

Structural and molecular basis for allosteric regulation and catalytic coupling of human phosphoribosylformylglycinamidase synthase

Corresponding Author: Professor Jarrod French

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript by Sharma and colleagues reports the structures of three complexes of human PFAS that elucidate different steps in the catalytic cycle. Importantly, these structures reveal the ammonia tunnel between the glutaminase and FGAMS domain and elucidate the gating mechanism. The structures also appear to contain a metastable intermediate. These are very important findings. The experiments are carefully performed and for the most part clearly described. A few issues could be further clarified.

Major points

1. The authors claim to capture the iminophosphate intermediate for the first time. However, they really only observe electron density that they have modeled as the iminophosphate intermediate. Given the resolution is only $\sim 3\text{\AA}$, can they really rule out other potential complexes? Fits to the electron density (e.g., Fig. S8) should be included in the main manuscript, with the intermediate and ADP displayed as they appear in the active site, not separately as in Fig. S8, so that the reader can assess the goodness of the fit. Alternative complexes should be included in the SI to show that other reasonable possibilities do not fit as well (FGAR•ADP•Pi, FGAR•ATP, also alpha FGAR). In the absence of other evidence confirming the iminophosphate intermediate, given the resolution, it might be good to moderate their claim.

2. ADP is a common contaminant of AMPPNP and ATP- have the authors confirmed that the apoenzyme contains the second ADP molecule?

3. Tunnel mutants. The key issue in these experiments is whether the glutaminase reaction has become uncoupled from the FGAMS reaction. This is very difficult to assess from the data as presented. One wants to directly compare the rate of ammonia production to the rate of FGAM production, which is most easily accomplished in a Table. What is the "relative basal glutamate production" in Fig. S18A? there is no wild-type basal NH_3 production...

Minor issues

1. The introduction refers to Fig. 1A, but never discusses IgPurLs and smPurLs.
2. The manuscript text should report k_{cat} for glutaminase reaction in addition to K_m . Note that the k_{cat} reported in Fig 1 legend has units of $\mu\text{M}/\text{min}$ - these are units for V_{max} . The value of k_{cat} should be in time^{-1} (e.g., s^{-1}): $V_{\text{max}} = k_{\text{cat}} \cdot [\text{E}]$
3. Fig. 1B would be easier to read if the structures had thicker lines/bold fonts.
4. Fig. 1C the y-axis should be converted to concentration of product (or the assay should be described since it is not clear what absorbs at 363 nm). The x-axis should be converted to minutes.
5. Fig. 1D legend- "kinetic analysis of the GD" can be taken to mean that the GD domain is assayed independently. Better to "kinetic analysis of the glutaminase reaction".
6. The $\text{IC}_{50} = 148 \mu\text{M}$ for FGAR requires more explanation- as written, it implies that the authors have developed a new assay for the FGAMS reaction. However, the new assay is for a promiscuous dephosphorylation reaction.

7. "Cryo-EM of the HSF GAMS ternary" section, 3rd line: "Glutamine" should be "Ammonia". Second to last line "formylglycinamide" is misspelled.
8. The two conformations of ADP should be discussed when the ternary complex is discussed.
9. More information about how the composition of the ammonia tunnel resembles the MS simulations would be welcome.
10. Gates 3 and 4 in Fig. 7 are not discussed in the text.
11. No red C-loop fragment is apparent in Fig. 2A although red loops fragments are visible in Fig. 2B.
12. Fig. 6 talks about Gates 1 and 2 controlling entry into the glutaminase site, but the ammonia should be leaving the glutaminase site and going into the tunnel. It might make more sense to number the gates in the order that the ammonia would encounter them.

Reviewer #2

(Remarks to the Author)

In this manuscript, Sharma et al. have structurally characterized catalytic cycle of human phosphoribosylglycinamide synthase (FGAMS). This is in line with previous results of Nandini Sharma on the topic of FGAMS enzymatic mechanism, e.g. Sharma et al. *Acs Catal* 2022 and *Science advances* 2020.

The manuscript describes detailed conformational steps in the enzymatic reaction performed by FGAMS on the example of the human enzyme. This brings insights into allosteric regulation and the related conformational changes of this class of enzymes. In particular, identification of the reaction intermediate, formation of the ammonia channel or the catalytic coupling of individual enzyme domains. The authors should however bring these results better in perspective to the current knowledge (largely produced by the first author) rather than claiming future possible use of the results in therapeutic development, which is not the scope of the manuscript.

The structural data is of good quality to answer the relevant biological questions. Structural figures are very informative. The final figure of the proposed model could be improved.

Overall, the study by Sharma et al. is undoubtedly a significant contribution to our understanding of amidotransferase enzymes and will be interesting to a broad audience of readers. However, some adjustments of the manuscript can be done to improve the interpretation of the very complex mechanism the authors describe.

