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The physiological target for LeuRS translational quality control is norvaline

Nevena Cvetesic, Andrés Palencia, Ivan Halasz, Stephen Cusack and Ita Gruic-Sovulj

Corresponding author: Ita Gruic-Sovulj, Faculty of Science University of Zagreb

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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 13 March 2014

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.

As you will see from the reports, all three referees highlight the quality and impact of your findings, although they also raise a few issues that should be addressed before they can fully support publication of the manuscript in The EMBO Journal.

I would ask you to particularly focus your efforts on the following points:

-> enhancing the clarity of the figure presentation as suggested by refs #1 and 2

-> discuss the effective concentration of amino acids in vitro and in vivo and its consequence for the observed specificity (ref#1)

-> either modulate the title to reflect the concerns raised by ref#3 or include additional data on cellular
consequences of norvaline charging (if available)

-> similarly, while we agree with ref#1 than structural insight on mutants M40A and M40P would further strengthen the manuscript, we realize that this may be beyond the scope of the current work and this will therefore not be an absolute requirement from our side. However, should such data be available we would encourage you to include it.

Given the referees' positive recommendations, we offer you the opportunity to submit a revised

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version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. Please do not hesitate to contact me if you have questions related to the review process and the requests made by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the 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://emboj.embopress.org/about#Transparent_Process

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. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

REFEREE REPORTS

Referee #1:

The manuscript by Cvetesic et al. reports a rigorous and comprehensive study of amino-acylation of tRNA(Leu) by E. coli LeuRS. Leu is similar to Ile, Val, norvaline and gamma-aminobutyric acid in its size and chemical nature and hence LeuRS uses an editing site to hydrolyze mis-charged aminoacyl tRNA to ensure fidelity of protein synthesis. Aminoacyl-tRNA synthetases catalyze amino-acylation of tRNAs by two step reactions. Firstly, ATP-dependent amino acid activation yields an aminoacyl-adenylate intermediate (AA-AMP) in the synthetic site. In the second step, either the 2'- or 3'-OH group of the terminal adenosine of the tRNA attacks the carbonyl carbon of AA-AMP, leading to transfer of the aminoacyl moiety onto the tRNA. Non-cognate AA-AMP is hydrolyzed in the synthetic site either tRNA dependent or non-dependent. If cognate aminoacyl-tRNA escapes this editing process, non-cognate amino acid moiety of mis-acylated tRNA is then transferred to the distant hydrolytic site by translocating the 3'-end of the mis-acylated tRNA and cleared by post-transfer editing at the specialized editing domain. Previous studies showed that the discrimination factors for activation of isoleucine by E. coli LeuRS is only from 630 to 5400. By using ultra-pure isoleucine the authors showed that isoleucine is mistakenly activated by LeuRS only with frequency of 1 in 31000: significantly lower than the observed error in protein synthesis (1 in 3300). The authors showed that the previous results are due to contamination of leucine in isoleucine. This suggests that editing of isoleucine is not essential and thus argues against the view that editing of isoleucine by LeuRS is a major defense against mistranslation of leucine codons as isoleucine in E. coli.

In order to investigate this problem more rigorously the authors determined kinetic parameters in amino acid activation by LeuRS. Using the wild type and a mutant which has a mutation in the editing site, the authors showed that isoleucine, but not norvaline, is significantly discriminated at the catalytic step (kcat is decreased 55-fold). They further investigate whether LeuRS also discriminates against isoleucine at the transfer step and found that a t rasfer of isoleucine is a 50-fold slower (1.3 s-1) as compared with leucine (58 s-1). In order to investigate the structural basis of isoleucine discrimination by LeuRS the authors determined the crystal structure of EcLeuRS bound to the cognate tRNALeu and a non-hydrolysable analogue of isoleucyl-adenylate (Ile-AMS) at 2.4 Å resolution. The structure shows that the conformation of isoleucine bound in the synthetic site corresponds to a 'gauche - like' conformation (X2 = 243 degrees) and that binding of the trans conformation, which is a predominant form in solution, is sterically prevented by the geometry of the LeuRS synthetic site, in particular by Met40. In contrast, isoleucine adopts the more favoured trans conformation when bound to the cognate IleRS synthetic site. The ability of EcLeuRS to discriminate against isoleucine is substantially lost in the Met40Ala mutant.

