UgpB Moonlights as a Bile-Responsive Chaperone

Changhan Lee, Patrick Betschinger, Kevin Wu, Dawid Zyla, Rudolf Glockshuber, and James Bardwell **DOI: 10.15252/embj.2019104231**

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Thank you for submitting your manuscript proposing a chaperone function for UgpB for consideration by The EMBO Journal. Please apologize the delay in communicating this decision to you, which was due to delays of the review process over the holiday period, as mentioned. We have now however received two reports on your study, which are included below for your information. I have also discussed these comments with other members of the editorial team and regret to inform you that we have come to the conclusion that we cannot offer further consideration for publication at The EMBO Journal at the current stage.

As you will see, both reviewers express an interest in the proposed chaperone function of UgpB, but are not convinced this is sufficiently supported by the data currently provided. I will not reiterate all of their specific concerns here, but both referees find that more mechanistic detail and experimental characterization of the chaperone function of UgpB would be needed, in particular regarding the role of G3P binding for this activity. Overall, we find that the major concerns raised by the referees would likely not be addressable within a normal single round of major revision and given that their unforeseeable outcome will affect the main conclusion of the study, we unfortunately cannot invite a revision at this time.

That being said, we appreciate that your findings will be of interest to the field, and I have therefore discussed your manuscript and the referee comments with my colleague Esther Schnapp, Senior Editor at our sister journal EMBO Reports. Esther would in principle be interested in considering your study further for potential publication in EMBO Reports, but would also require a substantial revision, in particular addressing referee #2's comments. She would be happy to directly discuss the requirements of a revision at EMBO Reports in case you are interested in a potential transfer (eschnapp@wiley.com).

I regret that I cannot be more positive, but hope that you will nonetheless find the comments of our reviewers helpful. Thank you again for giving us the opportunity to consider your manuscript.

REFEREE REPORTS

Referee #1:

This manuscript reports the identification of UgpB, a glycerol-3-phosphate binding periplasmic protein, as a novel chaperone protecting periplasmic proteins from bile salts-induced protein aggregation. The first part of the manuscript describes the elegant approach, combining TnSeq analysis and the use of a tripartite fusion, used by the authors to uncover this novel function of UgpB. In the second part, the authors investigate the chaperone properties of UgpB and conclude that it functions as a chaperone when it does not bind glycerol-3-phosphate. Overall, I find the paper well written and interesting, the approach used very clever and the identification of UgpB as a new periplasmic chaperone exciting. The results presented in the first part are solid, but I'm less convinced by those reported in the second part and the conclusions that were drawn, as explained in the comments below. I think that the authors need to clarify the role of G3P in UgpB chaperone activity and also provide some mechanistic explanations for the chaperone activity.

1: the authors report that purified UgpB has 90% G3P occupancy. They propose that the acid denaturation of UgpB that occurs when bacteria pass through the stomach causes UgpB to lose G3P, which would activate its chaperone function. However, in the growth assays that were done (for instance with the various pts mutants), periplasmic UgpB likely binds G3P, which should keep its chaperone function off. Yet, the authors show that it does prevent the aggregation of the tripartite fusion. This needs to be clarified.

2: the authors also explain that UgpB has an extremely high affinity for G3P (117 nM). However, adding 120 µM (1,000 fold excess) does not turn off the chaperone function of UgpB, which is very surprising. They only start seeing an effect when they add 1,2 mM. This also needs to be clarified. Could it be that G3P, when present at high concentrations, causes the aggregation of the substrate?

3: If the authors are right and that removing G3P activates UgpB as a chaperone, then what is the mechanism? Does free UgpB unfold? What do we know regarding the structure of this protein?

4: the authors also make confusing statements regarding G3P. They first explain that "the anti-aggregation of UgpB is tightly modulated by its binding to G3P". Later, they conclude that "UgpB only functions as a chaperone when it is G3P free". However, the results in Figure S11 do not support this second statement: UgpBG3P does protect MDH from aggregation, although less than the free protein.

5: Fig. S11 is an important one and should be moved to the main manuscript.

6:Figure 6 is beautiful and fancy. However, it is only very partially supported by (mostly in vitro) experimental data. I would move it to the supplementary information. Also, what evidence do they have that UgpB refolds after acid denaturation?

7:In the Results section, the authors describe UgpB as a "glyceraldehyde-3-phosphate" binding protein. This is incorrect: UgpB binds glycerol-3-phosphate. Are they sure they used glycerol-3-phosphate and not glyceraldehyde-3-phosphate in their experiments?

8:E. coli BL21 is not a K12 strain

9:Figure 4: the concentration of CHO used should be added in the Figure legends or in the figure itself.