Comments:

1. The authors discuss reaction pathway of the FGAR to FGAM conversion and structural confirmation of reaction intermediate, described also in figure S10. The discussion should clearly mark which pathway is supported by the structural data. In figure S10 it should be marked which intermediate is captured in the cryo-EM structure. From my understanding of results in ref. 57, the O18 labeling experiments only confirms the transfer of amide oxygen of FGAR to the released phosphate group, not the reaction intermediate itself.
2. The authors claim 'structural elucidation of an ammonia channel connecting the active sites of the glutaminase and synthase domains'. It might be beneficial to compare their finding with the previously observed ammonia channels, e.g. in work of Stephane Mouilleron (*J. Biol. Chem.* 2006).
3. In the final model Figure 8 there are many individual residues marked, however, their function is not mentioned in neither the discussion or figure legends. It might be beneficial to mention their function while describing the figure. Overall the figure could better depict the individual conformational changes and their respective functions. Omit marking of individual domains in individual states but rather describe what they do/how they change in individual steps. E.g. glutamine entry gating is completely omitted in the Figure 8.

Minor comments:

4. The authors should include line and page numbering in the submitted manuscript.
5. The first part of the introduction is dedicated to relevance of purine metabolism to disease, which is not the main topic of the study. Part of the introduction should be dedicated to the current knowledge of 'allosteric regulation and catalytic coupling' of amidotransferase-like enzymes. The 'Increased biosynthesis of (d)NTPs is a universal, and highly druggable, feature of cancer.' and 'The transfer of ammonia between the two active sites is facilitated by a putative ammonia channel.' sentences should be referenced.
6. The manuscript should avoid 'first time' claims as they do not add any useful meaning.
7. The first paragraph of results should be largely transferred to methods section.
8. The 'as a basis for future drug discovery efforts' should be omitted, as there is no reference to practical drug discovery in the manuscript. Or the authors should make some discussion on the possible drug discovery in the discussion section.
9. In the second paragraph of the results, authors might add some details to the description of the observed structure. Particularly to comment about the ligand combinations.
10. In the third paragraph of the results the sample preparation and cryo-EM details should be transferred into methods section.
11. Figure S4B is referenced but does not exist.
12. Is glutamine correct in this sentence: 'Glutamine liberated in the GD is added to FGAR in an ATP-dependent reaction. The mechanism of the synthase reaction is proposed to proceed via an activated iminophosphate intermediate (51).'??
13. Is this a surprise or rather expected outcome: 'Surprisingly, the resulting maps showed clearly resolved coulombic potential for a trapped iminophosphate intermediate, and ADP molecule, and a well ordered C-loop (Figs. 2C, 2D, 3C and S8).'??
14. Sentence 'This is consistent with previous computational studies that implicated these two residues in a gating mechanism for transient channel formation (54).' probably needs to be introduced to be in context with the text before.
15. The authors used the non-uniform refinement implemented in the cryoSPARC software, which generated over sharpened regions of the obtained cryo-EM maps. To mitigate this phenomenon they utilized the DeepEMhancer post-

processing. Please verify individual half-maps were used as inputs to DeepEMhancer post-processing. The authors should also mention DeepEMhancer utilization in any cryo-EM map figures, e.g. Figure S3 or S8 or S12. The authors should also make sure they deposit the raw cryo-EM maps along with the DeepEMhancer post-processed maps into the EMDB database.

16. Did the authors use any data aberration correction algorithms, reference-based motion correction and frame weighing to improve the final cryo-EM maps?

17. Could authors comment on the very low Q-scores of some residues of the deposited PDB model? In some places the model could be improved to correlate better with the obtained cryo-EM map. Please consider adding all side chain residues.

18. Some water molecules are modelled. Are the modeled water molecules confirmed by occurrence in multiple cryo-EM structures? The water molecules are not visible in the DeepEMhanced maps.

19. In the cryo-EM methods the authors mention utilization of Gatan K3 camera. In other parts they mention Falcon 4i detector. Please check this discrepancy.

Minor comments to Figures:

1. In Figure 1 the sticking of PAGE analysis should be marked.

2. In Figure 2 the overall cryo-EM maps might be omitted, as they do not add to much additional information. They look in different orientation in respect to the presented models.

3. In Figure 4C it might be helpful to directly compare details of C-loop in ternary complex and quaternary complex. Not only referencing to TmPurL.

4. Figure 7 legend. In line 5, there is probably a word missing after 'The' in the beginning of sentence.

5. In Figure 8, the titles of described states should be referred to observed structures.

6. In Figure 8, the position of release ammonia should correlate with the predicted position in the structure study.

Reviewer #3

(Remarks to the Author)

See attached review

[Editorial Note: See end of file]

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed all my concerns.

Reviewer #2

(Remarks to the Author)

The authors have addressed all concerns raised. They have improved the discussion section and the description of the final model in Figure 8. In particular, the revised manuscript provides a clearer description of the catalytic cycle of human FGAMS and the associated structural reorganizations of the enzyme.

The cryo-EM methodology follows standard approaches for 3D structure determination.

Minor comments:

The authors should refrain from making a "first time" claim in the discussion section (line 393).

In the superposition figures (e.g., Figures 5 and S17), it would be helpful to specify which residues were used for the structural alignment.

Reviewer #3

(Remarks to the Author)

The authors have responded appropriately to my comments.

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Point-by-point responses to reviewer comments.
(Reviewer comments are shown as *italicized* text and our responses are given in blue text.)

