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Molecular insights into protein synthesis with proline residues

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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 31 July 2016

Thank you for the submission of your research manuscript to our journal. I am sorry for the slight delay in getting back to you, which is due to the fact that I asked for a fourth opinion on your study and the three referee reports that are not exactly in agreement. The set of comments is pasted below.

As you will see, the referees acknowledge that the topic of the manuscript is important. However, they disagree on whether the manuscript provides sufficient novel insight for publication here. After consulting with another expert in the field, we decided to give you the opportunity to revise your study if additional biochemical assays can be performed to demonstrate that proline can indeed act as an A-site substrate for peptide transfer, and at what rate it does so compared with non-proline substrates. While a paragraph on eIF5A should also be added, it would not be necessary to solve a structural complex that includes eIF5A. All other referee concerns must be addressed.

Given these constructive comments, we would like to invite you to revise your manuscript. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. Given your 2 main figures, I suggest that we publish the study as a short report. For short reports, the revised manuscript should not exceed

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25,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. Please move at least one of the supplementary figures to the main text, along with the suggestion by referee 3. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. The entire materials and methods must be included in the main manuscript file. Please note that supplementary data and tables are called expanded view now.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where in the manuscript the requested information can be found. The completed author checklist will also be part of the RPF (see below).
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
- a separate PDF file of any Supplementary information (in its final format)

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

Melnikov and colleagues use x-ray crystallography to gain functional insights into how eukaryotic 80S ribosome utilizes tRNAs charged with proline amino acid as substrates. Unlike other proteinogenic amino acids, proline contains a secondary amine group. This dramatically affects its chemical and structural properties and makes it a poor nucleophile in transpeptidation reaction. First, it has the highest alpha-amine pKa out of 20 amino acids (10.60). Second, its 5-membered ring structure restricts proline's flexibility in phi-psi space on the Ramachandran plot, which is expected to compromise its nucleophilic properties further. Melnikov and colleagues provide a direct evidence of proline adapting a confirmation unfavorable to efficient transpeptidation in the context of 80S peptidyl transferase center. The conformation of proline in the context 80S-bound non-hydrolysable analogs of mono- and diprolyl-tRNAs is identical to that assumed by proline in PII-helixes formed in the context of proteins. However, I am not sure that the paper in its current form

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provides sufficient biological insight to warrant its publication in EMBO Reports for two reasons.

First, structures of stalled ribosomal complexes with P-site tRNA bearing a proline residue connected to a stalling peptide have already been reported either, e.g. Bischoff et al. Cell Reports (2014), Matheisl et al. NAR (2015), Zhang et al. Elife (2015). Neither of these reports are mentioned or discussed.

Second, both bacteria and eukaryotes have evolved a specialized system for overcoming proline stalling: elongation factors EF-P and eIF5A, respectively. Though the authors do refer to papers describing the system (references 10, 11, 12, 20, and 22), they do not mention EF-P and eIF5A in the main text. Moreover, Melnikov and colleagues have recently published a structure of eEF5A bound to 80S (Melnikov et al. JMB 10.1016/j.jmb.2016.05.011) - it is rather surprising that this paper is never mentioned in the current manuscript. In order to make the current data biologically relevant, one should assemble and solve a structure complex that simultaneously contains eIF5A and non-hydrolysable prolyl-tRNAs analogue.

Minor remarks:

Page 6, 3d paragraph: formatting issues ('[20,21{Elgamal, 2014 #85,22].')

Referee #2:

The structures described in this short mms address two intriguing issues, namely why prolyl tRNA is a poor substrate for protein synthesis, and why runs of proline codons in mRNAs are associated with arrest of protein synthesis. The crystals used in this study were obtained by soaking ACCA-amido-Pro, AAC-puromycin, ACCA-amido-diPro, and ACCA-amido-Leu-Phe into preformed crystals of yeast 80S ribosomes. The first two compounds are A-site substrate analogs, and the latter two are P-site substrate analogs. Structures were obtained by molecular replacement using an 80S structure lacking bound substrates as the starting structure. Thus these experiments are closely similar in character to the A-site and P-site substrate analog experiments that were done with H. marismortui large subunits over a decade ago (e.g. Schmeing et al. (2002) Nature Structural Biology 9, 225-230).
The results are easily summarized. First, AAC-puromycin binds to the 80S A site and ACCA-amido-Leu-Phe binds to the 80S P site in manner all but perfectly identical to what was reported in the past for H. marismortui. Second, ACCA-amido-diPro binds to the P site in a conformation suggestive of the possibility that oligo-Pro runs in nascent peptides might have trouble negotiating their passage through the exit tunnel properly, although the use of sparsomycin to stabilize that complex leaves room for some uncertainty in this regard. Third, and by far the most important of the observations reported, ACCA-amido-Pro binds to the A site of these ribosome with its Pro moiety "backwards". Its nitrogen is pointed away from carbonyl carbon atom it must attack if peptide transfer is to take place, rather than towards it, with the side chain carbon atoms of the Pro between its nitrogen and that carbonyl carbon atom. It is inconceivable that transfer of a peptide from a peptidyl tRNA in the P site to the proline moiety of a prolyl tRNA in the A site would ever occur with the proline moiety bound to the ribosome this way. However, references 8 and 9 indicate that the rate of this reaction should be only ~ 10 times slower than the rate of transfer to other aminoacyl tRNAs. What is going on here?

