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Structural insights into cognate vs. near-cognate discrimination during decoding

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 02 December 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below. You will see that the referees are generally supportive regarding publication of the paper here. Still, referees 1 and 3 point to a number of limitations of the approach used that need to be addressed in an adequate manner in a revised version of the manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

Agirrezabala et al. describe a cryo-EM reconstruction of the ribosome with a tRNA bound in the A/T state on a near-cognate codon and compare it to the (already known) structure of the cognate complex. The authors address one of the fundamental questions in understanding the fidelity of translation, namely the structural basis of the induced fit. The quality of the cryo-EM reconstruction is excellent and the paper is very well written. The reconstruction of the near-cognate complex was achieved by classification of the heterogeneous particles pool which contained as little as 8% ribosomes to which the ternary complex was bound. Due to this very low ribosome occupancy by the near-cognate tRNA, the resolution of the near-cognate structure is not very high, 13.2 Å, which is on the other hand very good considering that only 28000 particles were used. The authors identify the differences between the cognate and near-cognate complexes and interpret them in terms of induced fit. However, as the differences are quite small, I am not sure the interpretation is valid in every case. Furthermore, recent crystal structures indicated that the kirromycin-stalled post-hydrolysis A/T state (used in the present work) differs from the pre-hydrolysis state (Voorhees et al., 2010; Schmeing, 2009). It is thus possible that the differences found in the present work are not relevant for the induced fit, which is expected to operate at an earlier, pre-hydrolysis state.

1. The resolution of the cognate and near-cognate structures are different, 8.25 Å and 13.2 Å, respectively. Would the apparent differences in the densities remain if the two structures are shown at the same resolution?

2. How sure are the authors - given the 13 Å-resolution - that the differences of 4-6 Å between the cognate and near-cognate complexes are real?
3. It is surprising that no differences in tRNA positions are mentioned, despite the displaced GAC.

Referee #2 (Remarks to the Author):

The manuscript by Agirrezabala et al describes the cryo-EM structure of the ribosome containing a near-cognate ternary complex. Since near-cognate ternary complexes bind weakly to the ribosome, they used image classification procedures to identify ribosomes with bound ternary complex (approximately 8% of the ribosomes). There analysis showed that near-cognate ternary complex binds differently to the ribosome compared to the cognate ternary complex. Importantly, the acceptor end of the near-cognate tRNA appears to be more dynamic. These changes in the structure lead to a sub-optimal configuration for the near-cognate ternary complex on the ribosome. The authors propose that the altered configuration of the near-cognate ternary complex on the ribosome is the basis for the reduced rate of GTPase activation. The results provide a structural explanation for discrimination of near-cognate ternary complex by the ribosome. Furthermore, it is highly unlikely that a high-resolution crystal structure of near-cognate ternary complex bound to the ribosome will be solved due to the low occupancy. Thus, this cryo-EM structure fills an important gap in our understanding of the process of tRNA selection. My only suggestion (may be for future work) is to try a different near-cognate ternary complex to see how general the observed structural differences are.

Referee #3 (Remarks to the Author):

The manuscript by Agirrezabala et al. entitled "Structural insights into cognate vs. near-cognate discrimination during decoding" analyses the mechanism by which the ribosomes distinguishes between cognate and near-cognate tRNA during the elongation process of protein synthesis. For this, an artificial 70S ribosome complex was formed for which a UGA stop codon in the mRNA is introduced in the A-site position by phasing the P-site AUG start codon with initiator tRNA, and Trp-tRNATrp is used to generate a near cognate situation in the presence of the kirromycin-blocked

elongation factor EF-Tu. As this represents an instable, non-functional and therefore discriminated complex, only a small subpopulation of the near-cognate tRNA/EF-Tu 70S ribosome complex is detected and analyzed by single particle cryo-EM and particle sorting. This approach is indeed interesting and may provide insights into the tRNA selection mechanism on the ribosome by comparing the cryo-EM structure with other known cryo-EM or crystal structures of 30S and 70S tRNA complexes (notably with cognate tRNAs such as the reference complex included here with a cognate UGG codon for the Trp-tRNATrp). An interesting finding is that the acceptor stem region and CCA end of the tRNA become disordered in the near-cognate complex (important message also for the abstract). There seems to be two limitations to the study in that an initiator tRNA is used as a P-site tRNA which is helpful to form the complex but possibly not entirely representative for an elongating complex; secondly and more importantly, for the particle sorting based on different 70S references it is unclear why a reference containing the ternary complex is used (possible bias, especially for a weakly populated state; would the sorting work also with two empty ribosome references added one after the next in the refinement procedure?). The first limitation is at least mentioned in the text, the second should certainly be validated and discussed.