Reviewer 1:

The manuscript by Sharma and colleagues reports the structures of three complexes of human PFAS that elucidate different steps in the catalytic cycle. Importantly, these structures reveal the ammonia tunnel between the glutaminase and FGAMS domain and elucidate the gating mechanism. The structures also appear to contain a metastable intermediate. These are very important findings. The experiments are carefully performed and for the most part clearly described. A few issues could be further clarified.

We thank the reviewers for their positive comments and constructive feedback. Our responses are provided for all issues raised.

1. The authors claim to capture the iminophosphate intermediate for the first time. However, they really only observe electron density that they have modeled as the iminophosphate intermediate. Given the resolution is only ~3Å, can they really rule out other potential complexes? Fits to the electron density (e.g., Fig. S8) should be included in the main manuscript, with the intermediate and ADP displayed as they appear in the active site, not separately as in Fig. S8, so that the reader can assess the goodness of the fit. Alternative complexes should be included in the SI to show that other reasonable possibilities do not fit as well (FGAR•ADP•Pi, FGAR•ATP, also alpha FGAR). In the absence of other evidence confirming the iminophosphate intermediate, given the resolution, it might be good to moderate their claim.

The observed coulomb potential at the binding site in that structure shows clear density that is consistent with the proposed iminophosphate intermediate. We have moved this figure panel to Figure 3 in the revised manuscript (shown below). In order to rule out other possibilities, we have fit the other possible ligand combinations and included these results in a new figure in the SI (shown below). As illustrated, neither of the putative alternative substrate combinations matches the observed density. Binding of the alpha anomer of FGAR has been previously ruled out (Stubbe *et al.* Biochemistry 1986, 25(8): 2256) and is similarly not consistent with the observed density.

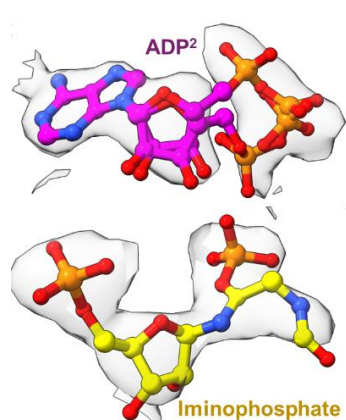
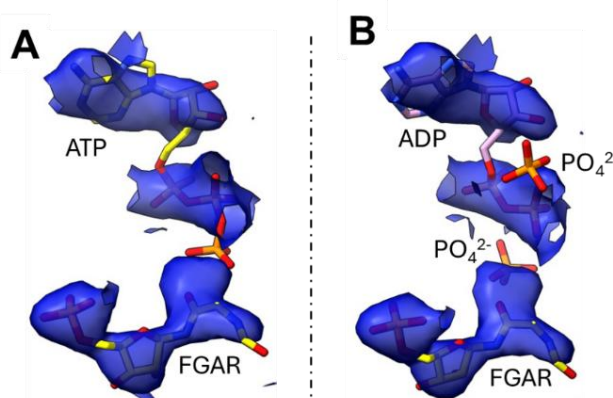


Fig. 3C from the revised manuscript showing the fit to the iminophosphate intermediate and ADP.



New SI Figure. Shows the fit to the alternative substrates, ATP and FGAR (A), and FGAR, ADP and phosphate (B).

2. ADP is a common contaminant of AMPPNP and ATP- have the authors confirmed that the apoenzyme contains the second ADP molecule?

As illustrated in the SI (Figs S3, S8 and S13), there is clear density consistent with an ADP molecule coordinated to three magnesium ions. This is also consistent with what was observed for homologous FGAMS proteins reported to bind ADP at this site. However, as the reviewer suggested, we have conducted additional experiments to confirm the identity of this molecule. New SI figure S5b shows results from HPLC analysis of denatured apo protein, purified in the absence of any additional nucleotides. There is a clear peak for a molecule present in the apo protein, and this elutes with a profile and UV spectrum matching that of the ADP standard.

3. Tunnel mutants. The key issue in these experiments is whether the glutaminase reaction has become uncoupled from the FGAMS reaction. This is very difficult to assess from the data as presented. One wants to directly compare the rate of ammonia production to the rate of FGAM production, which is most easily accomplished in a Table. What is the “relative basal glutamate production” in Fig. S18A? there is no wild-type basal NH₃ production...

We thank the reviewer for this suggestion. To better make the point, as the reviewer suggested we have added a table to Fig. S18 that shows the ratio of ammonia production to FGAM production. We have also changed the plots to all have the same scale, which additionally helps to clarify the point. Regarding the question about relative glutamate production in S18A, we have updated the description to now be “% Basal glutamate production”, which is defined as the rate of glutamate production in the absence of co-substrates as compared to the WT rate in the presence of all substrates (set to 100%). As suggested by the reviewer, this new representation of the data clearly shows a substantial reduction (20- to 100-fold) in coupling efficiency.

Minor issues

1. The introduction refers to Fig. 1A, but never discusses IgPurLs and smPurLs.

We have included additional text in the introduction discussing the distinction between large PurL and small PurL protein variants.