Biochemical issues:

It would be extremely helpful if authors were to do some biochemical experiments to find out if ACCA-amido-Pro is capable of acting as an A-site substrate in their system, and if so, at what rate compared to similar substrates where the amino acid is something other than Pro.

Crystallographic issues:

1. Table S1. The Rmeas statistics reported for the data sets the authors used are absolutely horrible, and totally inconsistent with the values claimed for Rwork and Rfree for the models obtained from those data sets. There is something seriously wrong here.

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2. As always when using ligands, parameter and topology files have to be provided so that structures including ligands can be refined. Some of the programs used for this purpose yield files that will hold ligand conformations rigid unless users modify them by hand appropriately. Were ligand torsion angles, bond lengths, and bond angles specified in those files in a way that would allow ligand structures to refine properly?

3. Some of the difference electron density maps shown for ligands are described as "unbiased". It would be helpful to specify exactly what is meant in this case. Is Fc the fully refined values for the structures in question, ligands included? Where did the phases come from? One would expect them to be the phases produced by rigid body refinement of the ligand-free test structure into the Fobs data set.

The answers to these questions all bear on the confidence one can have that the authors properly understand the conformation of their A-site bound prolyl moiety. If upon reflection they believe they have got it right, they need to include some comments in their mms that explain their thinking about why peptide bond formation can occur with pro in the A site.

Referee #3:

Incorporation of proline residues into the growing polypeptide chain on the ribosome has always been surrounded with lots of questions, because (i) unlike all other proteinogenic amino acids proline does not possess α-amino group and instead has α-imino group, which is a weaker nucleophile, and (ii) proline has very special geometry due to its rigid cycle. It becomes even a bigger problem for the ribosome when several consecutive prolines need to be incorporated into the protein. The major significance of the current work is that it addresses this very problem and specifically answers the question, for which we had rather guesses before - "why the translation stalls on polyproline tracts?"

In this manuscript by Melnikov et al., the authors report four new X-ray crystal structures of eukaryotic 80S ribosomes from yeast in complex with short tRNA-analogues that carry either amino acids or dipeptides. Comparison of the proline and tyrosine structures reveals that proline adopts a unique conformation in the A site of the ribosome. And this is, perhaps, the most significant finding of the current work because it explains the poor reactivity of proline as a substrate and also breaks the postulate that all 20 amino acids bind to the active site of the ribosome in the same uniform way. Furthermore, by comparing the structure of diprolyl-tRNA analogue with the structure of Leu-Phe-tRNA analogue in the P site of the ribosome authors revealed that diprolyl peptide adopts an unusual conformation, in which it looks towards the wall (and not the lumen) of the exit tunnel. Authors suggested that addition of an extra proline residue to such nascent chain would cause a clash with the wall of the exit tunnel, making subsequent rounds of elongation impossible. I think it is reasonable and is also very important because it explains why ribosome is unable to translate polyproline sequences without help from a dedicated translation factor EF-P/eIF5A.

In summary, authors provided a concise and clearly written manuscript, which was easy and interesting to read and that was supplied with nice figures. Authors were careful not to over-interpret their results and discussed their findings in respect to the previous biochemical studies. In my opinion, this work represents a significant conceptual advance in the field with important results and implications. It is a long awaited logical extension of the previous studies published by the same and other groups recently. Overall, this work is perfectly suited for the EMBO Reports Journal and I recommend it for publication after few corrections/modifications as suggested below.

Comments, suggestions and questions to the authors:

Major points:

1. The unusual conformation of proline in the A site of the ribosome does not fully explain its poor reactivity. Proline is generally less reactive simply because its α-imino group is a weaker nucleophile compared to the α-amino group (it is less willing to share its electrons with other atoms). However, I think authors just overlooked the actual explanation that they already have in their data. By looking at the structure of the proline in the A site of the ribosome (Figure 1, panel B),

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it becomes clear that the orientation of the lone pair of electrons of the α-imino group relative to the carbonyl carbon is unfavorable for the nucleophilic attack - and, in my opinion, this is the main reason why proline has poor reactivity on the ribosome. On the other hand, the standard amino acid tyrosine (Figure 1, panel B) has conformation of the α-amino group that is fully favorable for the nucleophilic attack. Therefore, I would like to strongly suggest to the authors to discuss this point in the appropriate sections of the manuscript. More specifically I would like to suggest the following changes to Figure 1:

- In both current panels A and B, add 2/1 hydrogens to the amino/imino groups, respectively, and draw lone pairs of electrons next to the highlighted nitrogen atoms. This way it will become clear to the reader that in the case of a normal amino acid (panel A) its lone pair of electrons is oriented towards the carbonyl carbon, while in the case of a proline (panel B) it is oriented away making nucleophilic attack unfavorable;

- In both current panels A and B, instead of drawing only the carbonyl carbon atom try drawing carbonyl group of the P-site substrate with the highlighted carbonyl carbon in the middle. This is important to illustrate that the α-nucleophile attacks carbonyl carbon perpendicular to the plane of the carbonyl group;

- In panel A, connect lone pair of electrons with the carbonyl carbon by an arrow, which will illustrate the direction of the nucleophilic attack. The same arrow in panel B would be simply impossible;

- Add either one or two additional panels, showing the entire substrates in the active site of the ribosome in the zoomed out view;

- Instead of showing nucleotides A2820/C2821 as surface try showing them as spheres. This way there would be no weird connections between the planes of the nucleotides that do not really exist.

