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Structural insights into initial and intermediate steps of the ribosome recycling process

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1st Editorial Decision 04 October 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the exceptionally long delay in getting back to you with a decision. Unfortunately, one of the referees was not able to return his/her report as quickly as initially expected. In addition, and after discussions within our editorial team, we have involved an external editorial advisor of suitable expertise on the conflicting reports of referees 1 and 2, and we have consulted with referee 2 once more. The referees' reports as well as the feedback from our advisor are pasted below.

As you will see while all three referees consider the study as potentially interesting in principle, referee 1 is concerned that the functional and physiological significance of your findings remain largely unclear, as it is known that the combination of the heterologous components used in this study is non-functional. Furthermore, he/she raises a number of further (partially related) concerns that affect the overall conclusiveness and insightfulness of the dataset. Referee 2 is clearly more positive and puts forward a number of points to strengthen the study and conclusions. Given this clear discrepancy between the reports, we have involved an expert editorial advisor (please see below). He/she can see where referee 1 comes from. Still, on balance he/she is still rather positive about the study and would support publication if all issues raised are addressed convincingly and part of the conclusions are toned down. Also, he/she puts forward a number of points/suggestions of his/her own to strengthen the study and presentation.

Taking together all issues raised, we would thus be prepared to consider a revised manuscript that addresses all points raised by the referees and the advisor to the full satisfaction of referee 2 (and our

advisor if needed). I should add that it is EMBO Journal policy to allow only a single round of revision, and that acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version as well as on a favourable re-evaluation.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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

Sincerely yours,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The paper by Agrawal, Kaji et al describes a cryo-EM structure of the ribosome recycling complex in the presence of RRF and EF-G. At the first glance, the result appears interesting, because the structure of the complex with both factors simulataneously bound to the ribosome is not available. However, in order to stall the complex the authors used a heterologous combination of RRF and EF-G (from T. thermophilus and E. coli, respectively), which is known to be inactive in ribosome recycling. While this allowed the determination of the structure, its physiological relevance is highly questionable. Arguably, stalling the complexes is necessary to obtain the structure. However, usually such stalled complexes are carefully characterized by biochemical methods in order to prove that the stalled complex is on the reaction pathway. In contrast, extensive work by Y. Nakamura et al. showed that heterologous factor combinations are inactive both in vitro and in vivo due to deficient interactions between RRF and EF-G.
The model derived from the data is in parts consistent with the bulk of existing data and not novel (Figure 7, states A,B,C), and in parts either speculative (intermediate D may not be on the pathway of normal recycling due to the use of inactive factors combination) or inconsistent with the current literature (state E; here a number of findings from different groups were ignored, and the present paper does not contribute new insights into the problem).
The details of structure interpretation are highly speculative. In particular, interpreting the delicate conformational changes of EF-G domains 4-5 into the density shown in Fig. 4 is really overdone. Interpretation of the blurred density around the tRNA anticodon as a movement towards the E site is not warranted at current resolution; this would require a careful sorting of complexes into those with the tRNA in the P/E and E/E state (if those really exist). Detailed molecular description of interactions among RRF, EF-G, and the ribosome (e.g. p. 8-9) is not warranted given the resolution.

Referee #2

Review of Yokoyama et al., "Initial and intermediate steps..."

This is a very well done cryo-EM study of the structure of a ribosome complex bound simultaneously with RRF and EF-G. In order to stabilize the normally unstable complex, the authors used the clever approach of using the heterologous RRF from Thermus thermophilus. This allowed

reconstruction of a new complex ("Complex II") in which RRF, EF-G and the ribosome are all found in conformational states that have not previously been observed. The authors propose that Complex II is an intermediate state following binding of EF-G to the RRF complex. This is a reasonable model that helps to enlarge on our understanding of the recycling process. The paper is very thorough and carefully written. I recommend publication, providing that the authors address the following points:

1. There is an important new high-resolution structure of an RRF complex from the Cate laboratory (Dunkle et al., 2011). This paper should be incorporated into the background and discussion.

2. On p. 7 (and elsewhere) there is reference to a mass of well-defined density ascribed to the CTD of protein L7/L12. Although it is hard to be sure, it looks to my eye that this density actually corresponds to protein L10 complexed with the N-terminal domains of the 4 copies of L7/L12. The authors should compare their density with the x-ray model of the EF-G complex from the Ramakrishnan lab where the latter is seen.

