, according to Yernol *et al.* (*Nature*, 2004). Gel filtration clearly illustrated a monomer and trimer. Selecting the trimer peak and oxidizing the protein before running it on a SDS gel, unambiguously showed the oligomeric species to correspond to a trimer (also verified by mass spectroscopy as a 88.7 kDa, vs. 29.4 kDa for monomer). Hence, because these two non-native cysteine residues readily form a disulphide-linked trimer, we concluded it was the biological unit.

   In response to the Referees query, we have cross-linked *native* $FeoB^{1-270}$ in solution with glutaraldehyde (Yernol *et al*. *Nature* 2004). This added experiment again clearly illustrates the protein to form a trimer in solution. The experimental approach and results have been added to Material and Methods and Supplementary Figure S3.

   In addition, we have highlighted in Supplementary Figure S1 key residues that are involved in hydrogen bonding between monomers, of which most are partially conserved (as proposed by Referee #3). Furthermore, we have now crystallized $FeoB^{1-270}$ in the same trimeric configuration in 4 different space groups ($C2$, $P2_1$, current work, $P2_12_12_1$, and $P3_1$ work in progress), again clearly illustrating the biological relevance of the trimer. We trust that this should provide ample proof for FeoB having a biological trimeric unit.

3. *The off rate remains high for the mutants when compared to small GTPases and Galpha, indicating that other differences must also be important. This should be discussed.*

   The referee correctly notes an intrinsically high GDP release rate in $FeoB^{1-270}$, even after we drastically reduced the rates by creating site-directed mutants in the G5 loop. This is an

intriguing aspect of the function of $FeoB^{1-270}$, which we have expanded on in the Discussion. We have added in the discussion (p.13) that other amino acids in the G5 motif, particularly two glycine residues (G153 and G155), could act as a 'hinge' for the motif and thus render it highly flexible. This flexibility, or ability to move to an "out" conformation, could hence be the reason for high basal off rates when compared to other GTPases. Studies are underway to investigate this, although we feel it falls outside the scope of this manuscript.

4. *p.13, discussion regarding G5 conformational changes in Galpha is speculative and should be eliminated.*

   We appreciate both referee #2 and #3's comments regarding the comparative analysis with Galpha, which referee #2 feels is too speculative while referee #3 comment us to *'do a good job of putting this structure into a broader context so that it will be of interest to a wide audience'*.

   We have therefore compromised and re-written the Discussion to maintain reference to the relevance of the G5 loop movement to Galpha proteins in general, but removed the specific discussion on the $G\alpha_{i1}$ subunit (deleting last paragraph under 'Mechanism of GDP release'). Also, in light of the changes we have removed Figure 3 and part of the last sentence in the introduction ('..,paralleled with a comparative analysis with the human Gα mutant causing testotoxicosis,..').

Referee #3

Response to comments:

1. *(a) It is not clear why the mant derivative of GMPPNP used for crystallization; (b) make it clear if its required for crystallization; (c) the mant group is not shown in any of the figures; (d) simulated annealing omit map would be more convincing of the nucleotide.*

   (a) and (b) See response to Referee #2 question 1. (c) We have amended figures to include the mant group, Mg^{2+} ion, and water molecules as requested by the referee. (d) We have also remade the figure to include a simulated annealing omit map for the mGTP molecule, exactly as requested by the referee.

2. *(a) Given this sequence, it is possible that disulfide bonds were formed before the gel filtration run. For the data to be convincing, the authors should show that the wild-type protein elutes at the same place as the cys mutant; (b) It would be nice to see the electrostatics of the face of the trimer that presumably interacts with the membrane; (c) The authors should indicate on the sequence alignment in the supplementary information which residues mediate the trimer interface.*

   (a) Please see response to Referee #2 question 2. (b) We agree with the referee that the potentials and charges of the $FeoB^{1-270}$ domain would be interesting to illustrate for the reader. The side of the trimer that is directed towards the membrane is likely directly interacting with loops situated between transmembrane helices of the membrane domain. However, on this side an interesting and clear pattern can be discerned. The center of the trimer (the pore region) is clearly negatively charged, while the outside of the pore region is defined by a mainly positive charge (which could reflect lipid interaction as the referee proposed). We have included this as Supplementary Figure S4, as we agree with the referee that this is useful information for the reader. In addition, as a comparison we have included similar surface rendering for the soluble domain of the K^+ channel KirBac1.1 and CorA, which have a similar change distribution (c) We have highlighted in Supplementary Figure S1 the residues that are involved in the hydrogen bonding network in the trimer interface, as suggested by the referee.

3. *The section on the fluorescence measurements should be expanded- (a) it is unclear why dissociation of mant-GTP and association of mant-GDP were not presented; (b)*

experiments support the involvement of S150 and R154 in binding, although it is not clear that these mutants substantiate the claim that G5 motif plays a role in nucleotide release.

(a) In response to the referee we have included the dissociation rate constants for mGTP in Table 2. However, as rate constants for association of mGDP are too fast to be reliably measured (as also noted in Marlovits *et al*., *PNAS* 2002), we have not included this data. In the Materials and Methods section, we have added under 'Stopped-Flow Fluorescence assay' the following sentence:

> 'Reactions were performed at 20 °C. At this temperature the rate constants $k_{obs,mGDP}$ >1000 s^{-1} were obtained, indicating that GDP binding and dissociation occurred on a time scale faster than what can be reliably measured by stopped-flow.'

(b) The data we have presented illustrates that binding rates are unaffected by the mutations, as well as the dissociation rate of mGTP. Release rate of GDP is the only parameter that is drastically altered. As the R154 residue is only involved in hydrogen bonding of the G5 "out" conformation and is not implicated in coordination of the nucleotide, we believe this provides support for the involvement of the G5 motif in GDP release, rather than being involved in GDP stability.

Author alterations

p.8 Added sentence 'Previous biochemical data has indicated this domain to be functioning as a Guanine Dissociation Inhibitor (GDI), although our structure indicate the domain to be situated too far away from the nucleotide binding site for a direct involvement in inhibition of nucleotide release.'

p. 16 Materials and methods (Expression and Purification), added '*Escherichia coli*'

p.18 Added 'The coordinates of both the apo-$FeoB^{1-270}$ and mGTP-$FeoB^{1-270}$ structures have been deposited in the Protein Data Bank with accession codes 3HYR and 3HYT, respectively.

