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A Monovalent Cation Acts as Structural and Catalytic Cofactor in Translational

Bernhard Kuhle and Ralf Ficner

Corresponding author: Bernhard Kuhle, Georg-August-University Göttingen

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Transaction Report:

(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.)

Editor: Anne Nielsen

1st Editorial Decision

21 May 2014

Thank you for submitting your manuscript for consideration by the EMBO Journal and once again my apologies for the unusually long duration of the review period in this case. Your study has now been seen by three referees whose comments are shown below.

As you will see from the reports all three referees find your observations intriguing and would support publication of a revised manuscript in The EMBO Journal. However, while refs #1 and #3 raise only minor concerns, ref #2 points out a number of critical issues related to the catalytic rate and the essential role for M⁺ that would have to be fully and extensively addressed (including presentation of all relevant data) before submission of a revised manuscript. This concern was furthermore shared by an additional expert advisor who we consulted prior to reaching an editorial decision. I would also ask you to perform the experiments suggested by ref#1 to extend the generality of your conclusions and to address all points raised by referee #3.

Given the referees' overall positive recommendations, I would thus invite you to submit a revised 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.

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.

Thank you for the opportunity to consider your work for publication and thank you again for your patience. I look forward to receiving your revision.

REFEREE REPORTS:

Referee #1:

This manuscript provides compelling evidence of an important role for monovalent ions in stabilizing the active GTP-bound conformation and contributing to the GTP hydrolytic mechanism of translational family GTPases. Crystal structures of the eIF5B core were determined with GTP-Na⁺, GTPγS-Na⁺, and GTPγS-K⁺, allowing comparisons of GTPγS vs. GTP and Na⁺ vs. K⁺. Additional structures from the PDB were also analyzed, and in some cases re-refined to account for M⁺ ions treated as waters in the original refinements. Effects of M⁺ ions on the temperature dependence of the binding enthalpy of GDP, GDPNP, and GTPγS were analyzed by ITC. The effects and specificity of M⁺ ions on intrinsic GTP hydrolysis and were analyzed for EF-Tu.

Overall, the study is well executed from a structural perspective and clarifies a number of confusing issues related to discrepancies between "active" structures of translational GTPases complexed with GTP, non-hydrolyzable, or poorly-hydrolyzable analogs. The biochemical evidence provided supports the main structural conclusions, although the generality of the predictions regarding the function of M⁺ ions in GTP hydrolysis could be further examined as noted below.

Specific comments:

The effect of M⁺ ions on GTP hydrolysis rates was measured for EF-Tu. The dependence on K⁺ or NH₄⁺ was substantially stronger than for Na⁺ or Li⁺, suggesting a preference for M⁺ ions with larger ionic radii. It would be interesting and relevant to perform similar measurements for the eIF5B construct used for the crystallographic analysis. Is a similar dependence and preference for K⁺/NH₄⁺? The additional experiments would help to clarify the generality of the role of M⁺ ions in the hydrolytic reaction.

Related to the previous point, it isn't clear why the experiments examining M⁺ dependence on GTP hydrolysis are presented as a supplemental figure, since the results provide important validation of the claim that K⁺ has an important role in hydrolysis.

P.11 - The greater coordinate error for the 2.84 Å structure should be noted when discussing coordination distances and comparing structures.

Minor:

P. 6 - "ansatz" is a bit obscure.

P. 11 - "owned to the increased". Typo. Presumably, the authors mean "owing".

Fig. 2B - the axis/tic labels are too small.

Referee #2:

This paper reports a new crystallographic analysis of translational GTPases, where the main point is that these enzymes seem to coordinate a monovalent cation between the beta and gamma phosphates when the true substrate (GTP) is bound. The authors solve structures of eIF5B in complex with GTP and GTP-gamma-S with Na⁺ and K⁺ ions, which yield distinct densities at this position. The arguments for ion binding are mainly made in terms of ligand distances, but the most convincing proof is the nature of the ligands in my opinion. As has been indicated earlier, it may well be the case that GDPND and GDPCP are not good GTP analogs, simply because they have a reversed polarity for the beta-gamma bridge. This would mean that they repel any cation that would otherwise interact with this position. It is indeed difficult to find perfect analogs of reactive compounds (even puromycin may be such a case as it has an amide instead of the ester link to the tRNA, also with reversed polarity) and this manuscript seems to prove the point. But even if this now is a specific cation site, the step towards proving that the cation is an essential catalytic element is rather big. The intrinsic EF-Tu activities shown in Fig. S5 are not entirely clear with regard to the catalytic effect. E.g., what does 5% GTP hydrolysis at physiological K⁺ (100mM) actually mean in terms of rate enhancement? Even if the authors argue against allosteric effects of ion binding this possibility does not seem to be totally excluded.