3. On the bottom of p. 7 it says "...see also Supplementary Figure S2A...", but I do not see the surrounding helices of 23S rRNA in Figure S2A.

4. At the top of p. 8 (and elsewhere) the authors discuss interactions involving individual amino acids and nucleotides. Since these individual residues are not resolved at the resolution of the method, these interactions should be described more circumspectly. For example, "...in the vicinity of Ser20 and Pro21 of protein L11..." or wording to that effect.

5. Similarly, at the bottom of p. 9, there is a description of rearrangement of the C-terminal alpha-helix of EF-G, referring to Fig. 4B. It is very difficult to see how one could reach this conclusion based on the low-resolution density shown in the Figure.

6. On p. 10 ("Dynamic movements..." paragraph and elsewhere), I infer that contacts between domain II of RRF and the SRL are disrupted and replaced by contacts with domain I of EF-G. Is this correct? If so, it should be mentioned.

7. On p. 13, paragraph 2, 9 lines from the bottom: The tip of EF-G interacts with the tRNA anticodon in the Gao et al. crystal structure. I gather that the tip of EF-G does not interact with tRNA in the RRF-EF-G structure. If this is correct, it should be stated.

Referee #3

The manuscript is, to my opinion, a valuable contribution to our
understanding of ribosomal recycling by RRF and EF-G. The reason is that structures in which both factors are bound to the 70S ribosome are rare, and such structures are exactly what we need.

Editorial Advisor:

Overall, the work looks interesting to me particularly with respect to the localisation of RRF and EF-G on the 70S, with some points if addressed/clarified in the text would be probably fine.
Despite the fact that combining E. coli & thermus factors is non-functional it at least allowed to trap a reaction intermediate of the translation post-termination/recycling process in which both RRF and EF-G are bound, but it is not entirely clear which intermediate is actually trapped especially regarding the conformational changes discussed (notably on EF-G).
It should be stressed in the text that the intermediate is non-functional, and as a result the discussion on the interactions between the factors should be limited to a minimum (the position of the global domains of the factors is likely to be the same for the corresponding E. coli & thermus complexes, but probably different for residues; a sequence analysis would be helpful here).

While the intermediate might not be precisely on the reaction pathway as referee 1 says, it is probably close to it. My main concern here is that intermediate D in the scheme implies an EF-G dependent translocation of the tRNA to the E/E state, but this is not that obvious from the map (as also referee 1 mentions); from the density it is not sure that this is a full E-state (Fig. 6). This aspect has obviously implications in the description of the intermediate in the scheme and on the discussion. There seems indeed to be little evidence for what is going on afterwards in the reaction pathway, i.e. dissociation of the ribosomal subunits and of the factors, step E should be better indicated with a question mark.
Fig. 4 should indeed be drawn more clearly (maybe use different viewing angle), the conformational change of EF-G is not convincing from the figure.
Finally, the comment on the level of molecular interactions is a more general one which could be addressed by discussing less details in the MS. This is important here because precise RRF/EF-G interactions cannot be described due to the functional context, but as I mentioned the global position of domains is probably fine. Resolution issues usually limit the discussion to which residues would be closeby (as also referee 2 says) but one cannot tell which ones interact. In any case, residue informations are interesting for designing functional tests (e.g. Fig. 2A/B).
Regarding referee 2 , I agree with his/her comments and I think they should all be taken into account.
The comments by referee 3 are not very helpful.

Some other points I spotted:
Fig.1: L7/L12 is indeed most likely L10 + possibly a part of L7/L12, as also described by Diaconu et al., Cell 2005.
Regarding the visualization of the mRNA (in dark blue in Fig. 1) I'm really not sure that this can be seen in a cryo-EM map around 10A resolution (especially because it is single-stranded); this would be very sensitive to the way the map is segmented. I would suggest to not attempt displaying the mRNA (its path is known from crystal structures anyway).
Fig.2: 70S will be too small in print; NTT label cut at right edge.
Fig.3: 70S will be too small in print.
Fig.4: display more clearly, check whether these EF-G differences can really be addressed in the map (this has obviously implications on the discussion).
Fig.6B: tRNA fit not convincing, and thus the conformational change that is proposed.
Thus, my main concerns are the precision by which tRNA and EF-G conformational changes can be addressed here and the type of intermediate the complex represents.
Finally, one should make sure the maps are deposited in the EM databank (the deposition is at least already visible on the website).