10:In Figure 4D, we do not see more MDH in the supernatant from the sample with UgpB. Because MDH is almost absent from the pellet, we would expect to see more protein in the supernatant.

11:Some of the Western Blot panels are so black that we can barely see the red bands. In Figure 2C, the levels of b-lactamase activity are similar in all three pts mutants. Why do we see much less Bla-Im7L53AI54A in the psts mutant in Fig. 2B?

Referee #2:

Periplasmic proteins from Gram-negative bacteria are strongly exposed to changes in growth parameters including salt concentration or pH. This is particularly relevant for enteric bacteria as they face diverse host defense barriers when passing through the gastrointestinal tract. For example bacteria are exposed to bile salts in the intestine. Bile salts (e.g. cholate) are amphipathic molecules with antibacterial potential by disrupting membranes or trigger protein unfolding and aggregation. How bacteria protect their proteome against such compounds is therefore interesting and medically relevant.

Here, the authors employed a β-Lactamase folding sensor whose stabilization by e.g. molecular chaperones leads to increased antibiotic resistance. This system allows if coupled to e.g. transposon mutagenesis to identify novel protein quality control factors and has been successfully applied by the authors before. Here, the they show that E. coli pstSCA mutants indirectly cause folding sensor stabilization by increasing the expression of the Glycerol-3-Phosphate (G3P) binding protein UgpB, which becomes the most abundant periplasmic protein. As ugpB expression can be induced by bile salts, the authors tested whether UgpB can protect model substrates from cholate induced unfolding and aggregation. They show that UgpB but no other periplasmic chaperones can protect proteins from bile-induced aggregation. UgpB chaperone activity is weakened in presence of its substrate G3P, raising the possibility that UgpB function as chaperone or G3P binding protein is controlled by e.g. substrate availability and can be switched depending on environmental conditions. Here, the authors speculate that G3P binding and dissociation will be modulated upon passage of enteric bacteria through a human body, allowing for specific UgpB chaperone activation upon exposure of bacteria to bile salts in the intestine.

The presented study is interesting, well executed and nicely presented. The model how specific environmental conditions (e.g. low pH in stomach) and substrate availability can control the diverse UgpB activities is intriguing, however, it remains largely speculative so far and is in need of further substantiation. Additional experiments seem required to validate the appealing model and should be provided in a revised manuscript.

Major points
- Fig. 5: the authors demonstrate that transient exposure to low pH causes dissociation of G3P from UgpB. Yet, this kind of substrate-free UgpB has not been tested in chaperone assays, which rely on UgpB that was initially denatured by high GdnHCl concentrations to trigger G3P removal. The suggested model would be strongly supported if UgpB chaperone function is increased upon pH-induced G3P dissociation and can be subsequently counterbalanced by G3P re-addition.

- The authors show that overproduction of UgpB confers increased resistance to bile salts but they did not test whether an ugpB mutant exhibits increased sensitivity. This seems relevant, as other periplasmic folding factors (e.g. Spy) might be sufficient to protect wild type bacteria from these compounds. As chaperones like UgpB and Spy might exhibit overlapping activities it is also recommended to study the effects of spy ugpB double mutants on bile salt sensitivity

- Does bile salt triggers protein aggregation in vivo and is the aggregation profile altered in ugpB mutant cells or upon UgpB overproduction?

- 90% of UgpB is substrate (G3P) bound upon native purification, yet the protein still exhibits (reduced) chaperone activity (Fig. S11A/B). Similarly, addition of high concentration of G3P only partially inhibited chaperone activity (Fig. 5C). This seems not entirely consistent with the model proposed by the authors.

Referee #1:

This manuscript reports the identification of UgpB, a glycerol-3-phosphate binding periplasmic protein, as a novel chaperone protecting periplasmic proteins from bile salts-induced protein aggregation. The first part of the manuscript describes the elegant approach, combining TnSeq analysis and the use of a tripartite fusion, used by the authors to uncover this novel function of UgpB. In the second part, the authors investigate the chaperone properties of UgpB and conclude that it functions as a chaperone when it does not bind glycerol-3-phosphate. Overall, I find the paper well written and interesting, the approach used very clever and the identification of UgpB as a new periplasmic chaperone exciting.

→We thank the reviewer for these kind comments.

The results presented in the first part are solid, but I'm less convinced by those reported in the second part and the conclusions that were drawn, as explained in the comments below. I think that the authors need to clarify the role of G3P in UgpB chaperone activity and also provide some mechanistic explanations for the chaperone activity.