2.A paragraph on the elongation factor EF-P/eIF5A - an essential translation factor, which is required for synthesis of proteins with polyproline tracts - should be included in the introduction section of this manuscript. Especially because the authors recently published a very relevant structural work, in which they revealed how eIF5A factor works and how it alleviates ribosome complexes stalled on consecutive proline tracts.

3. One very important figure is missing from the main text - a figure which will show the electron densities for the substrate analogs in the active sites of the ribosome for all four structures reported in the current work. Current figure S2 could be that figure with the addition of a panel showing overall structure of the yeast ribosome. Including such figure as Figure 1 will make it immediately clear which structures were determined in this study and which active sites of the ribosome are occupied with the substrate analogs in each of these structures.

Minor points:

4. Page 4, paragraph 1: Perhaps, it would be more informative to change "ACCPuromycin" to "ACC-Puromycin (ACCmA-mTyrosine)".

5. Page 4, paragraph 2: It will read better if "...supplemented with 300 μM the antibiotic sparsomycin. Sparsomycin was used to stabilize..." is changed to "...supplemented with 300 μM of the antibiotic sparsomycin, which was used to stabilize...".

6. Page 5, paragraph 4: Perhaps, authors meant to say "...A76 sugar pucker..." and not "...A76 sugar pocket..."?

7. Page 5, paragraph 4: This whole paragraph could be removed. It was already explained in the previous section of the manuscript that the antibiotic sparsomycin was used to force ACCA-dipeptide into the P site of the ribosome. No need to come back to the same point again, especially that this is relatively weak point and it just distracts the reader from the important findings.

8. Page 6, paragraph 2: I would suggest to possibly reword the following sentence from "This

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proline conformation suggests that the poor reactivity of proline as a peptidyl acceptor originates from two factors. These factors being the displaced α-amine position, and the proline side chain, both of which may prevent the optimal alignment of substrates in the peptidyl transferase center of the ribosome." to "The observed proline conformation suggests that the poor reactivity of proline as a peptidyl acceptor originates from two factors: (i) the displaced position of α-amine and (ii) the unusual location of the side chain. Both of these factors can prevent the optimal alignment of substrates in the peptidyl transferase center of the ribosome that is required for the efficient nucleophilic attack to take place."

9. All throughout the text the panels of the figures are referenced with the small letters. However, in the actual figures the panels are labeled with capital letters.

10. Figure 2:
- Instead of showing nucleotides G2872/G2403 as surface try showing them as spheres (the same comment is valid for Figures 1 and S3);
- Arrows are barely visible;
- It actually doesn't matter a lot where the alpha-amino group of the P-site substrate is located, since it is not a reactive group. Location of the carbonyl carbon matters more.
- Authors might wish to label the P-loop of the 25S rRNA and show H-bonds between C74/C74 and G2620/G2619, respectively.

11. Figure S2:
- Make grid spacing the same in each panel;
- Make mesh a little thicker and the sticks of the model thinner, so that it will be more clear how well the model corresponds to the density;
- It might be more accurate to change the labels at the top of each panel to include ACCA. For example, ACCA-Leu-Phe.
12. Figure S3, legend: Change "archeal" to "archaeal".

1st Revision - authors' response 05 September 2016

Dear Editorial Board of EMBO reports,

First of all, we thank both the reviewers for their thoughtful comments and you for considering our work for potential publication in EMBO reports.

Below we provide point-by-point response to the reviewers' comments.

Referee #1:
Melnikov and colleagues use x-ray crystallography to gain functional insights into how eukaryotic 80S ribosome utilizes tRNAs charged with proline amino acid as substrates. Unlike other proteinogenic amino acids, proline contains a secondary amine group. This dramatically affects its chemical and structural properties and makes it a poor nucleophile in transpeptidation reaction. First, it has the highest alpha-amine pKa out of 20 amino acids (10.60). Second, its 5-membered ring structure restricts proline's flexibility in phi-psi space on the Ramachandran plot, which is expected to compromise its nucleophilic properties further. Melnikov and colleagues provide a direct evidence of proline adapting a confirmation unfavorable to efficient transpeptidation in the context of 80S peptidyl transferase center. The conformation of proline in the context 80S-bound non-hydrolysable analogs of mono- and diprolyl-tRNAs is identical to that assumed by proline in PII-helixes formed in the context of proteins. However, I am not sure that the paper in its current form provides sufficient biological insight to warrant its publication in EMBO Reports for two reasons.