1st Revision - authors' response 13 December 2011

Response to referees' comments.

Referee #1:

1. However, in order to stall the complex the authors used a heterologous combination of RRF and EF-G (from T. thermophilus and E. coli, respectively), which is known to be inactive in ribosome recycling. While this allowed the determination of the structure, its physiological relevance is highly questionable. Arguably, stalling the complexes is necessary to obtain the structure. However, usually such stalled complexes are carefully characterized by biochemical methods in order to prove that the stalled complex is on the reaction pathway.

There are two lines of direct evidence that the intermediate described in this study is indeed biochemically active. First, the published data by Raj and coworkers (Raj *et al*, 2005, RNA ***11***, 275-284) shows that the combination is slightly active (compare Fig. 1a with 1c, where the amount of polysome is slightly reduced with the increase of 70S in c; that result is highly reproducible). Second, when we increased the amount of ttRRF and ecEF-G, the reaction proceeds almost as efficiently as with the same-species combination. These results will be published separately, as we strongly feel that these new findings are beyond the realm of current structural study, and inclusion of additional data will make the manuscript unusually long and will lose the focus. In the revised

version, we have now clarified these points (last two sentences on page 4, continued onto page 5; and line 5 on page 5).

2. In contrast, extensive work by Y. Nakamura et al. showed that heterologous factor combinations are inactive both in vitro and in vivo due to deficient interactions between RRF and EF-G.

We presume that this reviewer is referring to a paper by Ito and coworkers (Ito *et al*, 2002, Mol Cell, ***9***, 1263-1272). It should be pointed out that those authors concluded from their studies "domain 1 of RRF and domain IV of EF-G seem to interact with each other"**,** which was found to be mechanistically incomprehensible based on RRF and EF-G mappings on the ribosome (Lancaster *et al,* Cell, 2002, ***111***, 129-140; Agrawal *et al*, 2004, PNAS ***101***, 8900-8905). Moreover, their conclusion that the combination of ecRRF and ttEF-G does not function was based on the results obtained with another heterologous combination of RRF and EF-G from *E. coli* and *M. tuberculosis* (Rao and Varshney, 2001, EMBO J ***20***, 2977-2986), and does not apply to the heterologus combination used in the present study.

3. The model derived from the data is in parts consistent with the bulk of existing data and not novel (Figure 7, states A,B,C)

First of all we did not intend to claim that A, B, and C are novel states. However, it must be emphasized that this is the first time we have captured RRF almost exclusively in the P1/IIa position, which further substantiates our earlier findings (Barat *et al*, 2007, Mol. Cell, ***27***, 250-261). Moreover, we described states B and C as preceding states for state D, which is the main focus of this study. We believe that most of the readers are not familiar with relationship between B or C states because positions P1/IIa has been published only once (Barat *et al*, 2007, Mol. Cell, ***27***, 250-261). It is therefore advisable to describe B and C as part of the overall scheme.

4. … and in parts either speculative (intermediate D may not be on the pathway of normal recycling due to the use of inactive factors combination)

We already addressed this issue under first two points.

5. … or inconsistent with the current literature (state E; here a number of findings from different groups were ignored, and the present paper does not contribute new insights into the problem).

It is true that the mechanism of the disassembly appears to be somewhat controversial, but careful extensive analysis of "the current literature" suggests that most of the data are consistent with the model described in the present study. What this referee calls the "current literature" is not so current anymore because the controversies were published around 2005. Most issues were subsequently resolved and discussed in a review article (Hirokawa *et al*, 2006, TIBS ***31***, 143-149), which was already cited in the introduction section (page 3, 1st paragraph, last sentence). There has been no obvious contradiction among the papers published within last four years.

6. The details of structure interpretation are highly speculative. In particular, interpreting the delicate conformational changes of EF-G domains 4-5 into the density shown in Fig. 4 is really overdone.