Other points:

- important point: the concept of the 30S closed conformation has been concluded from 30S complexes, however it doesn't seem to apply to 70S complexes in the same way

- maps and coordinates should indeed be deposited in the appropriate data bases

- disorder of the tRNA CCA end: despite of interactions with EF-Tu? Is EF-Tu well ordered only because of the presence of kirromyin?

- Suppl. Fig. 2B is interesting, maybe it is worth including in the main figure 2

- discussion on the switch I loop region (page 7): can this region be sufficiently well resolved to discuss this?

- comparison of EF-Tu complexes: how are the structures aligned, with respect to the full 70S or to EF-Tu? Fig. 3C: EF-Tu seems shifted altogether which makes it difficult to compare its conformational differences if any

- table 1: while the relative values indeed allow to discuss the structural differences, it could be mentioned in the legend what the absolute values mean (normalization etc.).

- page 14: what is meant by "structures... were trimmed down"?

- page 15: reason for selecting pdb 2i2u as a reference for the structural comparisons?

- figures: put colored labels to identify the complexes (especially when using superpositions, Fig2/4); Fig. 2C: maybe a transparent tRNA would be better for the comparison; Fig. 2D: label switch II; Fig 3A: colors make model and map hard to distinguish (especially in print); suggestion to figure 3B: mark CCA binding site; figure 3C: stereo effect weak (requires ~6° rotation of the structure), label domains in stereo.

1st Revision - authors' response 05 January 2011

Reviewer #1

Agirrezabala et al. describe a cryo-EM reconstruction of the ribosome with a tRNA bound in the A/T state on a near-cognate codon and compare it to the (already known) structure of the cognate complex. The authors address one of the fundamental questions in understanding the fidelity of translation, namely the structural basis of the induced fit. The quality of the cryo-EM reconstruction

is excellent and the paper is very well written. The reconstruction of the near-cognate complex was achieved by classification of the heterogeneous particles pool which contained as little as 8% ribosomes to which the ternary complex was bound. Due to this very low ribosome occupancy by the near cognate tRNA, the resolution of the near-cognate structure is not very high, 13.2 Å; which is on the other hand very good considering that only 28000 particles were used. The authors identify the differences between the cognate and near-cognate complexes and interpret them in terms of induced fit. However, as the differences are quite small, I am not sure the interpretation is valid in every case. Furthermore, recent crystal structures indicated that the kirromycin-stalled post-hydrolysis A/T state (used in the present work) differs from the pre-hydrolysis state (Voorhees et al., 2010; Schmeing, 2009). It is thus possible that the differences found in the present work are not relevant for the induced fit, which is expected to operate at an earlier, pre-hydrolysis state.

We thank the referee for this insightful comment. We agree with him/her in the notion that the analysis of different near-cognate ternary complexes might provide definitive evidence on how general the differences observed in the $tRNA^{Trp}$ case are. Examination of different near-cognate structures will show whether the overall mechanism of aminoacyl-tRNA selection is the same in every case, highlighting the structural and binding features that are universally valid for all types of aa-tRNAs, or if there are features that might be unique to particular species. We have changed the text accordingly (page 12, 1st paragraph).

We also agree that the kirromycin-stalled complexes correspond to a state in which the GTP has already been hydrolyzed. Furthermore, we acknowledge that the antibiotic itself might induce modifications in the structural arrangement of the factor that cannot be ruled out. However, as documented in several publications, the kirr-stalled structure does resemble an earlier pre-hydrolysis GTPase-activated state and, as such, has been widely interpreted in multiple studies, including previous structural analyses. We consider this state as a state that comes after, but is still very close to, the GTP hydrolysis transition state. Indeed, we already included a line of reasoning (and a relevant related citation) in the text to clarify and support this point. We have now rewritten this part for clarity (page 4, 3rd paragraph).