→ To address this concern, we added new results which provide evidence for our mechanistic explanation of the molecular mechanism of UgpB's chaperone activity and also the role of G3P in the regulation of UgpB's chaperone activity. Our key results showed that (1) UgpB variants that fail to bind G3P binding are constitutively active as chaperones independent of the presence or absence of G3P. This result strongly suggests that the dissociation of G3P is a prerequisite for UgpB's chaperone activity and (2) that the residues located in the cleft between two domains that is exposed upon G3P dissociation is involved in UgpB's chaperone activity (new figures 4GH, figure 5 and S13-16). We obtained crystals of UgpB frustratingly just two days prior to the COVID-19 triggered shutdown of my lab. However, due to the kindness of Rudi Glockshuber, we able to gain access one of the only synchrotrons in the world accepting non-COVID-19 related samples namely the Swiss Light Source at the Paul Scherrer Institute. Operating remotely, we were able to obtain high resolution data that enabled us to determine the 1.25 Å resolution structure of the ligand-free form of UgpB. This structure shows that G3P release induces conformational change that opens up a deep groove in the structure of UgpB that easily docks a variety of peptides derived from the substrate MDH. The closed form buries residues of UgpB that are implicated in binding protein substrates, the G3P-bound form of UgpB is not able to function as a chaperone.

1: the authors report that purified UgpB has 90% G3P occupancy. They propose that the acid denaturation of UgpB that occurs when bacteria pass through the stomach causes UgpB to lose

G3P, which would activate its chaperone function. However, in the growth assays that were done (for instance with the various pts mutants), periplasmic UgpB likely binds G3P, which should keep its chaperone function off. Yet, the authors show that it does prevent the aggregation of the tripartite fusion. This needs to be clarified.

→ For the purification of UgpB to be of high yield, we found it to work better to express the protein in the *E. coli* cytoplasm by using a UgpB construct without signal sequence. Since G3P is synthesized by reducing dihydroxyacetone phosphate (DHAP), a glycolysis intermediate, G3P is abundant in the cytosol (Lemieux et al, 2004). Therefore, purification of cytoplasmically expressed UgpB results in high G3P occupancy. We did not make this point clear enough in the original submission, this deficiency has now been rectified.

To directly estimate G3P occupancy of UgpB that is located in the periplasm, we now performed experiments where bacterial cells were grown in the same growth conditions which we used for various *in vivo* experiments (see methods), we extracted the periplasmic fraction from the *pstS* single deletion and *pstS ugpB* double deletion strains, and then examined how the tryptophan fluorescence spectrum of this extract changes upon the addition of G3P. By these means we were able to estimate that periplasmic UgpB from the *pstS* deletion strains has ~36% G3P occupancy. Since we expect some G3P to be released during the preparation of the periplasmic extracts by polymyxin B treatment, this value likely represents an upper limit. In any case, these experiments indicate that periplasmic G3P contains a large proportion of UgpB in the apo state, which we postulate is the chaperone active configuration. This data is now shown in a new supplementary figure (10E).

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2: the authors also explain that UgpB has an extremely high affinity for G3P (117 nM). However, adding 120 µM (1,000 fold excess) does not turn off the chaperone function of UgpB, which is very surprising. They only start seeing an effect when they add 1.2 mM. This also needs to be clarified. Could it be that G3P, when present at high concentrations, causes the aggregation of the substrate?

→ We agree that a surprisingly high concentration of G3P is apparently required to compete with MDH. First, we now have a completely independent line of evidence that G3P binding interferes with UgpB's chaperone activity. As we describe above, we targeted residues known to be involved in G3P binding and showed that these mutants which failed to bind G3P were constitutively chaperone active. However, to further examine this reviewer specific point, as the reviewer

suggested, we also wondered if G3P induced aggregation might be interfering with our results. We thus examined whether G3P can enhance bile salt - induced aggregation of MDH. To do this we performed a supernatant/pellet solubility assay at various G3P concentrations for MDH only, UgpB only and also MDH in combination with UgpB. These results are shown in a new supplement figure (S11E). The solubility of MDH or UgpB itself was not affected by the addition of G3P. To verify why such a high concentration of G3P is needed to compete with MDH, we need to determine the binding affinity between bile salt denatured MDH and UgpB. However, this is technically difficult to measure because MDH is aggregation prone in the presence of bile salts.

3: If the authors are right and that removing G3P activates UgpB as a chaperone, then what is the mechanism? Does free UgpB unfold? What do we know regarding the structure of this protein?