Unfortunately, one of the most critical tests of the hypothesis that the ion is essential, namely mutation of the key ligand Asp-MC, is not done by the authors but instead referred to as unpublished results by Rodnina (personal communication). What does "negative impact" on hydrolysis mean in quantitative terms? It would have been very useful to be able to judge the effects of Asp-MC on the activity on the ribosome. The authors argue that the M⁺ ion is a constitutive component of the intrinsic GTPase activity, but that it is not enough for high catalytic rates, where His-cat is the critical switch which triggers the reaction. The analogy to RasGAP and mutations of the glutamine in Ras good I think, but the question whether the ion is really essential on the ribosome is rather difficult to answer without mutation data.

While I believe that the authors mechanistic idea may be correct the description of the hypothesized cation mode of operation is below standards. There is no developing negative charge on the gamma phosphate as said on p. 20, but rather the opposite. By attack of a water molecule on the gamma phosphate, one unit of negative charge would be transferred from gamma to beta. In this respect, Fig. 6 is also weird. The histidine has no protons on it while a water molecule is drawn with covalent bonds to the phosphate and only two arbitrarily chosen positive charges are shown (there is also an Mg⁺⁺) between beta and gamma. Although I understand what the authors mean this is not satisfactory from a chemical point of view if this is a paper about chemical mechanism.

Referee #3:

In this manuscript, Kuhle and Ficner provide compelling structural and biochemical evidence that a monovalent cation in the active site is required for conformational switching in a wide range of translational GTPases with the exception of those that employ the so-called "Arg finger" for stabilisation.

In my view, this is an exceptional paper, which will have a significant impact on the field. The experiments have been thoroughly carried out and the data are well-presented and convincing. And although the manuscript is quite long, it is generally very well written and a pleasure to read.

I have a few points below, which require the authors' attention, but overall I think the paper should be published with as little delay as possible.

Main points:

The arguments towards the presence of a monovalent ion in the active site of eIF2B are quite strong but could be strengthened significantly if the ion could be visualised by anomalous scattering.

Neither Na⁺, K⁺, nor NH₄⁺ have anomalous scattering properties, but have the authors considered crystallisation in the presence of Rb⁺?

The argument on p. 8 that the lack of crystallisation of eIF5B(517-858) in the presence of GDPNP indicates that this modified nucleotide is not sufficient to induce the GTP-bound conformation is weak. Lack of crystallisation could be due to any number of things, including lower affinity towards GDPNP, which is indeed also the case as shown later. What happens if higher than 3 mM concentrations of GDPNP are used? Can crystallisation then be induced?

Regarding the argument of Na⁺ versus H₂O on p. 14 in past structures: Please note that Na⁺ has 10 electrons exactly like H₂O, so a positive difference peak cannot be taken as evidence for the presence of the ion.

The stimulation of GTPase activity in Figure S5B lacks a control to show that the effect is not simply due to increased catalytic activity at higher ionic strength. Could a divalent cation be used as control? And in the same figure, why does Na⁺ not stimulate when you argue from the structure that it binds and organises switch I?

Why were two residues (859-860) removed from the protein for this paper compared to the previously reported structures? Does the longer construct not crystallise in the presence of the same nucleotides?

How was Hepes used for crystallisation titrated (p. 24)? If KOH was used as mentioned above for the purification, how can you be sure that Na⁺ is present in the crystal, as Hepes and NaOAc are both present at 0.1M?

Further comments:

Some references should be given to the preferred coordination numbers of Na⁺ and K⁺. In the paper, it is taken for granted that penta-coordination (Na⁺) and hepta-coordination (K⁺) is the norm.

Please show the calculations of VDW distances (p. 8 and 11).

ASA, TC etc, there are several undefined abbreviations throughout the ms.

In most of the figures Mg²⁺ is omitted. It would be useful to have the ion shown and labeled in those views where it would be visible.

The first paragraph in the section starting on p. 13 is somewhat repetitive.

The description of the effect on EF-Tu on p. 19 ("specifically stabilizes a conformation of switch 1 in which helix A" is 'attracted' to the GTP molecule") is unclear. Please rephrase.

On p. 21 of the discussion you state that the presence of the M⁺ ion itself does not stimulate GTPase activity. But isn't that exactly what you observe in Figure S5?

Figure 1C - it would be useful to show the GDP conformation for comparison.

Table I - please include CC1/2 as well for resolution cut-off.

Table I - The R and R_{free} are suspiciously close - were the free reflections transferred correctly from the previous refinement or chosen anew? If the free reflections were not transferred, the R_{free} value will be biased and essentially useless. In this case, the refinement should be repeated with the correct free set.