Unlike isoleucine the non-standard amino acid norveline is not stringently discriminated against in the synthetic site and the editing site plays a crucial role in preventing mis-incorporation of

norvaline into protein. They demonstrated that a LeuRS editing-deficient E. coli strain grows normally in non-physiologically high concentrations of isoleucine but, unlike wild-type, not under oxygen deprivation conditions when norvaline accumulates to substantial levels. These findings support previous observations that combination of amino acid specificity and editing mechanism provide an adaptive response mechanism under stress conditions.

The experiments are well designed and executed and the results are presented in a logical way. The introduction section explains the background well and in sufficient details.

There are a few points, which may be considered to improve the manuscript.

(1) The suggestion that AARS selectivity mechanisms are originated from preferential binding of higher-energy conformers is very interesting. However, in this regard it might be useful to solve the structures of the mutants M40A and M40P, to clarify the mechanism of eukaryotic LeuRS. Considering its importance, Figure 3 could be presented better (larger with map?) either in main part or as supplementary material. If only a minor conformer, the gauche form of isoleucine, can bind to the synthetic site, this reduces effective concentration of isoleucine (to 15%). Does the structure suggest that even the gauche form binds with much reduced affinity than Leu? It is striking that a loss of one methyl group in Nva affects Km by 100-fold.
(2) For in vivo experiment is the cellular level of isoleucine is as high as in the medium or regulated to a certain level when the isoleucine level in culture medium is high?
(3) His533 should be labelled in Figure 3B,C.

Referee #2:

The adaptive response to oxygen downshift in Escherichia coli entails editing of norvaline by LeuRS Cvetesic et al

Editing by aaRS is a crucial point of quality control during protein synthesis, and the specifics of each potential misaminoacylation event are important to fully understand the biological and technical implications of this process.

Why LeuRS mischarged Ile so efficiently was a puzzle in the field, given the obvious topological differences between both side chains.

Moreover, the development of boron-based antibacterials, and their apparent demise at recent clinical trials, made the issue of editing by LeuRS of general interest.

The authors of this paper show very clearly that previous data was tarnished by Leu contamination of Ile samples. This solves the riddle of the previously observed mischarging, and helps understand the rapid emergence of resistances in bacteria treated with editing domain inhibitors.

Moreover, the data obtained now with better amino acid preparations shifts the focus to norvaline, and a very compelling argument is proposed to explain why editing by LeuRS would have evolved mainly to prevent norvaline mischarging.

All in all I find this paper remarcable in its clarity, and important in its relevance to the field. The writing is excellent, and I only have two suggestions:

a) some of the figures are slightly substandard. I would probably help to have a better graphical representation of the side chains explored in the paper.

b) As far as I remember, one of the first demonstrations of the biological relevance of editing in vivo in E. coli was in a paper by Beebe, published in 2001 in Embo Journal. This paper should be cited in the second paragraph of page 12.

Referee #3:

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The manuscript reports the purification of leucine from isoleucine contaminants, and establishes that EcLeuRS does not discriminate against isoleucine by editing. However the authors demonstrate that EcLeuRS does discriminate against norvaline by editing. The experiments are convincing, using a range of different approaches to establish kinetic and thermodynamic parameters and structure. While I am not an expert in every method used the experiments appear well done and well controlled.

The authors go on to demonstrate that an editing deficient mutant of EcLeuRS grows poorly on norvaline but not isoleucine, providing indirect in vivo evidence for the role of editing. These experiments are followed with experiments showing a similar growth defect of the editing deficient mutants of LeuRS at low oxygen where norvaline accumulates.