First, structures of stalled ribosomal complexes with P-site tRNA bearing a proline residue connected to a stalling peptide have already been reported either, e.g. Bischoff et al. Cell Reports (2014), Matheisl et al. NAR (2015), Zhang et al. Elife (2015). Neither of these reports are mentioned or discussed.

We have now mentioned and discussed these studies in the following sections of the manuscript:

Introduction (page 3, paragraph 4)
Extensive kinetic studies of peptide bond formation with proline suggested that proline impedes the rate of protein synthesis by increasing entropy of peptide bond formation [7]. Furthermore, cryoelectron microscopy analysis of ribosome complexes with stalling peptides revealed the position of proline residues in the ribosomal P site during translational stalling [15-17]. These studies profoundly extended our understanding of protein synthesis chemistry with proline. However the conformation of proline residues in the peptidyl transferase center is still unknown and, therefore, it has remained unclear how proline slows down the rate of protein synthesis.

Second, both bacteria and eukaryotes have evolved a specialized system for overcoming proline stalling: elongation factors EF-P and eIF5A, respectively. Though the authors do refer to papers describing the system (references 10, 11, 12, 20, and 22), they do not mention EF-P and eIF5A in the main text. Moreover, Melnikov and colleagues have recently published a structure of eEF5A bound to 80S (Melnikov et al. JMB 10.1016/j.jmb.2016.05.011) - it is rather surprising that this paper is never mentioned in the current manuscript. In order to make the current data biologically relevant, one should assemble and solve a structure complex that simultaneously contains eIF5A and non-hydrolysable prolyl-tRNAs analogue.

We agree that this structure will help understanding how the stalling is resolved, and future studies will provide this understanding. Meanwhile, we have added a paragraph discussing translation factor eIF5A:

Introduction (Page 3, Paragraph 3)
"In a living cell, ribosome stalling by polyproline sequences is resolved by a universally conserved translation factor, known as eIF5A in eukaryotes and EF-P in bacteria [10-12]. In eukaryotes, eIF5A alleviates ribosome stalling by contacting the acceptor stem of the P-site tRNA, using a mechanism that is not yet fully understood [13,14]. The presence of eIF5A in eukaryotic cells enables synthesis of proteins containing polyproline motifs. This factor is essential because polyproline motifs are highly abundant in eukaryotic proteomes. Human proteome, for example, contains ~10,000 motifs with three or more consecutive proline residues, with some proteins having up to 27 consecutive prolines [1,2]."

Minor remarks:
Page 6, 3d paragraph: formatting issues ('[20,21{Elgamal, 2014 #85,22].')

This formatting error has been corrected.

Referee #2:
The structures described in this short mms address two intriguing issues, namely why prolyl tRNA is a poor substrate for protein synthesis, and why runs of proline codons in mRNAs are associated with arrest of protein synthesis. The crystals used in this study were obtained by soaking ACCA-amido-Pro, AAC-puromycin, ACCA-amido-diPro, and ACCA-amido-Leu-Phe into preformed crystals of yeast 80S ribosomes. The first two compounds are A-site substrate analogs, and the latter two are P-site substrate analogs. Structures were obtained by molecular replacement using an 80S structure lacking bound substrates as the starting structure. Thus these experiments are closely similar in character to the A-site and P-site substrate analog experiments that were done with H. marismortui large subunits over a decade ago (e.g. Schmeing et al. (2002) Nature Structural Biology 9, 225-230).

The results are easily summarized. First, AAC-puromycin binds to the 80S A site and ACCA-amido-Leu-Phe binds to the 80S P site in manner all but perfectly identical to what was reported in the past for H. marismortui. Second, ACCA-amido-diPro binds to the P site in a conformation suggestive of the possibility that oligo-Pro runs in nascent peptides might have trouble negotiating their passage through the exit tunnel properly, although the use of sparsomycin to stabilize that complex leaves room for some uncertainty in this regard. Third, and by far the most important of the observations reported, ACCA-amido-Pro binds to the A site of these ribosome with its Pro moiety "backwards". Its nitrogen is pointed away from carbonyl carbon atom it must attack if peptide transfer is to take place, rather than towards it, with the side chain carbon atoms of the Pro between its nitrogen and

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that carbonyl carbon atom. It is inconceivable that transfer of a peptide from a peptidyl tRNA in the P site to the proline moiety of a prolyl tRNA in the A site would ever occur with the proline moiety bound to the ribosome this way. However, references 8 and 9 indicate that the rate of this reaction should be only ~ 10 times slower than the rate of transfer to other aminoacyl tRNAs. What is going on here?

Having received this feedback, we realized that the current view shown in Figure 1 gives the false impression that proline's amine group is not accessible for peptide bond formation. Indeed, from this angle, it appears that proline's side chain occupies the position between the reacting amine and the carbonyl, which would prevent peptide bond formation. This impression, however, stems from our unfortunate view selection and from the lack of depth in this 2D image.

We therefore corrected Figure 1 to show the ligands in a way that shows both the side chain conformation and the α-amine conformation. It can be seen in this orientation (views from the nascent peptide tunnel) that the proline side chain is bound on a side rather than between the proline's amine and the P-site carbonyl.