→ To show that G3P removal activates the chaperone function of UgpB, we now provide evidence that UgpB variants that are defective in binding G3P are constitutively active as a chaperone (figure 4GH). G3P free UgpB does not unfold, but has a more open structure containing a deep groove as revealed by the protein crystal structure we have now solved of the ligand-free form of UgpB. In terms of structure, the dissociation of G3P from UgpB induces the conformational change from 'closed state' to 'open state' and consequently a deep groove is exposed. We showed that two tryptophan residues (W169, W172) located in the groove are involved in chaperone activity as well as G3P binding. These results are added as figure 5 and S13-16.

4: the authors also make confusing statements regarding G3P. They first explain that "the anti-aggregation of UgpB is tightly modulated by its binding to G3P". Later, they conclude that "UgpB only functions as a chaperone when it is G3P free". However, the results in Figure S11 do not support this second statement: UgpBG3P does protect MDH from aggregation, although less than the free protein.

→ In figure S11, UgpB$_{G3P}$ has ~90% G3P occupancy. Therefore, some chaperone activity is expected from the ~10% of UgpB in this preparation that lacks G3P. Indeed the chaperone activity of this preparation is 10% of that shown by apo UgpB (Figure 4C, S11AB). This fits very nicely with our assertion that ~10% of UgpB should be in the active chaperone form.

5: Fig. S11 is an important one and should be moved to the main manuscript.

→ We agree that the result shown in Fig S11E is important enough to move to the main manuscript (now fig 4F) The remainder of figure S11 has many panels. We would therefore prefer to mainly keep it in the supplement, though we note we are flexible in regards to this.

6:Figure 6 is beautiful and fancy. However, it is only very partially supported by (mostly in vitro) experimental data. I would move it to the supplementary information. Also, what evidence do they have that UgpB refolds after acid denaturation?

→ Thank you for the complement. As discussed above we think we do now have stronger support for the model, including, as discussed above, the observations that mutants of UgpB that fail to bind G3P are constitutively chaperone active and that the crystal structure of one such mutant opens up a groove suitable for peptide binding and also new data which show that pH transition from 2 to 7 can activate the anti-aggregation activity of UgpB and that the addition of G3P can re-inactivate its chaperone function (new figure 6C). We have also now shown that after an acid to base pH transition, UgpB is now again able to bind G3P and also is reactivated as a chaperone, This provides evidence that UgpB is refolded after acid denaturation (figure 6BC).

To further accommodate the reviewer, we have also softened the claims of the model by changing the perhaps too declarative title of the model from "The Activity of the Anti-Bile Chaperone UgpB Is Responsive to Small Molecules Present in the Digestive Tract' to be less declarative as 'Speculative Model of UgpB in the Digestive Tract'

7:In the Results section, the authors describe UgpB as a "glyceraldehyde-3-phosphate" binding protein. This is incorrect: UgpB binds glycerol-3-phosphate. Are they sure they used glycerol-3-phosphate and not glyceraldehyde-3-phosphate in their experiments?

→ Thank you for noticing this typographical error which has now been corrected to glycerol-3-phosphate.

8:E. coli BL21 is not a K12 strain

→ Correct, we have modified the text to make this clear.

9:Figure 4: the concentration of CHO used should be added in the Figure legends or in the figure itself.

→ The information has now been added in the figure legend

10:In Figure 4D, we do not see more MDH in the supernatant from the sample with UgpB. Because MDH is almost absent from the pellet, we would expect to see more protein in the supernatant.

→ The pellet fraction is concentrated (5-fold). So, the absolute amount of the aggregated MDH is
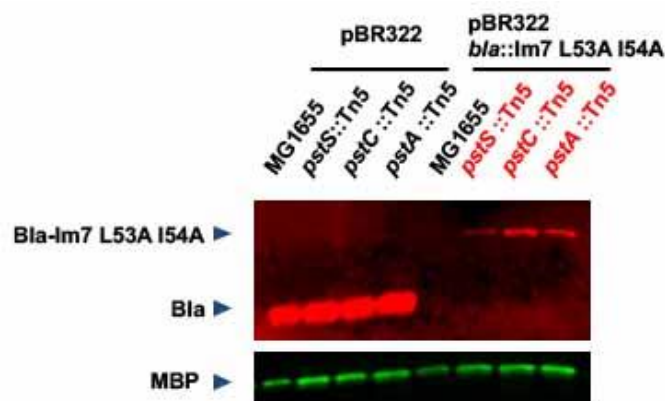
relatively low in the original reaction mixture. Hence, the increase of protein intensity in the supernatant fraction is subtle and is hard to see.

11:Some of the Western Blot panels are so black that we can barely see the red bands.

→ The Western Blot panels color brightness has been altered to make the figures less black

12: In Figure 2C, the levels of b-lactamase activity are similar in all three pts mutants. Why do we see much less Bla-Im7L53AI54A in the pstS mutant in Fig. 2B?