Figure S2 - Please have all x-axes on the same scale to allow comparison of affinities.

Referee #1:**Specific comments:**

The effect of M⁺ ions on GTP hydrolysis rates was measured for EF-Tu. The dependence on K⁺ or NH₄⁺ was substantially stronger than for Na⁺ or Li⁺, suggesting a preference for M⁺ ions with larger ionic radii. It would be interesting and relevant to perform similar measurements for the eIF5B construct used for the crystallographic analysis. Is a similar dependence and preference for K⁺/NH₄⁺? The additional experiments would help the clarify the generality of the role of M⁺ ions in the hydrolytic reaction.

Reply:

As suggested by the referee, we included measurements for the GTPase activity of eIF5B in dependency of the presence or absence of different M⁺ ions. The result of these experiments support the idea that the GTPase activity in eIF5B, like that of EF-Tu, depends on the species as well as the concentration of M⁺ ions. However, the preferences are slightly different, as eIF5B is most stimulated by K⁺ and Na⁺, those cations that have also been found in the crystal structures. Moreover, we incorporated mutational data, demonstrating that Asp-MC in the P-loop is critical for the M⁺-dependent intrinsic GTPase activity in eIF5B, as this activity is abolished by the exchange of Asp-MC for alanine or arginine, but retained with asparagine (which coordinates the M⁺ ion in the M⁺-dependent GTPase MnmE). In order to show that these effects are not due to a reduced binding affinity of the mutants to the substrate GTP, we also included steady-state fluorescence measurements with mant-labeled GTP (presented in Figure E5).

Important additional support for our interpretation is provided by three newly determined crystal structures of three eIF5B mutants: By using crystals of the wild-type protein as nucleation seeds, we were able to induce the crystallization of the eIF5B Asp-MC mutants D533A, D533R and D533N bound to GTP (or GTPγS), which do not crystallize on their own in common grid screens or in our own optimization screens (the detailed procedure by which we obtained these crystals was incorporated in the Expanded View Materials and Methods section). In line with the observation that mutation of Asp-MC to Asn (D533N) has only little effect on the GTPase activity of eIF5B in the presence of K⁺ or Na⁺, the D533N crystal structure still contains the M⁺ ion bound in the active site. By contrast, the alanine mutation (D533A), which shows reduced and M⁺ independent GTPase activity does not contain an M⁺ ion but instead a water molecule bound close to the original M⁺ binding site of the wild-type protein. Finally, the D533R mutant coordinates neither an M⁺ nor a water molecule next to the GTP molecule.

We would like to add here that the fact that we were able to obtain crystal structures of GTP/GTPγS-bound eIF5B in the GTP-conformation despite the absence of the M⁺ ion does not refute a role of the M⁺ ion as important structural cofactor. Our model does not propose that the M⁺ ion is the only contribution but one among several important contributions, e.g. the gamma-phosphate itself and the Mg²⁺ ion, that stabilize switch 1 in its 'on' state. Thus, according to our model, the loss of the contribution of the M⁺ ion shifts the equilibrium between 'on' state and 'off' state in GTP-bound eIF5B toward the latter, resulting in a smaller fraction but not the complete absence of eIF5B/GTP in the active conformation in solution. According to our crystallization experiments, the reduced fraction of eIF5B in the GTP-conformation is insufficient to promote crystallization in the case of the mutants, however, sufficient only if crystallization nuclei are provided by microseeding with seeds from wild-type crystals (in which switch 1 is additionally stabilized in the 'on' state by crystal contacts).

Related to the previous point, it isn't clear why the experiments examining M⁺ dependence on GTP hydrolysis are presented as a supplemental figure, since the results provide important validation of the claim that K⁺ has an important role in hydrolysis.

Reply:

The reason why we decided to move these data into the supplemental figure was that they were not actually new but merely a repetition and confirmation of previous data, published by Parmeggiani and coworkers. However, for the revised version of the manuscript, we performed new, original experiments on the intrinsic GTPase activity of wild-type EF-Tu as well as its Asp-MC mutant, some of which are presented in Figure 4 (D/E). Only some of the data (dependency of the Asp-MC mutant on salt species) are now still presented in the supplemental information (now Expanded View) (Fig. E6).

P.11 - The greater coordinate error for the 2.84 Ang. structure should be noted when discussing coordination distances and comparing structures.

Reply:

Since solving the first structure of the K⁺ bound eIF5B/GTPγS, we were able to improve crystals leading to a significantly higher resolution of this structure, which is now 2.28 Ang.

Here we would like to mention that using the improved crystals the new structure at higher resolution was solved and refined in space group P4(1)2(1)2 with two molecules per asymmetric unit instead of the original C222(1) with four molecules per asymmetric unit. This, however, had no effect on the structure of the crystallized protein, which is virtually identical to the model that had been obtained in C222(1).