We have now revised the Figure 4 by adding a new panel, where transparencies of the cryo-EM densities have been switched to more clearly reveal the conformational changes associated with EF-G domains IV and V as compared to that observed in the absence of RRF. In addition, we have now removed the description of the conformational change associated C-terminal alpha-helix of EF-G.

7. Interpretation of the blurred density around the tRNA anticodon as a movement towards the E site is not warranted at current resolution; this would require a careful sorting of complexes into those with the tRNA in the P/E and E/E state (if those really exist).

The separation of populations with different positions of anticodons when rest of the ribosome adapts a unique conformational state (an unratcheted state in this case) has not been possible by either supervised classification or maximum-likelihood methods. This was already described in the Methods section (page 16, lines 3-9 from bottom). However, we see distinct densities for P/E and E/E states, which has now been shown more clearly in new Supplementary Figure S8 (cited on pages 12 [line 5], 13 [line 8] and 28 [line 9]).

8. Detailed molecular description of interactions among RRF, EF-G, and the ribosome (e.g. p. 8-9) is not warranted given the resolution.

We have now toned down the description of interactions, by replacing the word like “contact” with “close proximity” or “close vicinity” on both pages 8 (lines 6-17) and 9 (1st complete paragraph, lines 1-7).

Referee #2:

There was no major concern. All minor points raised are addressed below.

1. There is an important new high-resolution structure of an RRF complex from the Cate laboratory (Dunkle et al., 2011,). This paper should be incorporated into the background and discussion.

This was an oversight, which has been corrected. We have now included the Dunkle *et al* paper throughout the manuscript (twice in Introduction section, four times in Results section, and once in Discussion section). We have also added the comparison with RRF position derived by Dunkle *et al* to Supplementary Fig. S5 (last row). However, none of these changes affect the original essence or conclusion of the present study.

2. On p. 7 (and elsewhere) there is reference to a mass of well-defined density ascribed to the CTD of protein L7/L12. Although it is hard to be sure, it looks to my eye that this density actually corresponds to protein L10 complexed with the N-terminal domains of the 4 copies of L7/L12. The authors should compare their density with the x-ray model of the EF-G complex from the Ramakrishnan lab where the latter is seen.

The density identified as CTD of protein L7/L12 is correct. However, in order to further clarify this issue, we have now highlighted the densities corresponding to extended stalk and CTD of protein L7/L12 in Figures 1D-E (and Supplementary Figure S1). In addition, we have now added a new Supplementary Figure S10, showing a side-by-side comparison with results from the Ramakrishnan lab. The new Figure S10 has been referred on page 27 (legend to Figure 5) in the main paper.

3. On the bottom of p. 7 it says "...see also Supplementary Figure S2A...", but I do not see the surrounding helices of 23S rRNA in Figure S2A.

Thanks for catching this mistake. We have now removed the citation of Supplementary Figure S2A from that sentence.

4. At the top of p. 8 (and elsewhere) the authors discuss interactions involving individual amino acids and nucleotides. Since these individual residues are not resolved at the resolution of the method, these interactions should be described more circumspectly. For example, "...in the vicinity of Ser20 and Pro21 of protein L11..." or wording to that effect.

As suggested, we have now toned down the description of interactions, by replacing the word like "contact" with "close proximity" or "close vicinity" on both pages 8 (lines 6-17) and 9 (1st complete paragraph, lines 1-7).

5. Similarly, at the bottom of p. 9, there is a description of rearrangement of the C-terminal alpha-helix of EF-G, referring to Fig. 4B. It is very difficult to see how one could reach this conclusion based on the low-resolution density shown in the Figure.

We have now removed the description of the conformational change associated C-terminal alpha-helix of EF-G from the text and the Figure 4 legend.

6. On p. 10 ("Dynamic movements..." paragraph and elsewhere), I infer that contacts between domain II of RRF and the SRL are disrupted and replaced by contacts with domain I of EF-G. Is this correct? If so, it should be mentioned.

Yes, this is correct. We have now added a sentence to describe this (page 10, 2nd last sentence of the "Dynamic movements..." paragraph).