2. The manuscript text should report *k*_{cat} for glutaminase reaction in addition to *K*_m. Note that the *k*_{cat} reported in Fig 1 legend has units of $\mu\text{M}/\text{min}$ - these are units for *V*_{max}. The value of *k*_{cat} should be in time ⁻¹ (e.g., s⁻¹): $V_{\text{max}} = k_{\text{cat}} \cdot [E]$

Corrected.

3. Fig. 1B would be easier to read if the structures had thicker lines/bold fonts.

We have updated Fig. 1B as suggested.

4. Fig. 1C the y-axis should be converted to concentration of product (or the assay should be described since it is not clear what absorbs at 363 nm). The x-axis should be converted to minutes.

We have included additional description to the online methods under the section ‘Assay for glutamate production’ to improve clarity, as suggested. The glutamate production of HsFGAMS was coupled with a standard glutamate dehydrogenase assay where glutamate produced from HsFGAMS is converted to α -ketoglutarate in the presence of NAD⁺. In the assay reported, we used 3-acetylpyridine adenine dinucleotide (APAD), in place of NAD⁺. APAD has a more

significant oxidation potential than NAD^+ and is more effectively reduced than NAD^+ . Also, the stability of its reduced product (which is monitored at 363 nm) is significantly higher than the reduced product of NAD^+ (NADH).

5. *Fig. 1D legend- “kinetic analysis of the GD” can be taken to mean that the GD domain is assayed independently. Better to “kinetic analysis of the glutaminase reaction”.*

Corrected.

6. *The $\text{IC}_{50} = 148 \mu\text{M}$ for FGAR requires more explanation- as written, it implies that the authors have developed a new assay for the FGAMS reaction. However, the new assay is for a promiscuous dephosphorylation reaction.*

We have corrected the main text to clarify these details about the new assay, as suggested.

7. *“Cryo-EM of the HSFGAMS ternary” sectin, 3rd line: “Glutamine” should be “Ammonia”. Second to last line “formylglycinamide” is misspelled.*

Corrected.

8. *The two conformations of ADP should be discussed when the ternary complex is discussed.*

We have included discussion of the two conformations of ADP in the revised manuscript.

9. *More information about how the composition of the ammonia tunnel resembles the MS simulations would be welcome.*

We have included two additional paragraphs of text in the results section (under the subheading “Catalytic coupling through transient ammonia channel formation”) further describing the ammonia channel and prior MD simulations.

10. *Gates 3 and 4 in Fig. 7 are not discussed in the text.*

We thank the reviewer for pointing this out. While we had discussed gates 3 and 4 in the text, they were not clearly defined as such. We have clearly defined gates 3 and 4 in the revised manuscript prior to the discussion of these gates.

11. *No red C-loop fragment is apparent in Fig. 2A although red loops fragments are visible in Fig. 2B.*

The red C-loop is now visible, and clearly labeled, in the revised figure 2A.

12. *Fig. 6 talks about Gates 1 and 2 controlling entry into the glutaminase site, but the ammonia should be leaving the glutaminase site and going into the tunnel. It might make more sense to number the gates in the order that the ammonia would encounter them.*

We chose to number the gates following the order that the ammonia source enters the glutaminase site via ammonia release from the glutaminase site followed by its entry to the synthase site for reaction. The gate 1 (loop L1; at the exterior of the protein), gate 2 (Y1202-F1117; next to the gate 1) allow the ammonia source (glutamine) to enter to the synthase site. Then the ammonia produced leaves the glutaminase site via rearrangements in gate 3 (R1300-D685; at the exit of glutaminase site) at GD and

enters to the synthase site via opening of the gate 4 (H311-Q485; at the entry of synthase site) at SD. We feel that this convention is the most suitable in this case.

Reviewer 2:

In this manuscript, Sharma et al. have structurally characterized catalytic cycle of human phosphoribosylglycinamide synthase (FGAMS). This is in line with previous results of Nandini Sharma on the topic of FGAMS enzymatic mechanism, e.g. Sharma et al. Acs Catal 2022 and Science advances 2020. The manuscript describes detailed conformational steps in the enzymatic reaction performed by FGAMS on the example of the human enzyme. This brings insights into allosteric regulation and the related conformation changes of this class of enzymes. In particular, identification of the reaction intermediate, formation of the ammonia channel or the catalytic coupling of individual enzyme domains. The authors should however bring these results better in perspective to the current knowledge (largely produced by the first author) rather than claiming future possible use of the results in therapeutic development, which is not the scope of the manuscript. The structural data is of good quality to answer the relevant biological questions. Structural figures are very informative. The final figure of the proposed model could be improved. Overall, the study by Sharma et al. is undoubtedly a significant contribution to our understanding of amidotransferase enzymes and will be interesting to a broad audience of readers. However, some adjustments of the manuscript can be done to improve the interpretation of the very complex mechanism the authors describe.

We thank the reviewer for their thorough evaluation of this work and recognition of its significance. We have attempted to address these suggestions, specifically regarding the scope of the work and model, as described in the point-by-point response below.