Minor:

P. 6 - "ansatz" is a bit obscure.

Reply:

“ansatz” has been exchanged for “assumption”.

P. 11 - "owned to the increased". Typo. Presumably, the authors mean "owing".

Reply:

The typo has been removed.

Fig. 2B - the axis/tic labels are too small.

Reply:

Done.

Referee #2:

The intrinsic EF-Tu activities shown in Fig. S5 are not entirely clear with regard to the catalytic effect. E.g., what does 5% GTP hydrolysis at physiological K⁺ (100mM) actually mean in terms of rate enhancement? Even if the authors argue against allosteric effects of ion binding this possibility does not seem to be totally excluded.

Reply:

For the revised manuscript, we repeated the experiments on the intrinsic GTPase activity in EF-Tu, this time for the wild-type protein as well as for the Asp-MC to Ala mutant. These experiments show that the M⁺-dependency observed for the wild-type protein is lost in the Asp-MC to Ala mutant, in line with the assumed key role of this residue in binding the M⁺ ion and its involvement in the GTPase reaction, and in line with the corresponding mutational and structural experiments for eIF5B (see response to referee #1). The corresponding rates and rate enhancements were included in the revised text as well as in the new Table 3 (for which we moved the original Table 2 with ITC data to the Expanded View (new Table E1)).

Unfortunately, one of the most critical tests of the hypothesis that the ion is essential, namely mutation of the key ligand Asp-MC, is not done by the authors but instead referred to as unpublished results by Rodnina (personal communication). What does "negative impact" on hydrolysis mean in quantitative terms? It would have been very useful to be able to judge the effects of Asp-MC on the activity on the ribosome. The authors argue that the M⁺ ion is a constitutive component of the intrinsic GTPase activity, but that it is not enough for high catalytic rates, where His-cat is the critical switch which triggers the reaction. The analogy to RasGAP and mutations of the glutamine in Ras good I think, but the question whether the ion is really essential on the ribosome is rather difficult to answer without mutation data.

Reply:

As described above, we incorporated experimental data on the intrinsic GTPase activity with the Asp-MC to Ala mutant of EF-Tu. Moreover, we included a biochemical as well as structural analysis for Asp-MC mutants of eIF5B. These data support the idea that eIF5B and EF-Tu bind an M⁺ ion in solution at physiological salt concentrations in a manner that is dependent on the presence of Asp-MC as key ligand and that directly contributes to the intrinsic GTPase activity (Figures 2C-F and 4D/E).

Concerning the personal communication with Marina Rodnina: The mutational data that we refer to will be published by Marina Rodnina and coworkers and we are therefore not entitled to make them public in our own manuscript. The original and new versions of the personal communication (see below) and the statements about the "significant reduction of the GTPase activity" (page 21) were written in direct agreement with Marina Rodnina. Concerning the effect of the Asp-MC mutation in quantitative terms, we can only say that it is in good agreement with the effect that was observed for mutations of the corresponding M⁺-coordinating residue (Asn226) in the M⁺-dependent GTPase MnmE (Scrima & Wittinghofer, EMBO J., 2006). The new version of the personal communication reads:

"Moreover, kinetic experiments indicated that mutations of Asp21 (Asp^{MC}) in *E. coli* EF-Tu result in a significant reduction of the GTPase activity in the EF-Tu-GTP·aa-tRNA complex in the presence of the correct codon on the ribosome (C. Maracci and M.V. Rodnina, personal communication). This observation as well is consistent with a role of Asp^{MC} as key ligand for a catalytic M⁺ ion in EF-Tu, involved in ribosome-dependent GTP hydrolysis."

While I believe that the authors mechanistic idea may be correct the description of the hypothesized cation mode of operation is below standards. There is no developing negative charge on the gamma phosphate as said on p. 20, but rather the opposite. By attack of a water molecule on the gamma phosphate, one unit of negative charge would be transferred from gamma to beta. In this respect, Fig. 6 is also weird. The histidine has no protons on it while a water molecule is drawn with covalent bonds to the phosphate and only two arbitrarily chosen positive charges are shown (there is also an Mg⁺⁺) between beta and gamma. Although I understand what the authors mean this is not satisfactory from a chemical point of view if this is a paper about chemical mechanism.

Reply:

We are thankful to the referee that he drew our attention to this point. However, we think that the raised concerns are based on a misunderstanding that we hope is clarified by the following explanation and the changes made in the revised manuscript.