→ If we compare the mean values of B-lactamase activity, the reviewer is correct in that the *pstC* mutant shows the highest average activity and *pstS* and *pstA* mutants show similar average activity, however since the *pstS* mutant measurements have larger errors compared to the others measurements the difference between *pstC* and the others is not statistically significant. For reviewer's consideration, the biological replicate of Western blot result is shown below.

Referee #2:

Periplasmic proteins from Gram-negative bacteria are strongly exposed to changes in growth parameters including salt concentration or pH. This is particularly relevant for enteric bacteria as they face diverse host defense barriers when passing through the gastrointestinal tract. For example bacteria are exposed to bile salts in the intestine. Bile salts (e.g. cholate) are amphipathic molecules with antibacterial potential by disrupting membranes or trigger protein unfolding and aggregation. How bacteria protect their proteome against such compounds is therefore interesting and medically relevant. Here, the authors employed a β-Lactamase folding sensor whose stabilization by e.g. molecular chaperones leads to increased antibiotic resistance. This

system allows if coupled to e.g. transposon mutagenesis to identify novel protein quality control factors and has been successfully applied by the authors before. Here, the they show that E. coli pstSCA mutants indirectly cause folding sensor stabilization by increasing the expression of the Glycerol-3-Phosphate (G3P) binding protein UgpB, which becomes the most abundant periplasmic protein. As ugpB expression can be induced by bile salts, the authors tested whether UgpB can protect model substrates from cholate induced unfolding and aggregation. They show that UgpB but no other periplasmic chaperones can protect proteins from bile-induced aggregation. UgpB chaperone activity is weakened in presence of its substrate G3P, raising the possibility that UgpB function as chaperone or G3P binding protein is controlled by e.g. substrate availability and can be switched depending on environmental conditions. Here, the authors speculate that G3P binding and dissociation will be modulated upon passage of enteric bacteria through a human body, allowing for specific UgpB chaperone activation upon exposure of bacteria to bile salts in the intestine. The presented study is interesting, well executed and nicely presented. The model how specific environmental conditions (e.g. low pH in stomach) and substrate availability can control the diverse UgpB activities is intriguing,

→We thank the reviewer for these kind comments.

However, it remains largely speculative so far and is in need of further substantiation. Additional experiments seem required to validate the appealing model and should be provided in a revised manuscript.

→ As described in detail in our response to reviewer 1 who had a similar criticism, we have added new results which strongly support our mechanistic model of the role of G3P in the regulation of UgpB's chaperone activity. In brief, we constructed the UgpB variants which cannot bind to G3P, and showed that these are constitutively active as chaperones. We also determined the 1.25Å crystal structure of the ligand-free form of UgpB. Structural and mutational analysis revealed that G3P release opens up a deep groove in UgpB that is implicated in chaperone function. We also showed that chaperone activity is regained after pH transition in support of our proposed mechanistic model. Though we feel we now have much more experimental support for our model, we toned down the perhaps too declarative title of the model shown in figure 7 from "The Activity of the Anti-Bile Chaperone UgpB Is Responsive to Small Molecules Present in the Digestive Tract' to be less declarative as 'Speculative Model of UgpB in the Digestive Tract'

Major points

- Fig. 5: the authors demonstrate that transient exposure to low pH causes dissociation of G3P

from UgpB. Yet, this kind of substrate-free UgpB has not been tested in chaperone assays, which rely on UgpB that was initially denatured by high GdnHCl concentrations to trigger G3P removal. The suggested model would be strongly supported if UgpB chaperone function is increased upon pH-induced G3P dissociation and can be subsequently counterbalanced by G3P re-addition.

→ As reviewer suggested, we performed experiments to prove pH transition can actually confer the activation of UgpB's chaperone activity. Indeed, pH transition from 2 to 7 activates anti-aggregation activity of UgpB and re-addition of G3P can completely inactivate the chaperone function. This new data is added in the figure 6C.

- The authors show that overproduction of UgpB confers increased resistance to bile salts but they did not test whether an ugpB mutant exhibits increased sensitivity. This seems relevant, as other periplasmic folding factors (e.g. Spy) might be sufficient to protect wild type bacteria from these compounds. As chaperones like UgpB and Spy might exhibit overlapping activities it is also recommended to study the effects of spy ugpB double mutants on bile salt sensitivity

→ UgpB deletion does show some sensitivity to bile salts, we show this in figure 4A. We agree that it is possible that UgpB and other periplasmic chaperones such as Spy or also SurA, Skp, DegP, OsmY and so on might have some overlapping specificity, but testing mutants of these chaperones both individually and in combination with UgpB mutants, though it could reveal interesting information, is outside the scope of this paper. We note that similar types of experiments have in the past, comprised the bulk of an entire paper e.g. (Weski & Ehrmann, 2012). In the specific case of Spy, we did not conduct these experiments because we thought it was unlikely that UgpB is functionally that close since Spy does not exhibit and *in vitro* anti-aggregation activity against bile salt induced aggregation (Figure 4D).