Comments:

1. The authors discuss reaction pathway of the FGAR to FGAM conversion and structural confirmation of reaction intermediate, described also in figure S10. The discussion should clearly mark which pathway is supported by the structural data. In figure S10 is should be marked which intermediate is captured in the cryo-EM structure.

The proposed intermediate captured in the cryoEM ternary complex is now highlighted in Fig. S10.

From my understanding of results in ref. 57, the 018 labeling experiments only confirms the transfer of amide oxygen of FGAR to the released phosphate group, not the reaction intermediate itself.

We thank the reviewer for catching this overstatement of the published data. We have removed this statement.

2. The authors claim 'structural elucidation of an ammonia channel connecting the active sites of the glutaminase and synthase domains'. It might be beneficial to compare their finding with the previously observed ammonia channels, e.g. in work of Stephane Mouilleron (J. Biol. Chem. 2006).

As suggested by the reviewer, we have included additional discussion of ammonia channel formation in bacterial glucosamine-6-phosphate synthase as described in Teplyakov et al (JMB 2001) and Mouilleron et al (JBC 2006).

3. In the final model Figure 8 there are many individual residues marked, however, their function

is not mentioned in neither the discussion or figure legends. It might be beneficial to mention their function while describing the figure. Overall the figure could better depict the individual confirmation changes and their respective functions. Omit marking of individual domains in individual states but rather describe what they do/how they change in individual steps. E.g. glutamine entry gating is completely omitted in the Figure 8.

To better depict the observed changes and their functions we have updated the figure and clarified the description of individual steps in the text. Specifically, we have removed the domain names, labeled the individual residues in the figure and labeled specific features, such as gates and loops, that are discussed more fully in the text. In addition, we have substantially expanded our discussion of the mechanism, clearly referencing the structural features in Fig. 8, and describing the relevant functional changes that accompany the structural reorganizations.

Minor comments:

4. *The authors should include line and page numbering in the submitted manuscript.*

Corrected.

5. *The first part of the introduction is dedicated to relevance of purine metabolism to disease, which is not the main topic of the study. Part of the introduction should be dedicated to the current knowledge of 'allosteric regulation and catalytic coupling' of amidotransferase-like enzymes.*

As suggested we have included an additional paragraph of text describing regulatory mechanisms of purine metabolic enzymes and, in particular, catalytic coupling of amidotransferases.

The 'Increased biosynthesis of (d)NTPs is a universal, and highly druggable, feature of cancer.' and 'The transfer of ammonia between the two active sites is facilitated by a putative ammonia channel.' sentences should be referenced.

Corrected.

6. *The manuscript should avoid 'first time' claims as they do not add any useful meaning.*

Corrected.

7. *The first paragraph of results should be largely transferred to methods section.*

As suggested, we have removed extraneous information about the purification from the first paragraph of the results section.

8. *The 'as a basis for future drug discovery efforts' should be omitted, as there is no reference to practical drug discovery in the manuscript. Or the authors should make some discussion on the possible drug discovery in the discussion section.*

Our group is currently using these results as a basis for drug discovery (anti-metabolite chemotherapy for liver cancer). We have included additional text near the end of the discussion section to clarify the importance of this protein target for drug discovery.

9. *In the second paragraph of the results, authors might add some details to the description of the observed structure. Particularly to comment about the ligand combinations.*

In the second paragraph of the results section, we introduce the three structures and describe the combinations of ligands bound for each. The text reads: “Using Cryo-EM, we solved the structures of 1) HsFGAMS with AMP-PNP bound (referred to as the binary complex); 2) HsFGAMS in complex with the iminophosphate reaction intermediate and adenosine diphosphate (ADP) (referred to as the ternary complex); and 3) HsFGAMS with glutamyl thioester, AMP-PNP and FGAR bound (referred to as the quaternary complex) (Fig. 2).” Detailed descriptions of each structure are provided in the sections following.

10. In the third paragraph of the results the sample preparation and cryo-EM details should be transferred into methods section.

As suggested, we have removed some Cryo-EM details to the methods section.

11. Figure S4B is referenced but does not exist.

Our apologies for the mistake. This has been corrected to cite the appropriate figure, Fig. S5A.

12. Is glutamine correct in this sentence: ‘Glutamine liberated in the GD is added to FGAR in an ATP-dependent reaction. The mechanism of the synthase reaction is proposed to proceed via an activated iminophosphate intermediate (51).’?

Our apologies for the mistake. This has been corrected to read “Ammonia liberated...is added to FGAR...”

13. Is this a surprise or rather expected outcome: ‘Surprisingly, the resulting maps showed clearly resolved coulombic potential for a trapped iminophosphate intermediate, and ADP molecule, and a well ordered C-loop (Figs. 2C, 2D, 3C and S8).’?

While, as the reviewer indicates, this result was what we were seeking to achieve with the experiment, the flexibility and dynamics of protein structures always makes solving a structure with a trapped intermediate a pleasant surprise. Having said that, given what was known about the protein, this result was not unexpected. We have removed the qualifier at the beginning of this sentence in the revised text. It now begins “The resulting maps showed...”

14. Sentence ‘This is consistent with previous computational studies that implicated these two residues in a gating mechanism for transient channel formation (54).’ probably needs to be introduced to be in context with the text before.