As pointed out by the referee, new data have been published (Voorhees *et al*., 2010) after this manuscript was submitted. This new X-ray structure, which represents a pre-hydrolysis state (the structure has been obtained using a non-hydrolysable GTP analogue) has shown a completely structured switch I loop and the activated conformation of His84. The structures from Schmeing *et al*., 2009 (a cognate kirr-stalled 70S-TC complex analyzed by X-ray), mentioned by the referee, were already reported in the table (2WRN, 2WRQ). The corresponding values of the structure reported by Voorhees and coworkers are as follows:

PDB	BP_{lopcl}	H_{tilt}	H_{opcl}	H_{swiv}
2XQD	4.45	1.69	1.79	2.5

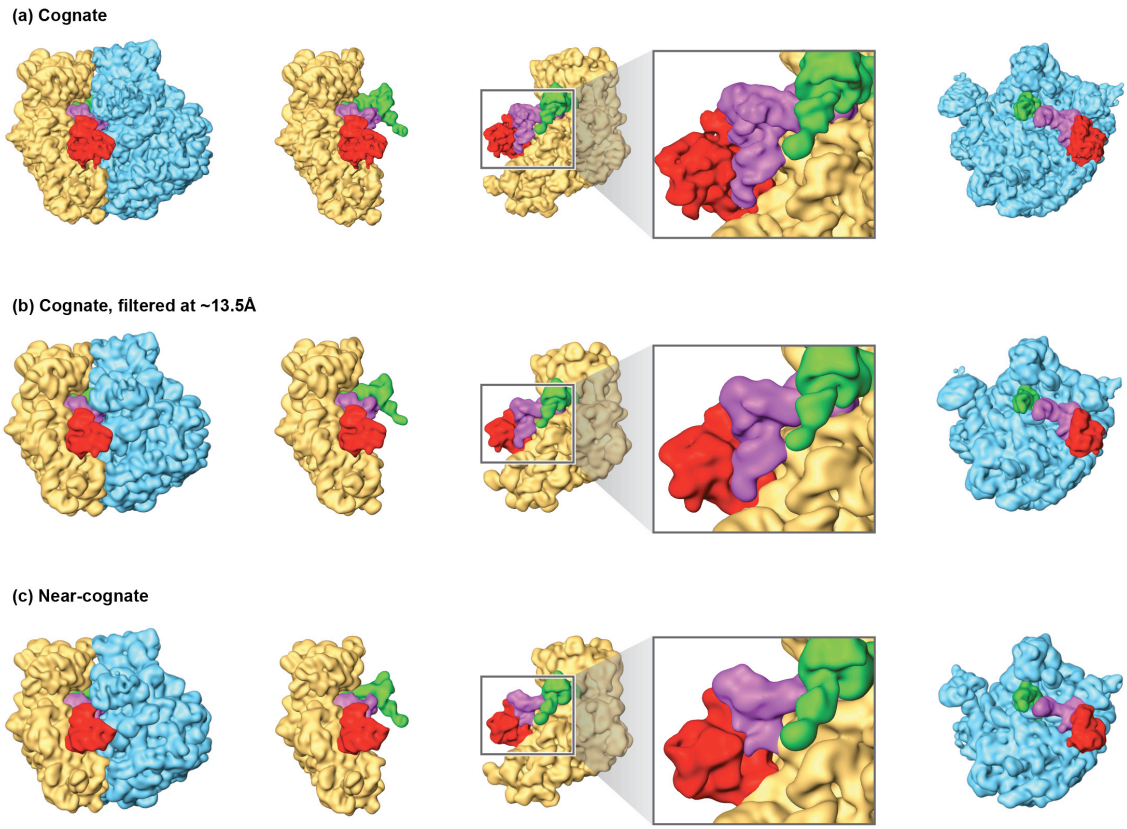
These values are very similar to the structure 2WRQ (and other cognate GDP-kirromycin structures, including ours), except that the body is closed to a larger extent. This closing may indicate a slight relaxation upon GTP hydrolysis. However, we would take this result with some caution. First, only one structure (at 3.2 Angstroms) is reported in Voorhees *et al*., 2010 (in Supp. Material, Voorhees