- Does bile salt triggers protein aggregation in vivo and is the aggregation profile altered in ugpB mutant cells or upon UgpB overproduction?

→ Yes, bile salt induces protein aggregation *in vivo* (Cremers et al, 2014). Diverse ribosomal proteins, metabolic enzymes, DNA/RNA binding proteins and membrane proteins are prone to be aggregated by bile salt treatment.

We thought of attempting to look to see if the aggregation profile is altered in *ugpB* mutant cells or upon UgpB overproduction but we are unable to monitor the aggregation of periplasmic proteins because of a technical issue. As we have reviewed previously (Quan et al, 2013), all methods for isolation of periplasmic proteins involve the permeabilization of cells, which allows

for the escape of periplasmic contents, followed by a centrifugation step that pellets the cells. Unfortunately, aggregates are extremely unlikely to be able to escape through the relatively small holes generated during the various permeabilization procedures, we have found that polymyxin treatments for instance preferentially allow the escape of proteins of small molecular weight.

- 90% of UgpB is substrate (G3P) bound upon native purification, yet the protein still exhibits (reduced) chaperone activity (Fig. S11A/B).

→ We expressed UgpB in the *E. coli* cytoplasm to get high yield of protein purification. G3P is abundant in the cytosol as it is produced by reducing dihydroxyacetone phosphate (DHAP), a vital glycolysis intermediate. (Lemieux et al, 2004). Therefore, UgpB purified from the cytosol has a high G3P occupancy, which we measured at 90%. When purified from the periplasm, we observed that UgpB has 36% of G3P occupancy which in our model indicates that the majority of periplasmic UgpB should be active (new supplementary figure 10E). Though $UgpB_{G3P}$ with~90% G3P occupancy does exhibit some chaperone activity, it is roughly ~10-fold lower activity than apo UgpB (Figure 4C, S11AB), perfectly consistent with our model.

Similarly, addition of high concentration of G3P only partially inhibited chaperone activity (Fig. 5C). This seems not entirely consistent with the model proposed by the authors.

→ We are not entirely sure why this is but we now have considerably more independent evidence for our model. First of all, now we have demonstrated, by a completely independent line of evidence acquired by mutational and structural studies, that G3P binding inactivates UgpB's chaperone activity. In summary, we targeted the resides known to be involved in G3P binding and showed that alteration of these residues makes UgpB a constitutively active chaperone (new figure 4GH). Based on structural analysis, ligand release induces an open state of UgpB which reveals a deep binding groove suitable for binding peptides. However, we were still wondering that why high concentration of G3P is required to compete with MDH. One possibility is that G3P can enhance bile salt-induced aggregation of MDH. To test this possibility, we performed a supernatant/pellet solubility assay at various G3P concentrations for MDH only, UgpB only and also MDH in combination with UgpB. The solubility of MDH or UgpB itself was not affected by the addition of G3P (new supplementary figure S11F). To further investigate bile salt induced aggregation reactions in vitro it would be useful to know the binding affinity of bile salt denatured MDH with UgpB. Unfortunately, this Kd value cannot be easily determined because MDH is aggregation prone in the presence of bile salt. We are not alone in facing these types of

issues, for instance others have had difficulty in determining the binding affinity of the ensemble of heat or chemically denatured proteins for their chaperones.

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Thank you again for submitting a revised version of your manuscript EMBOJ-2019-104231, which we had recently rejected post-review. As already mentioned, given the additional experiments included in this version, we decided to send the manuscript back to the initial two referees. Please excuse the delay in communicating the decision to you, which was due to a delayed report on account of the current pandemic. We have now however received both referee's comments, which are included below for your information.

As you will see, the referees appreciate that the data added to the revised version, in particular the crystal structure, has improved the study and overall find that their initial concerns have been largely addressed. Referee #2 raises some remaining points, which should be addressed in a revised version of the manuscript. The suggested analysis of a mutant that is deficient in chaperone function, but not G3P binding, can possibly in part be addressed by the additional data you have meanwhile acquired, and we would ask you to include these experiments. Please also revise the model and discussion according to the referees remaining concerns. Once these issues are resolved we will be happy to consider the study further for publication.