We have updated this text to put it better into context with the previous statements.

15. The authors used the non-uniform refinement implemented in the cryoSPARC software, which generated over sharpened regions of the obtained cryo-EM maps. To mitigate this phenomenon they utilized the DeepEMhancer post-processing. Please verify individual half-maps were used as inputs to DeepEMhancer post-processing.

Individual half-maps were used as inputs to DeepEMhancer. We have clarified this in the text.

The authors should also mention DeepEMhancer utilization in any cryo-EM map figures, e.g. Figure S3 or S8 or S12. The authors should also make sure they deposit the raw cryo-EM maps along with the DeepEMhancer post-processed maps into the EMDB database.

We have revised the captions for these figures to describe that individual half-maps were used. The raw maps and post-processed maps have been deposited to the EMDB database.

16. Did the authors use any data aberration correction algorithms, reference-based motion correction and frame weighing to improve the final cryo-EM maps?

No. No further improvements were made beyond what is mentioned in the text.

17. Could authors comment on the very low Q-scores of some residues of the deposited PDB model? In some places the model could be improved to correlate better with the obtained cryo-EM map. Please consider adding all side chain residues.

We built the model into the map using the iterative process described in the methods section until, in our estimation, no further residues or side chains could be definitively built into the maps. The fit to the maps is given in SI figures S3, 8 and 13. While additional side chains could be added, it is our convention to only add side chain density when the placement of the side chain is unambiguous. As discussed, we have deposited the raw data for the maps and the models to the EMDB for later analysis by anyone interested.

18. Some water molecules are modelled. Are the modeled water molecules confirmed by occurrence in multiple cryo-EM structures? The water molecules are not visible in the DeepEMhanced maps.

Yes, we have confirmed that the ordered water molecules placed in the maps are accurate and consistent across multiple structures. The enhancement algorithm used by DeepEMhancer often suppresses the signals from non-protein features.

19. In the cryo-EM methods the authors mention utilization of Gatan K3 camera. In other parts they mention Falcon 4i detector. Please check this discrepancy.

Thank you for catching this typo. We have corrected and further clarified this in the text (the Images were collected on a 300kV Titan Krios G2 microscope equipped with K3 Summit detector).

Minor comments to Figures:

1. In Figure 1 the sticking of PAGE analysis should be marked.

We have updated this in the revised manuscript.

2. In Figure 2 the overall cryo-EM maps might be omitted, as they do not add to much additional information. They look in different orientation in respect to the presented models.

The models are presented in the exact same orientation with respect to the maps. We appreciate the reviewer's viewpoint, but respectfully disagree that the maps should be omitted. It is our opinion that they are a vital accompaniment to the models by providing support for the quality of the data.

3. In Figure 4C it might be helpful to directly compare details of C-loop in ternary complex and quaternary complex. Not only refencing to TmPurL.

We thank the reviewer for the suggestion and have included in the revised Fig. 4 the comparison with both the ternary and quaternary complex.

4. Figure 7 legend. In line 5, there is probably a word missing after 'The' in the beginning of sentence.

Corrected.

5. In Figure 8, the titles of described states should be referred to observed structures.

Corrected.

6. In Figure 8, the position of release ammonia should correlate with the predicted position in the structure study.

We have modified the position of the ammonia release in the figure to more accurately reflect the position in the structure.

Reviewer 3:

The manuscript describes structures of the human de novo purine biosynthetic enzyme phosphoribosylglycinamide synthase (hFGAMS) from cryo-EM analysis.

The 3-domain monomeric enzyme (>1300 residues) catalyzes a multi-step reaction: ATP-dependent formation of an imino-phosphate intermediate of the FGAR substrate in a synthase active site, release of ammonia by glutamine hydrolysis in a glutaminase active site, diffusion of ammonia through a channel in the enzyme, and attack of ammonia on the imino-phosphate to release the FGAM product. The synthase and glutaminase active sites are >20 Å apart. A long-standing question has been the coordination of the catalytic steps, as no glutamine hydrolysis occurs in absence of the FGAR substrate.

The authors have considerable experience with FGAMS, and here they cryo-trapped 3 substrate complexes on EM grids by judicious choices of reaction conditions. The EM maps are of high quality (resolution 3.1 – 3.3 Å by 0.143 FSC). They include
binary complex of AMP-PNP in the synthase active site,
ternary complex with the imino-phosphate intermediate & ADP in the synthase active site,
quaternary complex with AMP-PNP & FGAR in the synthase active site and the acyl-enzyme intermediate (Cys-Glu thioester) in the glutaminase active site.

Most of the Results section is devoted to a detailed analysis of small structural features of the 3 complexes. Nearly all the structure regions described have good density in the EM maps. FGAMS is well conserved from bacteria to humans, so some of the substrate poses can be validated in published higher-resolution crystal structures of prokaryotic homologs. This aspect of the interpretation seems very solid. The changes in structure with addition of substrates are very small. Visualizing the human enzyme in several catalytic states is a substantial advance in our understanding of catalytic coupling in hFGAMS, but – in my view – less of an advance than the authors claim.