7. On p. 13, paragraph 2, 9 lines from the bottom: The tip of EF-G interacts with the tRNA anticodon in the Gao et al. crystal structure. I gather that the tip of EF-G does not interact with tRNA in the RRF-EF-G structure. If this is correct, it should be stated.

Yes, this is correct. We have now added this information (page 13, lines 5-6 from bottom).

Referee #3:

There was no criticism.

Editorial Advisor:

1. Overall, the work looks interesting to me particularly with respect to the localisation of RRF and EF-G on the 70S, with some points if addressed/clarified in the text would be probably fine.Despite the fact that combining E. coli & thermus factors is non-functional.

As stated in our response to referee # 1, this combination is functional, but the reaction is slow. We have now clarified these points (last two sentences on page 4, continued onto page 5; and line 5 on page 5).

2. ..it at least allowed to trap a reaction intermediate of the translation post-termination/recycling process in which both RRF and EF-G are bound, but it is not entirely clear which intermediate is actually trapped especially regarding the conformational changes discussed (notably on EF-G).

The question is not totally clear to us. The structure captured in our study represents an intermediate state of the recycling process that is stalled by fusidic acid immediately after EF-G-dependent GTP hydrolysis. We have added this statement on page 12 (lines 4-5 from bottom). Furthermore, our study reveals that EF-G can adopt more than one conformational state on the ribosome in post-GTP hydrolysis state. We have added such a statement on page 10 (lines 5-6).

3. It should be stressed in the text that the intermediate is non-functional.

See our response to the first point above and first point of referee # 1. We strongly believe that it is a functional intermediate, but would refrain from including the new biochemical data because it is beyond the realm of this paper and will be published separately. As stated above, the evidence that it

is slowly active already exists (Raj *et al*, 2005, RNA, ***11***, 275-284), though it was not so inferred in previous publication.

4. …and as a result the discussion on the interactions between the factors should be limited to a minimum (the position of the global domains of the factors is likely to be the same for the corresponding E. coli & thermus complexes, but probably different for residues; a sequence analysis would be helpful here).

We have now toned down the descriptions of interactions at the residue level by replacing the word like "contact" with "close proximity" or "close vicinity" on both pages 8 (lines 6-17) and 9 (1st complete paragraph, lines 1-7). Furthermore, we now include corresponding *E. coli* residues in bracket, both in the main text and the Supplementary Table S1. In addition, we now provide a new Supplementary Figure S4, showing sequence alignment of ttRRF and ecRRF with highlighted equivalent points of possible contacts with EF-G. The new Figure S4 has been cited on page 8 (line 9) in the main text.

5. While the intermediate might not be precisely on the reaction pathway as referee 1 says, it is probably close to it. My main concern here is that intermediate D in the scheme implies an EF-G dependent translocation of the tRNA to the E/E state, but this is not that obvious from the map (as also referee 1 mentions); from the density it is not sure that this is a full E-state (Fig. 6). This aspect has obviously implications in the description of the intermediate in the scheme and on the discussion.

We are extremely confident about the shift in the tRNA position in a major population of the complex. Moreover, we have now added a Supplementary Figure S8, showing stereo representation of the marked movement of tRNA based on fitting of atomic structure of tRNA in the strongest cryo-EM densities. The new Figure S8 has been cited in the main text on pages 12 (line 5), 13 (line 8) and 28 (line 9). In addition, we have revised the Supplementary Movie S3 showing a clear movement of tRNA.

6. There seems indeed to be little evidence for what is going on afterwards in the reaction pathway, i.e. dissociation of the ribosomal subunits and of the factors, step E should be better indicated with a question mark.

We agree that there is little evidence of actual sequence of events for step D to E. Therefore, we simply show the known end products rather than sequence of events in the panel E. However, we have now added a sentence to reflect this fact in the Figure legend (page 28, lines 4-5 from the bottom), and have toned down the description of Figure 7E.

7. Fig. 4 should indeed be drawn more clearly (maybe use different viewing angle), the conformational change of EF-G is not convincing from the figure.

We have now revised the Figure 4 by using a slightly different viewing angle and by adding a new panel, where transparencies of the cryo-EM densities have been switched to more clearly reveal the conformational changes associated with EF-G domains IV and V as compared to that observed in the absence of RRF.