The central conclusion of our manuscript is that an M^+ ion and thus an additional positive charge is introduced into the active site, which is in direct contact with all three phosphates of the bound GTP molecule. The necessary consequence is that it interacts with and thus stabilizes negative charges that are present or develop during the hydrolysis reaction either in the non-bridging α - and γ -phosphates or the β - γ -bridging oxygen, which is particularly relevant for the stabilization of the transition state. The mechanistic relevance of this observation is deduced in part from the effect of M^+ ions on the GTPase activity in trGTPases and in part by analogy to the virtually identically positioned catalytic arginine-finger in Ras-RasGAP and the catalytic M^+ ion in other M^+ -dependent GTPases. Consequently, our contribution to the elucidation of the hydrolysis mechanism lies entirely in the answer to the question how its second step, the stabilization of the transition state, is achieved, and not the question whether the reaction follows an associative, dissociative or concerted mechanism or whether the hydrolytic water molecule is activated by a single or double-protonated catalytic histidine. Both of these latter questions would decide over the way in which partial negative charges and the imidazole moiety have to be presented in figure 6B/D. As they are not the central issue of our manuscript and as they are still under debate, we originally intended to keep Figure 6 as simple as possible to avoid a distraction from the main point of our argument. Moreover, the way of presentation in figure 6B/D was directly adopted from the corresponding figures by Bos et al (Cell, 2007) and Rodnina (PNAS, 2009). However, we do understand the concern of the referee regarding the chemical correctness of the presentation and therefore included the proton on the imidazole moiety and changed the presentation of the bonds of the γ -phosphate to the attacking water and the leaving group. Moreover, we now presented the positive charge of the guanidino group as delocalized. However, we decided not to include the Mg^{2+} ion, simply because we would like to leave the emphasis on the analogy between the positions of the M^+ ion in trGTPases and the arginine-finger in Ras-RasGAP, and because it would additionally raise the question why only the P-loop lysine (the third critical positive charge) is left out. Again, we do not propose a specific mechanism for the GTP hydrolysis reaction that would require a detailed reactions scheme; we propose that the M^+ ion provides electrostatic stabilization for the transition state of the reaction, which would be true for an associative, as well as for a dissociative mechanism.

Concerning our statement about the developing negative charges: To our knowledge, the way in which partial negative charges are shifted to reach the transition state of the GTP hydrolysis reaction depends on whether the reaction follows a more associative or dissociative pathway. In the dissociative mechanism the negative charge accumulates on the β - γ -bridging oxygen (which is in direct contact with the M^+ ion) and thus in the leaving group. Although the mainly associative reaction as well ultimately results in the transfer of one unit of negative charge to the leaving group, an accumulation of charge – or at least the transient formation of partial negative charges – is as well assumed for the non-bridging oxygens in the transition state of the transferred phosphoryl group. This view was adopted from a number of works, e.g. Schweins et al (Nature, 1995), Li & Zhang (J. Mol. Biol., 2004), Maegley et al (PNAS, 1996), Pasqualato & Cherfils (Structure, 2005). Moreover, the formulation “...neutralize developing negative charges in the γ -phosphate and the designated leaving group” (p.20) does not necessarily imply a net transfer of charge toward the γ -phosphate but instead is supposed to account for the possibility that a partial negative charge developing in the transition state during/after the attack of the hydrolytic water on the non-bridging γ -phosphate oxygen is stabilized electrostatically by the M^+ . Again, the critical information is that the M^+ ion is in a position that would allow an electrostatic stabilization of any negative charge in the γ -phosphate oxygens or the β - γ -bridging oxygen, regardless of whether the transition state may be of mainly associative or dissociative character, similar to the situation in Ras-RasGAP (Kamerlin et al, Quarterly Reviews of Biophysics, 2013). However, in order to account for this inaccuracy, we changed the passage in question, which now reads:

“Following the ribosome-induced activation of His^{cat} (which leaves the coordination shell for the M^+ ion intact (Fig. 3B) (Voorhees et al, 2010)), the M^+ ion is in a suitable position to neutralize negative charges of the transition state in the γ -phosphate as well as the designated leaving group (GDP).”

Referee #3:

Main points:

The arguments towards the presence of a monovalent ion in the active site of eIF2B are quite strong but could be strengthened significantly if the ion could be visualised by anomalous scattering. Neither Na⁺, K⁺, nor NH₄⁺ have anomalous scattering properties, but have the authors considered crystallisation in the presence of Rb⁺?