The structural details were challenging to understand, even after superposition of the 3 models, because several elements of structure are not defined (loop 1, loop 4, C-loop, catalytic triad, oxyanion loop, Ω-loop, 3-helix bundle, gate 1, gate 2, gate 3, gate 4). This makes it very challenging to look at the structures and evaluate the authors' model for catalytic coupling and ammonia travel.

To address this point and help clarify the catalytic coupling mechanism, we have made substantial changes to the model figure and included additional text in the figure caption and body of the manuscript. In particular (as suggested by this and the other reviewers), we have included additional labels on the

figure, changed the titles of the states and clearly described each of the labeled structural elements in the caption and body of the manuscript. We feel that this improved figure and additional details provide a much more thorough and clearer description of the mechanism and features that drive it.

Moreover, the structural differences among the 3 complexes are very small – this is apparent in the superposition. For example, the salt bridge between D685 and R1300 occurs in all 3 complexes, belying the claim that it “reorganizes to form the opening of the channel at the glutaminase active site”. The very small structural changes may be amplified by use of a too-small probe sphere (radius not stated) in the CAVER calculation of an interior channel surface.

We have included a detailed description of the methods used to identify the transient ammonia channel, including the probe size (0.9 Å), in the revised online methods section. We have also updated the figure showing the proposed gating residues to include bond distances. With respect to the scale of the difference between the complexes, the maps were of sufficient quality to clearly place the sidechains of the residues lining the ammonia channel (including D685 and R1300) in all three complexes with a high degree of certainty. Comparison of the quaternary structure to that of the ternary structure shows a clear 1 Å shift in the gating residue (D685) coordinated with a shift in R1300. This opens the distance in the channel from 3 Å to greater than 4 Å (as shown in figure 7B – reproduced below). While this is a relatively small change, considering the effective Van der Waals diameter of ammonia is estimated to be 3.7 Å, the scale of the change is certainly consistent with the role of these residues in controlling access to and through this transient channel. Note also that, as mentioned in the text, the involvement of these residues was predicted and then confirmed by MD simulations and mutagenesis studies, respectively, in the homologous *S. typhimurium* enzyme StPurL (Science Advances, 2020, 6, eaay7919; ACS Catalysis 2022, 12: 1930).

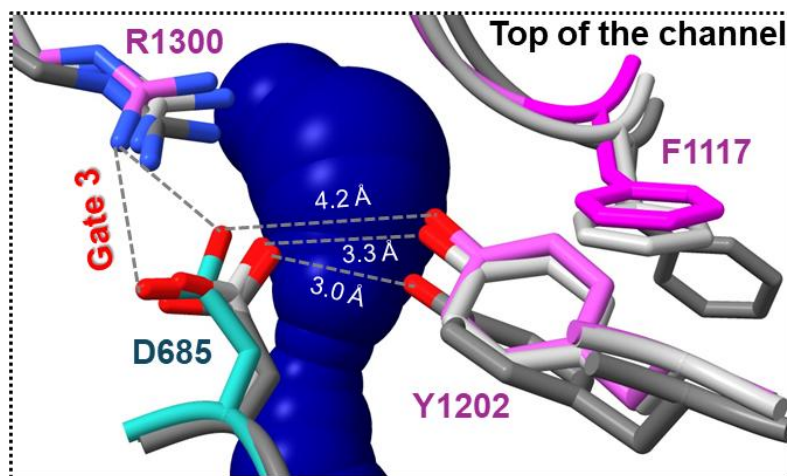


Fig. 7B: A superposition of the HsFGAMS structures shows changes in the gating residues at the top of the channel. The binary complex is colored white, ternary complex is colored gray and for the quaternary complex, the GD residues are colored magenta, SD residues are cyan. The ammonia channel is shown by dark blue spheres.

The ammonia channel is certainly near where the authors have placed it, but close examination of the structures does not support a unique position for the channel. Mutagenesis of residues lining the proposed ammonia channel (especially those in secondary structures where substitution is less likely to alter other structure elements) would be a good test of the proposed channel location.

As mentioned in the previous comment and described in the text, previous computational and mutagenesis experiments using StPurL are consistent with the description of the ammonia channel provided herein. The channel lining residues are highly conserved from human to *S. typhimurium* (as illustrated in SI Fig. S18). Additional structural and mutagenesis experiments in StPurL on residues lining the channel similarly demonstrated a profound impact on activity (ACS Chem Biol 2014, 10: 698). The further mutagenesis work described herein provides additional strong evidence for the location of the channel and role of the residues lining this structure. While additional mutations across the enzyme could be evaluated, considering the preponderance of structural and functional data, all consistent with the model proposed herein, we feel that further mutagenesis experiments will not substantially alter the outcome and, as such, are beyond the scope of this current work.

The mutagenesis data in Fig S18 are a nice addition to the paper. It would be interesting to determine whether the loss of catalytic coupling in some of the variants affects only glutamine-dependent FGAM production (can hFGAMS use ammonia directly?).

HsFGAMS does not accept ammonia directly as a substrate for the synthase activity and, as such, it is not possible to determine a rate for glutamine independent FGAM production. Note however, that mutations that effect the gating residues impact the coupling efficiency (how effectively the synthase site uses the ammonia produced at the glutaminase site), as described in the revised figure S19.