8. Finally, the comment on the level of molecular interactions is a more general one which could be addressed by discussing less details in the MS. This is important here because precise RRF/EF-G interactions cannot be described due to the functional context, but as I mentioned the global position of domains is probably fine. Resolution issues usually limit the discussion to which residues would be close by (as also referee 2 says) but one cannot tell which ones interact. In any case, residue informations are interesting for designing functional tests (e.g. Fig. 2A/B).

See our response to point 4 above.

9. Regarding referee 2, I agree with his/her comments and I think they should all be taken into account.

We have adequately addressed all comments of referee 2.

10. Fig.1: L7/L12 is indeed most likely L10 + possibly a part of L7/L12, as also described by Diaconu et al., Cell 2005.

See our response to comment # 2 of referee 2. We have now clarified this by showing the L7/L12-CTD and the extended stalk in Fig. 1D and E (and Supplementary Fig. S1). In addition, we have included a Supplementary Figure S10 to further distinguish the positions of L7/L12-CTD and L10 components of the stalk. However, since the new Figure S10 does not directly impact the main findings of our paper, it has been referred only in the legend to Figure 5 (page 27) of the main paper.

11. Regarding the visualization of the mRNA (in dark blue in Fig. 1) I'm really not sure that this can be seen in a cryo-EM map around 10A resolution (especially because it is single-stranded); this would be very sensitive to the way the map is segmented.

We are very confident about the mRNA segmentation in both our maps. Densities corresponding to mRNA are so obvious that not showing them would mean that we are refusing to fully interpret the maps. In fact, we see a longer 3' ends of mRNA than that was observed in the X-ray structure. This could be simply because we have a very high occupancy of mRNA in our PoTC and that mRNA are rigidly held in unique position on the ribosome to provide sufficient signal for capture by averaging more than 100,000 single-particle images. We now provide a Supplementary Figure S3 to indicate what portions of the mRNA were directly visible and what portions were extracted after subtracting the ribosomal components. This supplementary Figure also shows that the independently segmented mRNA density matches closely with the mRNA conformation derived by X-ray crystallography. This new Figure S3 has been cited on pages 7 (line 8 from bottom), 13 (line 5), and 25 (line 19) in the main text.

12. I would suggest to not attempt displaying the mRNA (its path is known from crystal structures anyway).

If we had any doubt about the mRNA density, we would have easily removed it from the manuscript to humbly comply with the suggestion, but that is not the case. Moreover, we are not saying that we have discovered the mRNA path, which is already known from crystal structure. Our analysis clearly shows that mRNA is still bound during this intermediate state of recycling, and that we are indeed looking at PoTC.

13. Fig.2: 70S will be too small in print; NTT label cut at right edge. Fig.3: 70S will be too small in print.

We have now increased the size of thumbnails not only in Figs. 2 and 3, but in all other figures, including Supplementary figures. We also fixed the NTT label in Fig. 2.

14. Fig.4: display more clearly, check whether these EF-G differences can really be addressed in the map (this has obviously implications on the discussion). Fig.6B: tRNA fit not convincing, and thus the conformational change that is proposed. Thus, my main concerns are the precision by which tRNA and EF-G conformational changes can be addressed here and the type of intermediate the complex represents.

Please see our response to points 6 and 7 of referee 1, and points 5 and 7 above. Also, see response to point 2 above.

15. Finally, one should make sure the maps are deposited in the EM databank (the deposition is at least already visible on the website).

Apparently, this point was missed by the Editorial Advisor. We had already deposited the maps in the EM databank and fitted atomic coordinates in the PDB before previous submission (now page 17, last paragraph). Depositions are already visible on the respective websites.

2nd Editorial Decision 22 December 2011

Thank you for sending us your revised manuscript. Our original referee 2 and our expert editorial advisor (referee 4) have now seen it again, and you will be pleased to learn that they now both support publication of the study here. Still, referee 4 puts forward a number of minor issues and suggestions that you may wish to address in an amended version of the manuscript.

Thank you very much again for considering our journal for publication of your work. We are looking forward to the final version of your manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2

The authors have taken care to address all of my concerns as well as those of Reviewer #1, in my opinion. This is an excellent paper that should be published in EMBO J.