In this view it is clear that the reactive amine has a similar position in proline and methyl-tyrosine. In this position, the reactive groups have space to react, although the proline side chain may affect the precise alignment of the ligands. Therefore, proline reactivity should not be dramatically different from the reactivity of other amino acids.

Biochemical issues:
It would be extremely helpful if authors were to do some biochemical experiments to find out if ACCA-amido-Pro is capable of acting as an A-site substrate in their system, and if so, at what rate compared to similar substrates where the amino acid is something other than Pro.

We used amido-variants of aminoacyl-tRNA analogs because they were shown to mimic natural ribosome substrates in their functionality. In particular, Schmeing et.al., 2005, Mol.Cell showed that amido-derivatives of aminoacyl-tRNA mimics adopt the same conformation in the peptidyl-transferase center as natural aminoacyl-tRNAs. Thus, there currently is no biochemical, chemical or structural evidence that an amido group may distort the ligand conformation in the ribosomal A site. We, therefore, assume that additional biochemical measurements of the proline reactivity would be a repetition of measurements by Pavlov et.al., 2009, PNAS.

To add this information in the text, we have the following sentences:

Results and discussion (Page 4, Paragraph 2)
"Our choice of amido-variants of aminoacyl-tRNA analogs was based not only on the fact that these analogs are hydrolysis-resistant and therefore, compatible with crystallization conditions, but also because they were shown to functionally mimic natural ribosome substrates. In particular, it was previously shown that amido-derivatives of aminoacyl-tRNA mimics adopt the same conformation in the peptidyl-transferase center as natural aminoacyl-tRNAs [20].

Crystallographic issues:
1. Table S1. The Rmeas statistics reported for the data sets the authors used are absolutely horrible, and totally inconsistent with the values claimed for Rwork and Rfree for the models obtained from those data sets. There is something seriously wrong here.

We understand the reviewer's concern about the dataset statistics. Indeed, in a traditional data collection strategy (which was historically developed for CCD detectors, but is also frequently used for Pilatus detectors) these statistics would reflect serious problems, such as severe radiation damage, lack of isomorphism, or errors in data processing etc.

In our case, however, these unusual statistics reflect a radically different data collection strategy. Since the year of 2010, we have followed a strategy proposed by engineers from *DECTRIS* for their new generation of Pilatus detectors, which we used to collect our data. We described this strategy in two consecutive papers (Ben-Shem et.al, 2010, *Science*, Ben-Shem et.al, 2011, *Science*). Later, a stand-alone paper was published to describe the rationale and details of this approach (Mueller et.al, 2012, *Acta Cryst D*).

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This reference, with the reference to our previous studies, can be also found on the official DECTRIS website: https://www.dectris.com/features-281.html
(see **Optimal signal-to-noise ratio**).

Unlike the traditional approach, this method exploits a highly attenuated beam (up to 99%) to collect highly redundant data in which a low signal-to-noise ratio is compensated for by data redundancy and low radiation damage. The benefits of this strategy, over traditional data collection, have been best illustrated by numerous studies in which the data were used as a source of anomalous signal to improve electron maps or to solve the phase problem for *de novo* structure solution. These cases were recently summarized, for example, in a review by Wayne Hendrickson, 2015, TiBS.

To better emphasis these differences in data collection, we extended our materials and methods section to describe data collection in a separate chapter. We also added a brief explanation note to the caption of the Table S1.

Materials and methods (**Page 10, Paragraph 1**)
Data collection and reduction. Diffraction data were collected from crystals cooled to 90^{o}K using 0.1^{o} oscillation range and the beam-line Proxima 1 at the Synchrotron Soleil (France). We used a data collection strategy developed at Swiss Light Source Synchrotron (Switzerland) which exploits the unique features of the single photon counting pixel detector PILATUS 6M [18, 23, 24]. During data collection, the beam was attenuated to 3-10% of its maximum flux so that radiation damage could be markedly reduced and a highly redundant data-set could be collected using several crystals and/or multiple spots of each crystal. Then, data were processed and reduced by the XDS suite [25] yielding the statistics displayed in the Table S1.

This data collection strategy results in unconventionally high Rmeas values, when it is compared to CCD-detector type data collection strategies (no beam attenuation, no fine ϕ-slicing, low data redundancy), but provides more accurate values of reflections' intensities and anomalous signal [24, 26]".

2. As always when using ligands, parameter and topology files have to be provided so that structures including ligands can be refined. Some of the programs used for this purpose yield files that will hold ligand conformations rigid unless users modify them by hand appropriately. Were ligand torsion angles, bond lengths, and bond angles specified in those files in a way that would allow ligand structures to refine properly?

Yes, we have now specified in the text that ligand geometry (torsion angles, bond lengths, and bond angles) was refined and not rigidly fixed during refinement. We have added the following sentence to the Materials and Methods Section:

Materials and methods (Page 10, Paragraph 4):
"Ribosome structure and ligand geometry (torsion angles, bond lengths, and bond angles) were refined using Phenix.refine [8]."

3. Some of the difference electron density maps shown for ligands are described as "unbiased". It would be helpful to specify exactly what is meant in this case. Is Fc the fully refined values for the structures in question, ligands included? Where did the phases come from? One would expect them to be the phases produced by rigid body refinement of the ligand-free test structure into the Fobs data set.