and coworkers state that the two crystal forms in the original asymmetric unit are related by a small translation of the ribosome, which leads to crystallographic symmetry and thus, the presence of only one ribosome molecule in the final, smaller asymmetric unit), and as the comparison of the values reported in Table 1 in the manuscript shows, the variation of structures within the same crystal can be significant (e.g., compare the entries 2J00 and 2J02). On the other hand, the structure reported by Voorhees *et al*. contains an A/T G24A Trp-tRNA^{Trp} (i.e., the miscoding Hirsh mutant, a tRNA^{Trp} variant that decodes both UGG and UGA stop codons), which apparently increased the reproducibility of crystal growth. It is well established that while this mutant tRNA, which shows a substitution in the D-arm, increases the GTP hydrolysis rate on the near-cognate codon, both wild-type and mutant species activate hydrolysis on the cognate codon to the same extent (Cochella and Green, 2005). However, it is possible that the D-arm substitution might have an effect on the deformability of the tRNA even in cognate environments and therefore, have an influence in the further closing of the body as measured in structure 2XQD. We note that Voorhees and coworkers state (see Suppl. Material) that "… crystal structures of G24A and tRNA^{Trp} and bound to the ribosome on the cognate codon are extremely similar (T. M. Schmeing, Voorhees, R.M., Kelley, A.C., and V. Ramakrishnan, Submitted)", however, the state in which the complexes are trapped (and then compared) is not known to us (i.e., pre-hydrolysis using the GTP analog or post-hydrolysis using GTP-kir).

In order to address this issue, we have included a note at the very end of the manuscript (page 13).

1. The resolutions of the cognate and near-cognate structures are different, 8.25Å and 13.2 Å, respectively. Would the apparent differences in the densities remain if the two structures are shown at the same resolution?

Based on the criticisms of this reviewer, we have performed a comparison using density maps filtered at ~13.5 Angstroms. As seen in the figure enclosed below, the main differences remain. We therefore do not believe that we overinterpret our data. We have added a sentence in the text reflecting the outcome of the test inspired by the referee's question: "These apparent differences … do persist when the two structures are displayed at the same resolution, 13.5Å (data not shown)" (page 6, 1st paragraph).



2. How sure are the authors -given the 13Å resolution- that the differences of 4-6Å between the cognate and near-cognate complexes are real?

Several authors have arrived at a consensus estimate that a docking precision of one order of magnitude, or at least a factor of five, above the nominal spatial resolution of an EM map is achieved using cross-correlation (Rossmann, 2000; Volkmann *et al*., 2000; Wriggers & S. Birmanés, 2001). Thus, we regard it as very unlikely that for a ~13 Angstroms map, movements in the range of 4-6 Angstroms reported by the docking algorithm could be the result of fitting errors.

3. It is surprising that no differences in tRNA positions are mentioned, despite the displaced GAC.

Several differences in the position of the tRNAs were addressed in the text, at least to a certain point. For further clarification, the new version of the manuscript now contains former Supplementary Figure 2 included in the main text as Figure 4, following the suggestion of Reviewer #3 (see below). However, there are outstanding questions that are interesting which we cannot address definitively. Due to the evident flexibility of the acceptor stem region of the near-cognate tRNA, we have tried to be cautious in regards to this issue to avoid over-interpreting the data.

Reviewer #2

The manuscript by Agirrezabala et al describes the cryo-EM structure of the ribosome containing a near-cognate ternary complex. Since near-cognate ternary complexes bind weakly to the ribosome, they used image classification procedures to identify ribosomes with bound ternary complex

(approximately 8% of the ribosomes). There analysis showed that near-cognate ternary complex binds differently to the ribosome compared to the cognate ternary complex. Importantly, the acceptor end of the near-cognate tRNA appears to be more dynamic. These changes in the structure lead to a sub-optimal configuration for the near-cognate ternary complex on the ribosome. The authors propose that the altered configuration of the near-cognate ternary complex on the ribosome is the basis for the reduced rate of GTPase activation. The results provide a structural explanation for discrimination of near-cognate ternary complex by the ribosome. Furthermore, it is highly unlikely that a high-resolution crystal structure of near-cognate ternary complex bound to the ribosome will be solved due to the low occupancy. Thus, this cryo-EM structure fills an important gap in our understanding of the process of tRNA selection. My only suggestion (may be for future work) is to try a different near-cognate ternary complex to see how general the observed structural differences are.