We realize that lab work worldwide is currently affected by the COVID-19/SARS-CoV-2 pandemic and that more extensive experimental revisions may be significantly delayed. We can extend the revision time when needed, and we have extended our 'scooping protection policy' to cover the period required for a full revision. However, it is nonetheless important to clarify any questions and concerns, and to discuss potential issues you may foresee as soon as possible.

Please also feel free to contact me should you have any other further questions. Thank you for the opportunity to consider your work for publication. I look forward to receiving your revised manuscript.

REFEREE REPORTS

Referee #1:

The authors have satisfactorily addressed my concerns. The revised manuscript is very nice. I have no further issues.

(peptide is bound in a deep groove IN the cleft region: IN is missing)

Referee #2:

In their revised version the authors have largely addressed my previous concerns. In particular, they determined the crystal structure of an UgpB mutant (W169S/W172S) that cannot bind its small molecule substrate G3P. This major addition is supporting the initial model of UgpB chaperone activity control, justifying publication of the revised study.
The UgpB variant is constitutively active in chaperone assays and no longer affected by G3P presence, supporting the authors model that G3P availability controls UgpB chaperone function. Structure determination revealed exposure of a groove that might allow binding of misfolded proteins, an assumption indirectly supported by bioinformatic analysis (peptide docking). Further biochemical validation of this potential binding site for misfolded proteins (by e.g. a crosslinking approach) would further increase the impact of the study, yet this is not considered essential for publication.

Further points:
It would be nice to characterize an UgpB groove mutant, that is deficient in chaperone function but not G3P binding. The reviewer is aware of the fact that this is not easily feasible as groove residues might be involved in both types of interactions.

The idea that UgpB becomes activated by low pH in the stomach is nice and is also partially validated by new in vitro experiments. The model however still remains speculative and respective statements in the discussion section should be softened. In figure 7 the cartoon depicting the journey of bacteria through the digestive tract should be removed.

We were gratified by the positive comments from both reviewers.

Attached is a point by point response to all of the reviewers' few remaining comments our responses are shown in blue font.

Response to Referee Reports.

Referee #1:

The authors have satisfactorily addressed my concerns. The revised manuscript is very nice. I have no further issues.
→ We thank the reviewer for the positive comment concerning our revision.

(peptide is bound in a deep groove IN the cleft region: IN is missing)
→ Thank you for noticing this error, which has now been corrected.

Referee #2:

In their revised version the authors have largely addressed my previous concerns. In particular, they determined the crystal structure of an UgpB mutant (W169S/W172S) that cannot bind its small molecule substrate G3P. This major addition is supporting the initial model of UgpB chaperone activity control, justifying publication of the revised study.
The UgpB variant is constitutively active in chaperone assays and no longer affected by G3P presence, supporting the authors model that G3P availability controls UgpB chaperone function. Structure determination revealed exposure of a groove that might allow binding of misfolded proteins, an assumption indirectly supported by bioinformatic analysis (peptide docking). Further biochemical validation of this potential binding site for misfolded proteins (by e.g. a crosslinking approach) would further increase the impact of the study, yet this is not considered essential for publication.
→ We thank the reviewer for the positive statements concerning our revision.

Further points:
It would be nice to characterize an UgpB groove mutant, that is deficient in chaperone function but not G3P binding. The reviewer is aware of the fact that this is not easily feasible as groove residues might be involved in both types of interactions.
→ As reviewer mentioned, we also agree that the isolation of mutant which is deficient in chaperone function but not G3P would be difficult, because (1) many of hydrophobic residues in the groove region are likely involved in the chaperone function and (2) the alteration of these residues might be also affecting the G3P binding. (3) The identity of residues that are directly or indirectly involved in chaperone action of even extremely well studied chaperones such as GroEL and Hsp70 or Hsp90 is still the subject of considerable debate despite hundreds or thousands of papers that have been published on these chaperones. To make these mutants we would first need to clearly define the residues directly involved in peptide binding, even the crosslinking experiments that the reviewer suggests above would likely only result in a partial or preliminary idea of which residues are directly involved in peptide binding. In contrast, the residues that are directly

involved in G3P binding for UgpB have been clearly defined by others by solving the co-crystal structure with G3P (4AQ4, 6R1B) and verified with biochemical experiments that show that mutating these residues eliminates G3P binding (1, 2). We consider that the fundamental basis of the reviewer's request is to isolate and characterize mutants that clearly distinguish between G3P and peptide binding