Technical comments

“formation of the ternary complex...results in a 90° flip of the oxyanion loop and creation of the oxyanion hole”, but the “flip” looks far less than 90°.

The change in angle was 67°, 75° and 120° for the three flipped residues, G1115, G1116 and F1117, respectively. As such, we reported this as the approximate average value of 90° for the loop residues. We have updated the text to reflect that this is an approximate value for the three residues.

I disagree that this “flip” of the 1115-1116 peptide, whatever its magnitude, forms the oxyanion hole. The oxyanion hole should consist of 2 H-bond donors, presumably the backbone NHs of G1116 and N1159. In the ternary complex where an oxyanion hole is proposed to exist, the G1116 NH is not pointed in a direction that can partner with the backbone NH of N1159 to stabilize a transient O⁻. However, this pair of NHs is beautifully positioned to stabilize an oxyanion in the binary and quaternary complexes.

The reviewer's latter point is correct. It was not very clearly stated in the manuscript, but the oxyanion hole is formed by two of these residues, including N1159 and one of the residues in the loop 1115-1117. The point that we wished to make was that there is a flexible loop region that is clearly involved in stabilizing the oxyanion that is formed during the reaction. Our structural data shows a transition from a closed/narrow to open to closed state as the reaction proceeds. While it is not possible to definitively assign which residues stabilize the transition state, the peptide flip is consistent with a reordering of this region to accommodate and stabilize the oxyanion. This type of oxyanion loop reorganization has been previously observed for other enzymes including glutamine amidotransferases and glycerol phosphate synthase (as described in the text). We have updated the text in the revised manuscript to more clearly describe this facet of the observed structural reorganization and putative implications for mechanism.

“an enantiomeric mixture of the substrate, α/β-FGAR” – shouldn't this be an anomeric mixture Would the density have been cleaner if a pure anomer (or stable analog thereof) were available?

The reviewer is correct; we have changed this to “an anomeric mixture”. Since the protein will only bind one anomer, it is unlikely that the purity of the anomeric mixture would impact the results.

“Glutamine liberated in the GD is added to FGAR” – should be ammonia liberated in the GD

Corrected.

Several waters have only a whisper of density – why are they in the models?

The water molecules in the model are fitted based on the density observed in the refined sharpened maps of the complexes. As described in our response to reviewer 2, these densities are not clearly observable in the deepEMhanced maps because the algorithm is trained primarily on protein-only datasets, making it unable to recognize or preserve the much weaker, diffuse signals characteristic of dynamic small molecules like water. The enhancement process prioritizes sharpening protein density to aid in modeling, which can actively suppress or remove the signals from non-protein features. As such, while we have modeled waters only into density that was clearly visible in the sharpened maps, the density may not be clear in the enhanced maps. Both the sharpened and deepEMhanced maps have been uploaded in the database for each structure and will be accessible for further analysis by all interested parties.

Response to reviewer comments (our response follows each reviewer comment):

The authors should refrain from making a “first time” claim in the discussion section (line 393).
We have removed all “first time” claims from the manuscript.

In the superposition figures (e.g., Figures 5 and S17), it would be helpful to specify which residues were used for the structural alignment.

We have now specified the numbering of the aligned residues and rmsd values in the figure captions of these respective figures.

The manuscript describes structures of the human *de novo* purine biosynthetic enzyme phosphoribosylglycinamide synthase (hFGAMS) from cryo-EM analysis.

The 3-domain monomeric enzyme (>1300 residues) catalyzes a multi-step reaction: ATP-dependent formation of an imino-phosphate intermediate of the FGAR substrate in a synthase active site, release of ammonia by glutamine hydrolysis in a glutaminase active site, diffusion of ammonia through a channel in the enzyme, and attack of ammonia on the imino-phosphate to release the FGAM product. The synthase and glutaminase active sites are >20 Å apart. A long-standing question has been the coordination of the catalytic steps, as no glutamine hydrolysis occurs in absence of the FGAR substrate.

The authors have considerable experience with FGAMS, and here they cryo-trapped 3 substrate complexes on EM grids by judicious choices of reaction conditions. The EM maps are of high quality (resolution 3.1 – 3.3 Å by 0.143 FSC). They include

- binary complex of AMP-PNP in the synthase active site,
- ternary complex with the imino-phosphate intermediate & ADP in the synthase active site,
- quaternary complex with AMP-PNP & FGAR in the synthase active site and the acyl-enzyme intermediate (Cys-Glu thioester) in the glutaminase active site.

Most of the Results section is devoted to a detailed analysis of small structural features of the 3 complexes. Nearly all the structure regions described have good density in the EM maps. FGAMS is well conserved from bacteria to humans, so some of the substrate poses can be validated in published higher-resolution crystal structures of prokaryotic homologs. This aspect of the interpretation seems very solid. The changes in structure with addition of substrates are very small. Visualizing the human enzyme in several catalytic states is a substantial advance in our understanding of catalytic coupling in hFGAMS, but – in my view – less of an advance than the authors claim.

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