We appreciate the reviewer's positive comments on the advance provided by this study. Regarding examining different near-cognate ternary complexes, a similar point was also raised by Reviewer#1 (please see our response above).

Reviewer #3

The manuscript by Agirrezabala et al. entitled "Structural insights into cognate vs. near-cognate discrimination during decoding" analyses the mechanism by which the ribosomes distinguishes between cognate and near-cognate tRNA during the elongation process of protein synthesis. For this, an artificial 70S ribosome complex was formed for which a UGA stop codon in the mRNA is introduced in the A-site position by phasing the P-site AUG start codon with initiator tRNA, and Trp-tRNATrp is used to generate a near cognate situation in the presence of the kirromycin-blocked elongation factor EF-Tu. As this represents an instable, non-functional and therefore discriminated complex, only a small subpopulation of the near-cognate tRNA/EF-Tu 70S ribosome complex is detected and analyzed by single particle cryo-EM and particle sorting. This approach is indeed interesting and may provide insights into the tRNA selection mechanism on the ribosome by comparing the cryo-EM structure with other known cryo-EM or crystal structures of 30S and 70S tRNA complexes (notably with cognate tRNAs such as the reference complex included here with a cognate UGG codon for the Trp-tRNATrp). An interesting finding is that the acceptor stem region and CCA end of the tRNA become disordered in the near-cognate complex (important message also for the abstract). There seems to be two limitations to the study in that an initiator tRNA is used as a P-site tRNA which is helpful to form the complex but possibly not entirely representative for an elongating complex; secondly and more importantly, for the particle sorting based on different 70S references it is unclear why a reference containing the ternary complex is used (possible bias, especially for a weakly populated state; would the sorting work also with two empty ribosome references added one after the next in the refinement procedure?). The first limitation is at least mentioned in the text, the second should certainly be validated and discussed.

We have followed the reviewer's suggestion and modified the abstract. It now includes the following sentence: "One of the interesting findings is that near-cognate tRNA's acceptor stem region is flexible and the CCA end becomes disordered."

As mentioned by the referee and stated in the Material & Methods section, the two 3D references employed differ only in the presence vs. absence of a ternary complex. Considering that the individual particle image is compared with both references and is then assigned to the most similar one (i.e., the reference that gives the highest correlation coefficient), the use of a reference containing a (cognate) ternary complex is necessary to isolate the individual ribosome images that also bear (near-cognate in this case) ternary complexes. We strongly agree with the referee that the question of the bias introduced by the initial templates has to be addressed very carefully. This is indeed why the selected particles were re-aligned using an "empty" ribosome (i.e., without the

ternary complex and P-site tRNA) as a starting reference (we understand that this is exactly what the referee had in mind) and refined iteratively during multiple rounds. This procedure allows us to obtain final structures that diverge from the initial reference on the basis of the intrinsic structural information of the given subset. While we acknowledge that this approach does not totally exclude the possibility of bias, it serves to minimize any the risk of reference bias in the final structure. As recommended by the reviewer, we have now discussed the issue in more depth and added text to make this point clear (page 5, 1st paragraph).

At this point, we want to express that our preferred solution in resolving the data heterogeneity at the level of image processing is to apply a general unsupervised classification method (maximum likelihood based techniques for example). However, due to the weak binding of the near-cognate ternary complex in the current near-physiological conditions, extremely large data sets are required, and the amount of computation time that this type of classification algorithm requires is very extensive. Therefore, we are unable to attempt this analysis within the time frame provided. However, future work will include the optimization of the binding of the near-cognate complex as well as analysis by maximum likelihood-based methodology.