The manuscript is well written and the experiments well executed and interpreted. While the majority of the data relates to the in vitro specificity of EcLeuRS with respect to norvaline and isoleucine, the title focuses on the adaptive response to oxygen. I would suggest tweaking the title to reflect the focus of the paper or adding additional experiments explicitly demonstrating that a deficiency in editing in vivo really leads to the accumulation of norvaline in proteins and misfolding. I do not think such experiments are necessary for the paper to be published in an excellent journal, but I do think it is important that the title capture the body of work on the molecular specificity of the synthetase.

1st Revision - authors' response 17 April 2014

Reviewer 1.

(1) The suggestion that AARS selectivity mechanisms are originated from preferential binding of higher-energy conformers is very interesting. However, in this regard it might be useful to solve the structures of the mutants M40A and M40P, to clarify the mechanism of eukaryotic LeuRS. Considering its importance, Figure 3 could be presented better (larger with map?) either in main part or as supplementary material. If only a minor conformer, the gauche form of isoleucine, can bind to the synthetic site, this reduces effective concentration of isoleucine (to 15%). Does the structure suggest that even the gauche form binds with much reduced affinity than Leu? It is striking that a loss of one methyl group in Nva affects Km by 100-fold.

It will be certainly interesting to solve the structures of M40A and M40P variants bound to Ile-AMS and Leu-AMS, as well as the structure of eukaryotic LeuRS. We plan to solve at least some of these structures in the future to complement kinetic analysis of the eukaryotic LeuRS specificity using highly purified isoleucine sample.
Figure 3 has been reorganized according to the suggestions of reviewers 1 and 2; E532 residue and water No 26 were removed and His 533 residue was labeled. Also, we have introduced a novel figure (Extended view figure 4) showing stereo diagram of unbiased m(Fo-Fc) difference electron density for residues M40, H533, base Ade76 of the tRNA, and Ile-AMS in the synthetic site of the *E. coli* LeuRS–tRNA complex.
The crystal structure indeed reveals the origin of discrimination between the isoleucine *gauche* –conformer and leucine. While the leucine side chain delta carbon atoms fit perfectly within the hydrophobic binding pocket and establish van der Waals contacts that contribute favourably to the binding energy, the isoleucine β-branched side chain occupies slightly different position which lacks the capacity to establish full repertoire of van der Waals interactions. On top of that, discrimination against the most abundant isoleucine conformer in solution additionally contributes to binding affinity. Thus, these two effects together establish very weak binding of isoleucine to LeuRS. Because of the reviewer's comment we became aware that the manuscript did not address both contributions to the origin of discrimination against isoleucine explicitly. Driven by a novelty in our finding regarding discrimination against different amino acid conformers, we did not address properly other contributions to LeuRS specificity against isoleucine. Therefore, in the revised version we added two sentences in the result section under *Structural basis of isoleucine discrimination by LeuRS* subsection (second paragraph pages 6 and 7) which describe different modes of isoleucine and leucine accommodation within the synthetic site as described here. We

have also expanded one sentence to address this issue in the Discussion as well (third paragraph, page 11).

It is well established that the lack of CH_3 group may induce 100-fold increase in K_m; AlaRS: K_m(Ala) = 0.42 mM; K_m(Gly) = 58.5 mM (Beebe et al. (2003) *EMBO J*, **22**, 668-675); IleRS: K_m(Ile) = 0.005 mM; K_m(Val) = 0.4 mM (Jakubowski & Fersht (1981) *Nucleic Acid Res* **9**, 3105-3117; Dulic et al. (2010) *J Biol Chem* **285**, 23799-23809). The difference in binding energy between the two amino acids that differ in one methylene group is about 3 kcal/mol, which is equivalent to a factor of 100-200.