Reply:

The crystallization of eIF5B(517-858) in the presence of Rb⁺ has been attempted for exactly this purpose. However, although our GTPase experiments indicate that Rb⁺ is bound by eIF5B·GTP and moderately stimulates GTP hydrolysis, high quality crystals could not be obtained: The protein was purified (desalted) in RbCl containing buffer and then tested for crystallization in our fine screens containing Rb⁺ instead of Na⁺ or K⁺. Only after microseeding with seeds from crystals obtained either with Na⁺ or K⁺ we obtained spherulite-like aggregates, which, however, were not suited for seeding themselves. Similarly, macroseeding or soaking was not successful, as crystals either broke or dissolved in conditions containing Rb⁺. The likely reason for this sensibility of the crystals is that in both of our crystal forms (with Na⁺ or K⁺) switch 1 is directly involved in crystal contacts with symmetry related molecules. Thus, we assume that crystallization with Rb⁺ is hampered as the slightly increased ionic radius changes the position of switch 1 relative to the rest of the protein in a way that is incompatible with the crystal packing.

The argument on p. 8 that the lack of crystallisation of eIF5B(517-858) in the presence of GDPNP indicates that this modified nucleotide is not sufficient to induce the GTP-bound conformation is weak. Lack of crystallisation could be due to any number of things, including lower affinity towards GDPNP, which is indeed also the case as shown later. What happens if higher than 3 mM concentrations of GDPNP are used? Can crystallisation then be induced?

Reply:

We agree that the lack of crystallization in the presence of GDPNP cannot be taken as a direct evidence for its inability to stabilize the GTP-conformation in eIF5B; however, we took it as an indication, for which the ITC data subsequently provided more direct support.

At a concentration of 3 mM GDPNP eIF5B should be nearly fully saturated (at least 95%) with nucleotide even at the highest tested protein concentration and even with the lower affinity. However, in the light of our new results for the crystallization of eIF5B-D533 mutants which seemed to allow the crystallization if the equilibrium was shifted toward the GTP-form by providing crystallization nuclei (see answer to point #1 of referee #1), we performed various additional crystallization trials for eIF5B·GDPNP, which are reported in the revised manuscript. These included crystallization trials in the presence of up to 10 mM GDPNP (& MgCl₂) and microseeding. We were able to obtain spherulites similar to those observed for the D533R mutant. However, unlike for D533R, our trials so far did not yield diffracting crystals suited for structure determination.

Regarding the argument of Na⁺ versus H₂O on p. 14 in past structures: Please note that Na⁺ has 10 electrons exactly like H₂O, so a positive difference peak cannot be taken as evidence for the presence of the ion.

Reply:

We are aware of this fact, and the main argument for the identification of the Na⁺ ion of course lies in the coordination geometry. However, it is additionally evident from the comparison of the electron densities of the catalytic water molecule or those associated with the Mg²⁺ ion that lie in

the center of the active site with that of the supposed water molecule (here redefined as Na⁺) that the latter has the strongest electron density. This would be surprising if it really was a water molecule, considering the fact that this latter position lies in the periphery of the active site, significantly more solvent exposed than the other three positions. We therefore assumed that the coordination of the molecule/ion in this position must be qualitatively different from the one of the water molecules in the center of the active site to allow the stronger electron density. Moreover, despite the fact that Na⁺ and H₂O share an identical number of electrons, Na⁺ also contains three more protons in its nucleus than oxygen. This means that the actual charge density around the nucleus should be considerably higher in Na⁺ than in H₂O, where the electron cloud is more diffuse. Accordingly, we indeed observe a stronger density for the Na⁺ ions than for the three H₂O molecules in the active site in our high resolution structures of GTP-bound eIF5B, which is not the case for the water molecule next to Ala533 in the eIF5B-D533A mutant (Fig. E5A). Moreover, we observe positive electron density when these Na⁺-containing high resolution structures are refined with H₂O in the position of the Na⁺ ions, suggesting a similar origin for the positive density observed for aEF1A refined with water.

The stimulation of GTPase activity in Figure S5B lacks a control to show that the effect is not simply due to increased catalytic activity at higher ionic strength. Could a divalent cation be used as control? And in the same figure, why does Na⁺ not stimulate when you argue from the structure that it binds and organises switch I?

Reply:

There are various problems associated with the use of divalent cations as control: On the one hand, Mg²⁺ is critical for the GTPase activity in all G proteins and thus certainly has a concentration-dependent effect on the intrinsic GTPase in eIF5B and EF-Tu. Ca²⁺ on the other hand has a similar ionic radius and similar coordination distances as Na⁺ and could thus indeed replace the latter at high, non-physiological CaCl₂ concentrations. Thus, it would be difficult to draw useful conclusions from such experiments. Moreover, when we did test experiments, we observed that Ca²⁺ (as does Mg²⁺) at concentrations above 100 mM results in the precipitation of GTP as well as the proteins themselves. Instead, as an alternative control we performed the GTP hydrolysis experiments for EF-Tu as well as for eIF5B with their respective Asp-MC mutants. As the side chain of Asp-MC constitutes one of the key ligands for the M⁺ ion, the loss of M⁺-dependency in the Asp-MC to Ala mutants supports the idea that the M⁺ dependency observed for the wild-type protein is indeed due to a direct and not merely an allosteric effect of the M⁺ ion.