Other points:

- Important point: the concept of the 30S closed conformation has been concluded from 30S complexes, however it doesn't seem to apply to 70S complexes in the same way.

As pointed out by the referee, the recently published 70S structure with near-cognate tRNA in the A site (Jenner et al., 2010) does not show a closed configuration of the 30S subunit. However, as shown in Table 1, the analysis of the remaining 70S structures (including our new structures) does demonstrate that such a rearrangement occurs. In fact, the comparison of empty and cognate states by Jenner and coworkers has also demonstrated changes related to the head closure movement of the 30S subunit. We have changed the text to make this point clear (page 12, 3rd paragraph).

From our analysis we can conclude that the closing of the small subunit is also observed within the relevant 70S structures. For instance, structures with an unoccupied A site are more open than those with an occupied one (compare 2OW8 to 2J00 or to 2WRN in case of *T. thermophilus*, or 2I2U to 3I21 in case of *E. coli*). Note that the absolute values reported for similar complexes vary, and may depend on species-specific structural details or on the presence of a full tRNA instead of an ASL within the ribosome. Thus, to provide a robust analysis, we compare trends only and not the particular values. At this point, we can only speculate about the absence of head closure in the Jenner *et al*. (2010) near-cognate structure as possibly being related to the presence of an initiation tRNA in the A site.

- Maps and coordinates should indeed be deposited in the appropriate data bases.

The electron density maps and derived atomic models of cognate and near-cognate complexes have been deposited in the 3D-EM database with accession codes EMD-1849 and EMD-1850, and in the Protein Data Bank (PDB) database. Because of the holiday season, PDB IDs have not yet been assigned to date. All maps and coordinates will be released at the time of publication.

- Disorder of the tRNA CCA end: despite of interactions with EF-Tu? Is EF-Tu well ordered only because of the presence of kirromyin?

The referee raises a really difficult question. We agree with him/her that this issue is quite intriguing. As suggested, kirromycin may be involved in the stabilization of the near-cognate EF-Tu structure. Alternatively, and in line with reviewers 1# and #2's suggestion, the nature of the tRNA species used in this work (Trp) may allow unique binding features. On the other hand, we are also aware that considering that (a) interactions between the activated amino acid of the aa-tRNA and certain residues of EF-Tu protect the ester bond between the amino acid and the 3' CCA string, and (b), the CCA end is totally smeared out in our reconstruction due to its mobility, an interesting question is if the labile ester bond is still preserved in the observed near-cognate environment. Regretfully, an answer to this type of questions cannot be obtained from the current data due to the limited resolution of the maps.

- Suppl. Fig. 2B is interesting, maybe it is worth including in the main figure 2.

Former Supplementary Figure 2 has now been included in the main text as Figure 4.

- Discussion on the switch I loop region (page 7): can this region be sufficiently well resolved to discuss this?

This is certainly a fair point. We believe that while our EM data are of insufficient resolution to weigh in conclusively on this issue, they are nevertheless worth bringing into the forum as a contribution to the discussion. We agree that a note of caution is appropriate, and we have included it in the text. We also have minimised our interpretation and modified the paragraph accordingly.

- Comparison of EF-Tu complexes: how are the structures aligned, with respect to the full 70S or to EF-Tu? Fig. 3C: EF-Tu seems shifted altogether which makes it difficult to compare its conformational differences, if any.

The EF-Tu complexes in Figures 2 and 3 are aligned with respect to the full 70S ribosomes. A new figure using aligned EF-Tu complexes (Supp. Fig. 2) has been added to facilitate comparison of conformational differences as suggested by the reviewer.

- Table 1: while the relative values indeed allow to discuss the structural differences, it could be mentioned in the legend what the absolute values mean (normalization etc.).

The absolute values represent the angle formed between one of the vectors of the inertia tensor of one domain with a vector of the inertia tensor of another domain. The difference between the measured angle and the reported angle is a constant offset, which corresponds to the measured value in the reference structure. In case of the opening/closing of the body with respect to the platform the

offset is 127.8˚. As requested, we added this explanation in the corresponding legend. In addition, a new figure (Supp. Fig. 3) has been added. This new figure shows the inertia tensors for the 16S and the individual domains. The corresponding legend contains information concerning the vectors used for the calculations.