This is a worthy goal, thus instead of defining and then mutating the residues involved peptide binding we simply isolated UgpB variants in residues that are involved in G3P binding and then tested them for their effects on chaperone function, this accomplishes the same goal of distinguishing chaperone function and G3P binding. We selected glutamate 66 and arginine 374 and substituted then to alanine and valine, respectively. These two residues contain charged side chains that are involved in G3P binding, and these specific substitutions are known to be severely defective in G3P binding without affecting the overall fold of the protein (1, 2). We expected that these variants to not affect the hydrophobicity of core cleft region of UgpB, which is involved in the chaperone activity, but only affect G3P binding. Indeed, the E66A and R374V variants of UgpB exhibit the same chaperone activity as G3P-free form of wildtype UgpB, suggesting these substitutions do not affect the chaperone activity. Importantly, the chaperone activity of these UgpB variants is constitutively active, as it is not affected by the presence of G3P in contrast to wild type UgpB. These results strongly support our hypothesis that G3P binding modulates the chaperone activity (New figures 4I and 4J).
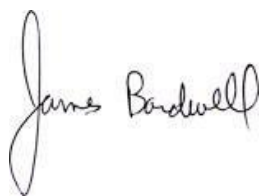
The idea that UgpB becomes activated by low pH in the stomach is nice and is also partially validated by new in vitro experiments. The model however still remains speculative and respective statements in the discussion section should be softened. In figure 7 the cartoon depicting the journey of bacteria through the digestive tract should be removed.
→ As reviewer #2 suggested the discussion part is had been softened to make it clear that our model is speculative and, figure 7 has been removed from the main text, and as suggested by reviewer 1 has been moved to the extended version. Since this figure, which reviewer #1 referred to as beautiful, is important to illustrate our model, for communication purposes, we would like to be able to refer to it and not remove it entirely, but will do so if reviewer #2 insists.

References

(1) Fenn JS, Nepravishta R, Guy CS, Harrison J, Angulo J, Cameron AD, Fullam E (2019) Structural Basis of Glycerophosphodiester Recognition by the Mycobacterium tuberculosis Substrate-Binding Protein UgpB. ACS chemical biology 14: 1879-1887
(2) Wuttge S, Bommer M, Jager F, Martins BM, Jacob S, Licht A, Scheffel F, Dobbek H, Schneider E (2012) Determinants of substrate specificity and biochemical properties of the sn-glycerol-3-phosphate ATP binding cassette transporter (UgpB-AEC2) of Escherichia coli. Molecular microbiology 86: 908-920

Sincerely yours,

[signature: James Bardwell]

Investigator, Howard Hughes Medical Institute
Rowena G. Matthews Collegiate Prof. of Molecular Cellular and Developmental Biology

Thank you again for submitting the revised manuscript. I am pleased to say that the referees now all support publication. Therefore I would like to ask you to address a number of editorial issues that are listed in detail below in a final revised version. Once these remaining issues are resolved, we will be happy to formally accept the manuscript for publication.

REFEREE REPORTS

Referee #2:

The authors have addressed my remaining minor concerns. I therefore support publication of the study in the EMBO Journal.

EMBO PRESS

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: James Bardwell
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2019-104231R-Q

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

➜ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
➜ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
➜ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
➜ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
➜ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

➜ a specification of the experimental system investigated (eg cell line, species name).
➜ the assay(s) and method(s) used to carry out the reported observations and measurements
➜ an explicit mention of the biological and chemical entity(ies) that are being measured.
➜ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

➜ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
➜ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
➜ a statement of how many times the experiment shown was independently replicated in the laboratory.
➜ definitions of statistical methods and measures:
  • common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  • are tests one-sided or two-sided?
  • are there adjustments for multiple comparisons?
  • exact statistical test results, e.g., P values = x but not P values < x;
  • definition of 'center values' as median or average;
  • definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

B- Statistics and general methods

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical method was used to pre-determine sample size. Sample size was chosen based on prior experience and standards in the field. Biological and biochemical experiments were generally repeated at least two times. The statistical analysis method, the number of repeats, the error and the data points is described in the figure legend.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Unpaired t-tests were performed and P-values were stated. The statistical analysis method, the number of repeats, the error and the data points is described in the figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Most experiments are biochemical analysis, so no specific tests were used to evaluate whether the variance of an independent experiment is normal distributed.

Is there an estimate of variation within each group of data?	We show for each sample the data points of independent repeats and the standard error of the mean (SEM) to show the variation within one sample
Is the variance similar between the groups that are being statistically compared?	N/A

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Anti-β-lactamase antibody (Millipore, AB-3738) and anti-maltose binding protein (MBP) (New England Biolabs, E8032S) were used. Anti-Im7 was made by Pacific Immunology and validated (Appendix, Fig. S14).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	PDB accession code for the protein structure is described in a Data Availability section.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A