According to the GTPase experiments, Na⁺ does stimulate GTP hydrolysis, however, at a significantly lower rate than do K⁺ or NH₄⁺. Thus, Na⁺ ions seem to have a lower affinity for the M⁺ coordination sphere in aEF1A, possibly be due to the – compared to the corresponding region in eIF5B – higher rigidity in helix A' of switch 1. However, this lower affinity apparently is compensated in the case of the aEF1A/pelota structure by the Na⁺ concentrations under which the crystals had been obtained.

Why were two residues (859-860) removed from the protein for this paper compared to the previously reported structures? Does the longer construct not crystallise in the presence of the same nucleotides?

Reply:

In the previously reported structure of eIF5B(517-860) the last two residues (Leu859 and Gln860) as terminal residues were only weakly defined in the electron density of both molecules in the asymmetric unit, indicating high flexibility. By contrast, Asp858 seemed to form a salt bridge to Arg838, thereby apparently contributing to the stability of the C-terminal alpha-helix. We removed residues 859 and 860, hoping that less flexibility at the very C-terminus would support crystallizability. Longer constructs (that is, constructs containing residues 859 and 860) were not tested for crystallization. We therefore do not know whether crystallizability actually improved. However, the newly determined structure of the shortened construct is virtually identical to the previous one, indicating that its behavior under the same conditions did not change to a significant extent.

How was Hepes used for crystallisation titrated (p. 24)? If KOH was used as mentioned above for the purification, how can you be sure that Na⁺ is present in the crystal, as Hepes and NaOAc are both present at 0.1M?

Reply:

The Hepes used for crystallization was titrated with NaOH (this information is now given in the revised manuscript).

Further comments:

Some references should be given to the preferred coordination numbers of Na⁺ and K⁺. In the paper, it is taken for granted that penta-coordination (Na⁺) and hepta-coordination (K⁺) is the norm.

Reply:

The reference of Harding (Acta Cryst. D, 2002) was additionally given on pages 7 and 10 of the revised manuscript.

Please show the calculations of VDW distances (p. 8 and 11).

Reply:

The values of VDW radius for sulfur (1.8 Å) and the effective ionic radii for Na⁺ (1.0 Å) and K⁺ (1.46 Å) that had been used to calculate the theoretical coordination distance are now given in the revised manuscript, together with the corresponding references (Bondi, 1964; Shannon, 1976).

ASA, TC etc, there are several undefined abbreviations throughout the ms.

Reply:

Done.

In most of the figures Mg²⁺ is omitted. It would be useful to have the ion shown and labeled in those views where it would be visible.

Reply:

Done.

The first paragraph in the section starting on p. 13 is somewhat repetitive.

Reply:

We rewrote the paragraph, which now reads:

“Most known structures of trGTPases that were reported to be in the GTP-conformation do not contain GTP but GDPNP or GDPCP that contain either an NH or a CH₂ group in lieu of the β-γ-bridging oxygen. Both prevent the coordination of the M⁺ ion as observed in structures of known M⁺-dependent GTPases. We therefore searched the PDB for structures of trGTPases that were cocrystallized with GTP and found two structures...”

The description of the effect on EF-Tu on p. 19 ("specifically stabilizes a conformation of switch 1 in which helix A" is 'attracted' to the GTP molecule") is unclear. Please rephrase.

Reply:

The sentence was rephrased and now reads as follows:

“...indicates that the M^+ ion specifically stabilizes a conformation of switch 1 in which helix A” is drawn toward the GTP molecule, which seems necessary for stable TC formation (Fig. 4C). Hence, in the absence of the M^+ ion, the aa-tRNA itself has to overcome the entropic penalty to arrange switch 1 in the correct conformation that would be paid by the M^+ ion in the correctly assembled active site with GTP.”

On p. 21 of the discussion you state that the presence of the M^+ ion itself does not stimulate GTPase activity. But isn't that exactly what you observe in Figure S5?

Reply:

In this paragraph, we intended to anticipate the possible counterargument that the constitutive presence of the M^+ ion as catalytic element in GTP-bound trGTPases would necessarily result in very high intrinsic GTP hydrolysis rates that would result in repeated futile GTP turnover cycles in the free trGTPase, thereby abolishing the function of the trGTPase in ribosomal translation. However, this is not the case, because the M^+ ion acts on the second step of the hydrolysis reaction by stabilizing its transition state and not on the first step which includes the activation of the catalytic water. Thus, although M^+ ions indeed stimulate the intrinsic GTPase activity of trGTPases this activity remains low as long as the first step of the hydrolysis reaction, the activation of the hydrolytic water molecule is not accelerated by the ribosome-induced reorganization of the catalytic histidine into its activated conformation.