- Page 14: what is meant by "structures... were trimmed down"?

As described in the manuscript, the subsystem used for the refinement simulations in explicit solvent contained the ternary complex and all the residue of the ribosome within 22 Å of the ternary complex. This "reduction" of the system size is referred to as "trimmed down". The sentence that prompted this comment has been modified. We now state: "To refine the local interactions of the ternary complexes with the ribosome, the system size was reduced, and…" (page 14, 3rd paragraph).

- Page 15: reason for selecting pdb 2i2u as a reference for the structural comparisons?

The only reason why we took 2I2U as reference is that we used this very same model in the fittings and the subsequent refinement simulations. In fact, one could use any other structure. Although the reported values of the measured angles vary slightly, their differences are very similar. For example, the difference in the opening/closing motion of the body with respect to the platform between the structures 2J00 and 2J02 is 0.58˚ using 2I2U (*E. coli*) as reference, while 0.57˚ is obtained using 2J00 (*T. thermophilus*) as reference.

- Figures: put colored labels to identify the complexes (especially when using superpositions, Fig2/4); Fig. 2C: maybe a transparent tRNA would be better for the comparison; Fig. 2D: label switch II; Fig 3A: colors make model and map hard to distinguish (especially in print); suggestion to figure 3B: mark CCA binding site; figure 3C: stereo effect weak (requires ~6 rotation of the structure), label domains in stereo.

Colored labels have been put in former Figures 2, 4 and S. Fig 2 (now, Figures 2, 5 and 3, respectively). Displaying two transparent tRNAs in Figure 2C at the same time could be confusing for readers as it makes difficult to distinguish the structures. If possible, we would like to keep it as it is. The transparency of cryo-EM densities was enhanced in Fig3A. Switch II has been labeled in Fig 2C. Marking the CCA binding site might introduce confusion, as figure 3B is already quite busy. Again, if possible, we would like to keep it as it is. We apologize for the defective stereo effect in Figure 3C. A corrected figure has been added. According to the aforementioned changes, we have included modifications throughout the corresponding legends.

2nd Editorial Decision 27 January 2011

Thank you for sending us your revised manuscript. Our original referees 1 and 3 have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Before this will happen, I was wondering whether you would like to consider addressing the minor

issues suggested by the referees (see below). Furthermore, I need to ask you to include the PDB accession details into the manuscript text at this final stage.

Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors answered all my questions and revised the text as to clarify the points raised by the reviewers. The paper is very well written and the results extremely interesting. Very recent biochemical data (Mittelstaet et al., 2011 JBC)strongly support the present structural interpretation; the authors might like to add a note to mention this.

Referee #3 (Remarks to the Author):

I went through the revised manuscript and find it overall fine now.

All points have been addressed or incorporated, with two exceptions:

- the discussion on the 30S head closure (MS pages 12/13) remains slightly unclear with respect to why head closure is unique or not to cognate codon interactions.

- the disorder of the tRNA CCA end while being in contact with EF-Tu (page 9 of the response to referees) would be worth discussing (or at least mentioning) in the final part of the discussion.
The figure on page 5 of the response to referees could be added as a Suppl.Fig.
The disordered (and thus missing) CCA region of the tRNA could be indicated in Fig.2B (by a star, or labelled); the fact that this part is missing in the map is hardly visible in Fig.2C (one should at least guess the bottom red edge).

Fig.3 still looks flat (is supposed to be a stereo representation), possibly wrong version of the figure panel?

Everything else seems fine, and it is stated that maps and coordinated will be made available in the data bancs upon publication which is good.

2nd Revision - authors' response 01 February 2011

We truly appreciate the constructive comments and suggestions from the reviewers, which have resulted in an improved manuscript. Following the final suggestion by Referee #1, we have now included a reference to the newly published work by Mittelstaet and coworkers, and noted the agreement with our results. We have also carefully considered the comments by Referee #3 and have modified the Discussion section in the manuscript accordingly. On his/her suggestion, we have included the rebuttal figure (resolution comparison) in the supplement. We have made sure that the

stereo figures work. Finally, the PDB accession details have been incorporated into the manuscript text.