Referee #4

The authors have addressed the main points raised I believe, in particular whether the chimeric complex using factors from E. coli and T. thermophilus can be considered as a functional intermediate of the ribosome recycling phase. The additional illustrations in the Suppl. Mat. are also useful. Some minor points and suggestions are listed below.

- abstract: wording: "unusal" conformation isn't very informative, the point is that RRF domain II changes position in the presence of EF-G
- clarify in the discussion: the movement of RRF towards the E-site (according to the distance travelled) appears to be a partial translocation only (because domain I sits already on the P-site position and moves only slightly towards the E-site), not a full translocation as usually for tRNAs. Also, as the P-site tRNA becomes translocated, the ribosome could be in a special conformation (in contrast to P-site tRNA complexes - which are also more stable with respect to dissociation of the subunits) which could explain why EF-G binding occurs in a non-ratcheted state in contrast to tRNA translocation complexes. In this context, it could be useful to show a figure of one of the published tRNA/EF-G complexes (e.g. as Suppl. Fig.) which would be helpful for the discussion on page 13 (comparison of EF-G complexes with RRF or tRNA, ribosome ratcheting).
- Fig.4C (referee 2 and me): the model displayed still contains an alternative conformation of the C-terminal helix of EF-G for the 2 fits - which one is the correct one? Even though this aspect is not discussed anymore because it cannot be addressed with the cryo-EM map, it would be useful for the reader to label the C-terminus.
- last comment to referee 2, page 10 in the MS: should this not say RRF domain II becomes occupied by domains III and V and part of IV of EF-G (visible in Fig. 1E)?
- my previous comment (#2 in the response), meant was: intermediate within the reaction pathway

2nd Revision - authors' response 11 January 2012

Response to minor points/suggestions of referee #4:
1. *Abstract: wording: "unusal" conformation isn't very informative, the point is that RRF domain II changes position in the presence of EF-G.*

We have now replaced the word “unusual” with “hitherto unknown”

2. *Clarify in the discussion: the movement of RRF towards the E-site (according to the distance travelled) appears to be a partial translocation only (because domain I sits already on the P-site position and moves only slightly towards the E-site), not a full translocation as usually for tRNAs.*

We mentioned “toward the E site”, which did not imply movement to the E site. Anyway, we have now added the word “slightly” along with “~8 Å” on page 12 (second sentence of the Discussion section) and have also added the word “slightly” on page 10 (last sentence).

3. *Also, as the P-site tRNA becomes translocated, the ribosome could be in a special conformation (in contrast to P-site tRNA complexes - which are also more stable with respect to dissociation of the subunits) which could explain why EF-G binding occurs in a non-ratcheted state in contrast to tRNA translocation complexes. In this context, it could be useful to show a figure of one of the published tRNA/EF-G complexes (e.g. as Suppl. Fig.) which would be helpful for the discussion on page 13 (comparison of EF-G complexes with RRF or tRNA, ribosome ratcheting).*

We believe that this comment is somewhat out of context, because non-ratcheting of the P-site bound ribosome in the absence of EF-G is primarily due to the presence of the peptidyl moiety on the CCA end of the tRNA, which is not the case with RRF (or ttRRF). Nevertheless, we have now added a new Supplementary Figure S9 to show a comparison of 30S subunit movement in Complex2 with that in a tRNA•EF-G-bound ribosome complex. This figure is referred on page 13 (2nd paragraph, line 6).

4. *Fig.4C (referee 2 and me): the model displayed still contains an alternative conformation of the C-terminal helix of EF-G for the 2 fits - which one is the correct one? Even though this aspect is not discussed anymore because it cannot be addressed with the cryo-EM map, it would be useful for the reader to label the C-terminus.*

We have added the label “C” referring to C-terminal α-helix, and have briefly mentioned this in the text (page 10, lines 2-3; and also to the figure legend) to indicate the fact that the result is based on flexible docking.

5. *Last comment to referee 2, page 10 in the MS: should this not say RRF domain II becomes occupied by domains III and V and part of IV of EF-G (visible in Fig. 1E)?*

Fig. 1E shows an overall view of the cryo-EM density. Whereas the description on page 10 refers to Figure 5, and is inferred after docking of RRF and EF-G atomic structures. The existing description on page 10 is accurate.