We are grateful for this commentary pointing to the use of jargon in our manuscript. Indeed, the phases were produced by rigid body refinement of the ligand-free test structure into the Fobs data set. We have added this information to both Materials and Methods and figure captions:

Fig. 1 (former Fig. S2) caption:
"The Fo-Fc maps were calculated using phases produced by rigid body refinement of the ligand-free test structure put into the Fo data set."

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The answers to these questions all bear on the confidence one can have that the authors properly understand the conformation of their A-site bound prolyl moiety. If upon reflection they believe they have got it right, they need to include some comments in their mms that explain their thinking about why peptide bond formation can occur with pro in the A site.

We addressed this question by providing a more informative view of the A-site substrates (Fig. 1) and additional text in the manuscript and Fig. 1 caption:

Results and discussion (Page 5, Paragraph 4)
"Notably, despite proline's side chain not entering the A-site cleft of the ribosome and adopting a highly unusual conformation, the α-amine of proline remains accessible for the peptide bond formation and has a position in the peptidyl-transferase center comparable to the ones observed for other amino acids, illustrating why proline remains reactive, although at an order of magnitude slower rate when it is compared to other proteinogenic amino acids [7-9].

Referee #3:
Incorporation of proline residues into the growing polypeptide chain on the ribosome has always been surrounded with lots of questions, because (i) unlike all other proteinogenic amino acids proline does not possess α-amino group and instead has α-imino group, which is a weaker nucleophile, and (ii) proline has very special geometry due to its rigid cycle. It becomes even a bigger problem for the ribosome when several consecutive prolines need to be incorporated into the protein. The major significance of the current work is that it addresses this very problem and specifically answers the question, for which we had rather guesses before - "why the translation stalls on polyproline tracts?"

In this manuscript by Melnikov et al., the authors report four new X-ray crystal structures of eukaryotic 80S ribosomes from yeast in complex with short tRNA-analogues that carry either amino acids or dipeptides. Comparison of the proline and tyrosine structures reveals that proline adopts a unique conformation in the A site of the ribosome. And this is, perhaps, the most significant finding of the current work because it explains the poor reactivity of proline as a substrate and also breaks the postulate that all 20 amino acids bind to the active site of the ribosome in the same uniform way. Furthermore, by comparing the structure of diprolyl-tRNA analogue with the structure of Leu-Phe-tRNA analogue in the P site of the ribosome authors revealed that diprolyl peptide adopts an unusual conformation, in which it looks towards the wall (and not the lumen) of the exit tunnel. Authors suggested that addition of an extra proline residue to such nascent chain would cause a clash with the wall of the exit tunnel, making subsequent rounds of elongation impossible. I think it is reasonable and is also very important because it explains why ribosome is unable to translate polyproline sequences without help from a dedicated translation factor EF-P/eIF5A.

In summary, authors provided a concise and clearly written manuscript, which was easy and interesting to read and that was supplied with nice figures. Authors were careful not to over-interpret their results and discussed their findings in respect to the previous biochemical studies. In my opinion, this work represents a significant conceptual advance in the field with important results and implications. It is a long awaited logical extension of the previous studies published by the same and other groups recently. Overall, this work is perfectly suited for the EMBO Reports Journal and I recommend it for publication after few corrections/modifications as suggested below.

Comments, suggestions and questions to the authors:

Major points:
1. The unusual conformation of proline in the A site of the ribosome does not fully explain its poor reactivity. Proline is generally less reactive simply because its α-imino group is a weaker nucleophile compared to the α-amino group (it is less willing to share its electrons with other atoms). However, I think authors just overlooked the actual explanation that they already have in their data. By looking at the structure of the proline in the A site of the ribosome (Figure 1, panel B), it becomes clear that the orientation of the lone pair of electrons of the α-imino group relative to the carbonyl carbon is unfavorable for the nucleophilic attack - and, in my opinion, this is the main reason why proline has poor reactivity on the ribosome. On the other hand, the standard amino acid tyrosine (Figure 1, panel B) has conformation of the α-amino group that is fully favorable for the

nucleophilic attack. Therefore, I would like to strongly suggest to the authors to discuss this point in the appropriate sections of the manuscript.

We are very grateful to the reviewer for pointing to this alternative explanation, which we have accommodated in the revised Results and Discussion section:

Results and discussion (Page 5, Paragraph 3)
"Additionally, compared to other amino acids, the α-amino group of the proline residue is displaced by ~1Å from the ribosomal the P site (**Fig. S3**). In this conformation, proline may have an unusual orientation of the reactive electron pairs in the α-amine group: the tetrahedral electron pair geometry and limited flexibility of the proline cycle suggest that proline's electron pair should deviate from the favorable position required for optimal nucleophilic attack (**Fig. 2b**)."