(2) For in vivo experiment is the cellular level of isoleucine is as high as in the medium or regulated to a certain level when the isoleucine level in culture medium is high?
There are two uptake systems for the branched chain amino acids in prokaryotes: i) LIV-II-secondary transport system (Na^+-coupled symport) which has a low substrate affinity, sets the basal level of amino acid uptake and is not significantly regulated and ii) LIV-I – primary transport system (binding protein dependent system) which has a high substrate affinity and may accumulate amino acid in the cell at the expense of ATP. Its expression is stimulated by low intracellular leucine concentration (Krämer (1994) *Arch Microbiol* **162**, 1-13). Isoleucine may also enter the cell by simple diffusion. Due to its hydrophobicity isoleucine diffuses through plasma membrane at significant rates. Hence, diffusion may play an important role in both influx and efflux directions. It has been also recognized that isoleucine excretion may occur through both passive diffusion and carrier-mediated excretion (Krämer (1994) *FEMS Microbiology Reviews* **13**, 75-94).
In our *in vivo* experiments we use high external concentration of isoleucine (up to 100 mM) which stimulates a diffusive preloading of *E. coli* cells. There is no indication that a high intracellular concentration of isoleucine inhibits its uptake; on contrary isoleucine may stimulate LIV-II system. On the other hand, high internal isoleucine concentration may induce the activity of the carrier-mediated excretion system. Nevertheless, it is highly unlikely that carrier-mediated excretion significantly contributes to the isoleucine net excretion under conditions of high external concentration of isoleucine, as the gradient of the transport solute itself represents a part of a driving force for the efflux. Indeed, the measurements showed that the efflux rate decreases with an increase in the external isoleucine concentration (Herman & Krämer (1996) *Applied and Environmental Microbiology* **62**, 3238-3244). In agreement, *Corynebacterium glutamicum* rapidly accumulates internal pool of about 32 mM, 75 mM, 145 mM or 195 mM isoleucine when the media contained 50 mM, 100 mM, 200 mM or 250 mM isoleucine, respectively. These findings strongly support our assumption that isoleucine roughly equilibrates between the medium and *E. coli* cytoplasm, and thus provide a considerable level of confidence in the reported high discriminatory power of LeuRS against isoleucine both *in vitro* and *in vivo*.
(3) His533 should be labelled in Figure 3B,C.
We did that.

Reviewer 2.

1) Some of the figures are slightly substandard. I would probably help to have a better graphical representation of the side chains explored in the paper.

Figure 3 has been reorganized according to the suggestions of reviewers 1 and 2; E532 residue and water No 26 were removed and His 533 residue was labeled.

2) As far as I remember, one of the first demonstrations of the biological relevance of editing in vivo in E. coli was in a paper by Beebe, published in 2001 in EMBO Journal. This paper should be cited in the second paragraph of page 12.

We included Beebe et al (2003) *EMBO J* **22**, 668-75 in Discussion (page 12; second paragraph) of the revised version of the manuscript. We did not find any paper by that author published in 2001 in the EMBO J. However, it is likely that the reviewer was actually thinking about Beebe et al (2003) paper which includes demonstration of the relevance of AlaRS editing *in vivo*. In that work the authors also demonstrated that AlaRS discriminates against glycine and serine only at the level of ground state binding (100-fold K_m effect and lack of the k_{cat} effect), and thus requires editing to eliminate translational error. This work thus further supports our conclusion that AARSs which are

incapable of exerting specificity at the catalytic steps generally require editing, and may be cited along with other papers.

Reviewer 3.

In response to reviewer's suggestion we change our title to *The physiological target for LeuRS translational quality control is norvaline, not isoleucine*.

Accepted 07 May 2014

Thank you very much for submitting the revised version of your manuscript. It has now been seen by two of the original referees who both find that all criticisms have been sufficiently addressed. I am therefore happy to inform you that your manuscript has been accepted for publication in The EMBO Journal.

Before we can transfer your manuscript for production, I would ask you to address the minor editorial points outlined below:

-> Please include information on the nature of the error bars in figures 5b, 7b, 7d, 7f and 8c in the figure legends (and please also double-check if error bars should be included in the image in fig 8c).

REFEREE REPORTS

Referee #1:

The authors have responded well to the points raised by the referee and the manuscript should now be published without further delay. Very interesting and excellent paper.

Referee #2:
All my comments have been addressed satisfactorily.

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