More specifically I would like to suggest the following changes to Figure 1:
- In both current panels A and B, add 2/1 hydrogens to the amino/imino groups, respectively, and draw lone pairs of electrons next to the highlighted nitrogen atoms. This way it will become clear to the reader that in the case of a normal amino acid (panel A) its lone pair of electrons is oriented towards the carbonyl carbon, while in the case of a proline (panel B) it is oriented away making nucleophilic attack unfavorable.
- In both current panels A and B, instead of drawing only the carbonyl carbon atom try drawing carbonyl group of the P-site substrate with the highlighted carbonyl carbon in the middle. This is important to illustrate that the α-nucleophile attacks carbonyl carbon perpendicular to the plane of the carbonyl group;
- In panel A, connect lone pair of electrons with the carbonyl carbon by an arrow, which will illustrate the direction of the nucleophilic attack. The same arrow in panel B would be simply impossible;
- Add either one or two additional panels, showing the entire substrates in the active site of the ribosome in the zoomed out view;

We have introduced this modification and added additional views of the zoom-out ribosome complex.

- Instead of showing nucleotides A2820/C2821 as surface try showing them as spheres. This way there would be no weird connections between the planes of the nucleotides that do not really exist.

We show these nucleotides as surface instead of sphere to illustrate the shape of the pocket. This isbecause surface representation more accurately reflects the shape of cavities in protein/RNA structures.

2.A paragraph on the elongation factor EF-P/eIF5A - an essential translation factor, which is required for synthesis of proteins with polyproline tracts - should be included in the introduction section of this manuscript. Especially because the authors recently published a very relevant structural work, in which they revealed how eIF5A factor works and how it alleviates ribosome complexes stalled on consecutive proline tracts.

We have added the following paragraph:

Introduction (Page 3, Paragraph 3)
"In a living cell, ribosome stalling by polyproline sequences is resolved by a universally conserved translation factor, known as eIF5A in eukaryotes and EF-P in bacteria [10-12]. In eukaryotes, eIF5A alleviates ribosome stalling by contacting the acceptor stem of the P-site tRNA, using a mechanism that is not yet fully understood [13,14]. The presence of eIF5A in eukaryotic cells enables synthesis of proteins containing polyproline motifs. This factor is essential because polyproline motifs are highly abundant in eukaryotic proteomes. Human proteome, for example, contains ~10,000 motifs with three or more consecutive proline residues, with some proteins having up to 27 consecutive prolines [1,2]."

3. One very important figure is missing from the main text - a figure which will show the electron densities for the substrate analogs in the active sites of the ribosome for all four structures reported in the current work. Current figure S2 could be that figure with the addition of a panel showing

overall structure of the yeast ribosome. Including such figure as Figure 1 will make it immediately clear which structures were determined in this study and which active sites of the ribosome are occupied with the substrate analogs in each of these structures.

To address this commentary, we have moved Fig. S2 to the main text and introduced the requested changes.

Minor points:
4. Page 4, paragraph 1: Perhaps, it would be more informative to change "ACCPuromycin" to "ACC-Puromycin (ACCmA-mTyrosine)".

We have renamed the ligand and used names suggested by the reviewer.

5. Page 4, paragraph 2: It will read better if "...supplemented with 300 μM the antibiotic sparsomycin. Sparsomycin was used to stabilize..." is changed to "...supplemented with 300 μM of the antibiotic sparsomycin, which was used to stabilize...".

We have introduced this change.

6. Page 5, paragraph 4: Perhaps, authors meant to say "...A76 sugar pucker..." and not "...A76 sugar pocket..."?

No, sugar pucker was used intentionally.

7. Page 5, paragraph 4: This whole paragraph could be removed. It was already explained in the previous section of the manuscript that the antibiotic sparsomycin was used to force ACCA-dipeptide into the P site of the ribosome. No need to come back to the same point again, especially that this is relatively weak point and it just distracts the reader from the important findings.

With this paragraph, we wanted to draw attention to the limitations of our experimental system to avoid over interpretation of our data by others. Therefore, we would like to keep this paragraph in the text.

8. Page 6, paragraph 2: I would suggest to possibly reword the following sentence from "This proline conformation suggests that the poor reactivity of proline as a peptidyl acceptor originates from two factors. These factors being the displaced α-amine position, and the proline side chain, both of which may prevent the optimal alignment of substrates in the peptidyl transferase center of the ribosome." to "The observed proline conformation suggests that the poor reactivity of proline as a peptidyl acceptor originates from two factors: (i) the displaced position of α-amine and (ii) the unusual location of the side chain. Both of these factors can prevent the optimal alignment of substrates in the peptidyl transferase center of the ribosome that is required for the efficient nucleophilic attack to take place."

We have rephrased this section accordingly.

9. All throughout the text the panels of the figures are referenced with the small letters. However, in the actual figures the panels are labeled with capital letters.

This formatting has been corrected.

10. Figure 2:
- Instead of showing nucleotides G2872/G2403 as surface try showing them as spheres (the same comment is valid for Figures 1 and S3);
- Arrows are barely visible;
- It actually doesn't matter a lot where the alpha-amino group of the P-site substrate is located, since it is not a reactive group. Location of the carbonyl carbon matters more.
- Authors might wish to label the P-loop of the 25S rRNA and show H-bonds between C74/C74 and G2620/G2619, respectively.

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Fixed and relabeled as suggested. Although, the surface representation was preserved to show the A-cleft shape.

11. Figure S2:
- Make grid spacing the same in each panel;
- Make mesh a little thicker and the sticks of the model thinner, so that it will be more clear how well the model corresponds to the density;
- It might be more accurate to change the labels at the top of each panel to include ACCA. For example, ACCA-Leu-Phe.

The grid spacing has been changed and the labels have been modified.

12. Figure S3, legend: Change "archeal" to "archaeal".
This typo was corrected. Thank you very much for noticing it!

2nd Editorial Decision 19 September 2016

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed comments from the referees that were asked to assess it.

As you will see, while referee 3 is staisfied with the revised study, referee 2 remains of the opinion that biochemical assays are required to demonstrate that the tRNA analogs used are biologically active. Given the discrepancy, I contacted an advisor to arbitrate, and the advisor agrees with referee 3 pointing out that the types of aminoacyl-tRNA analogs used have been shown in the past to bind to the ribosome as native substrates do and to be biochemically active. We can therefore in principle accept your study for publication.

For the final version of the manuscript please upload all EV figures as separate files and add the figure legends to the end of the main manuscript file. Please relabel S to EV figures, also in the main text. Please also remove the main figures from the manuscript file. Given the total of 3+5 figures, 1 or 2 EV figures could be changed into main figures, if you agree.

Please also address referee 2's second comment.

Please remember to add PDB accession codes to the main manuscript file, eg to the methods section and main text.

Please rename table S1 to table EV1 and change it to black and white colors.

Please also send us a COI and author contribution statement.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #2:

The current version of this mms is an improvement over its predecessor. Two issues remain. First, and most important, the authors have chosen not to accept the recommendation that biochemical assays be done to demonstrate that the substrate analogs they have used are capable of engaging in the peptidyl transferase reaction, preferring instead to assume that because experiments of this kinds have been done successfully with similar analogs that included ordinary alpha amino acids, their

analogs must be active too. The fact is that they are trying to explain why proline adds to nascent chains about 10 times slower than other amino acids, which is not a big difference, and their structures show that their proline analogs do not bind to the ribosome the same way as similar analogs containing other amino acids. If it were found that their compounds are indeed active, then their findings would gain a lot of credibility. If they are inactive, of course, the conclusion would be that the structures are physiologically irrelevant. The one Angstrom displacement of the imino group of proline from the positions occupied by the alpha amino groups of other amino acids, which is much more clearly illustrated in this version of the mms than it was in the original, is a lot when it comes to reactivity. Second, and much less important, it is still hard to understand the crystallographic statistics provided by the authors. If the average merging R-factors for their data are as high as they report, then the only way they can obtain decent R-factors for their structures, which they appear to have done, is if the multiplicity of those data is high. A multiplicity of ~ 5, which is what they report, won't do it. A multiplicity of 40 might.

Referee #3:

In my opinion, the authors done an excellent job correcting their original manuscript, making it much more clear not only to the specialists in the field, but also to a general reader. The authors addressed all of the critical comments and I think that the manuscript could be accepted for publication in its new revised form.

2nd Revision - authors' response 28 September 2016

Dear Editorial Board of EMBO reports,

First of all, we thank the reviewer 2 for his/her thoughtful comments. Below is our response to the comment about our statistics.

Referee #2:
Second, and much less important, it is still hard to understand the crystallographic statistics provided by the authors. If the average merging R-factors for their data are as high as they report, then the only way they can obtain decent R-factors for their structures, which they appear to have done, is if the multiplicity of those data is high. A multiplicity of ~ 5, which is what they report, won't do it. A multiplicity of 40 might.

High Rmeas values in our data sets primarily stem from not from redundancy, but from high attenuation of the beam. This is described in detail in the papers we've cited in the revised manuscript. In simple terms, beam attenuation results in the increased signal-to-noise ratio and increased error for each individual measurement. This is reflected in high Rmeas values. However, because the attenuated beam causes only a very subtle radioactive damage, the **average** values of intensities are highly accurate. In this data collection strategy we need redundancy to neutralize noise in our data.

We also want to stress that this data collection strategy is not new. As stated in the revised manuscript, we first described this strategy in 2010, and since then we used it to determine more than 50 crystal structures, including those published in (Ben-Shem et al, Science 2010), (Ben-Shem et al, Science 2010), (Demeshkina et al. Nature 2012) and (Garreau-de-Loubresse et al Nature 2014). And most importantly, this strategy was co-developed with engineers from Dectris – developers of Pilatus detectors, – and their work is also cited in the revised manuscript.

To stress the effect of beam attenuation on Rmeas, we have modified our data collection section:

Materials and methods/Data collection and processing
Due to highly attenuated beam**,** this data collection strategy results in unconventionally high Rmeas values, when it is compared to CCD-detector type data collection strategies (no beam attenuation, no fine ϕ-slicing, low data redundancy), but provides more accurate values of reflections' intensities and anomalous signal [24, 26]".

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Accepted 30 September 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Marat Yusupov
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-42943V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
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  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
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  - exact statistical test results, e.g., P values = x but not P values < x;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	5dtv, 5lyb, 5tga, 5tgm. These codes are now listed in page 11 of the revised manuscript, in the acknowledgement section.
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