Figure 1C - it would be useful to show the GDP conformation for comparison.

Reply:

We deliberately left out the GDP-conformation in these images in order to keep them as simple as possible. In our opinion a third structure for comparison would only make sense, if it is presented in the identical manner as the GTP structures; however, in this particular case the GDP form (at least the relevant switch regions) would not be visible at all, as they point in the opposite direction away from the nucleotide binding pocket (in the GDP structure, Gly-MC lies about 30 Ang. from the GDP molecule). Thus, we would have to show considerably more of all three structures in order allow a comparison with the GDP form, which, however, would be a distraction from the main point: the position of the MC-loop in dependency of the bound nucleotide. Instead, we do show the GDP-conformation in Figure 5A.

Table I - please include CC1/2 as well for resolution cut-off.

Reply:

CC(1/2) values have been included in the revised manuscript (Tables 1 and E2).

Table I - The R and Rfree are suspiciously close - were the free reflections transferred correctly from the previous refinement or chosen anew? If the free reflections were not transferred, the

***R*free value will be biased and essentially useless. In this case, the refinement should be repeated with the correct free set.**

Reply:

The free reflections were transferred from previous rounds of refinement. The fact that they are apparently unusually close might be due to the quality of the data: the resolution cutoff was chosen relatively high at a CC(1/2) of 75, 82 and 91% and R-factors (Rsym) of ~50%.

Figure S2 - Please have all x-axes on the same scale to allow comparison of affinities.

Reply:

Unfortunately, we are not sure in what way this could be accomplished. Either we would have to broaden the lower panel of figure E2B to roughly twice its current width, which would result in the necessity to significantly reduce the overall size of all images, or we would have to compress figures E2A and B to an extent that would not allow a sufficient resolution of the inflection point or the individual data points. Additionally, one has to keep in mind that in either case the change in the x-axis for the isotherms (lower panel) would result in the change of the scale for the time course for the titration curve (upper panel), as well. Moreover, in our data presentation we simply followed the convention in previous ITC studies. Due to these considerations, we think that a change of the x-axes would have a negative impact on the clearness of figure E2, which induced us to leave it as it was in the original version.

With these modifications and our responses to the referees' comments, we hope that the revised version of this manuscript is now acceptable for publication in The EMBO Journal. We would like to take this opportunity to thank the three anonymous referees for their comments and valuable suggestions on our manuscript.

2nd Editorial Decision

30 July 2014

Thank you for submitting your revised manuscript to The EMBO Journal and my apologies for the slight delay in communicating our decision to you. The study has now been seen by two of the original referees (comments included below) and as you will see they both support publication of the revised manuscript (although ref#2 does express concern that the initial impact of M+ on catalysis no longer appears as strong as in the original version of the study). I have also consulted with the third referee as this person equally supports publication of your work in The EMBO Journal.

However, before we can officially accept your study for publication and transfer all relevant files for production, I would ask you to address the following editorial points in a final revised version of the manuscript:

-> Please make sure that you specifically list and deposit all PDB coordinates for the new structures presented in this paper.

-> Please restructure the discussion to remove the 'concluding remarks' section since I am afraid this does not fit the article format in EMBO J (see author guidelines on our website). In addition, I would encourage you to shorten the discussion by 15-20%, if possible, to more clearly bring out the main conclusion of your study.

-> Please also expand the Materials and Methods section of the article file to include further description of crystallization conditions and ITC measurements. In accordance with our guidelines,

all experiments central to the paper need to be included in full in the main manuscript and only a minor section of highly specialized descriptions can be moved to the supplemental materials section. In this respect, I would like to mention that we have stopped inferring strict size restrictions on the overall manuscript length.

-> As of Jan 1st 2014 every paper published in The EMBO Journal will include a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis will include a short standfirst - written by the handling editor - as well as 2-5 one sentence bullet points that summarise the paper and are provided by the authors. These bullet points should be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion of key acronyms and quantitative information. I would therefore ask you to include your suggestions for bullet points

-> In addition, I would encourage you to provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels. You are welcome to send such a synopsis image to me by email.

Thank you again for submitting your work to The EMBO Journal, I look forward to receiving this final version of the manuscript.

REFEREE REPORTS:

Referee #1:

The manuscript has been satisfactorily revised.

Referee #2:

The authors have made substantial improvements of their manuscript and I think it is now OK. As for the the AspMC mutation effect on the ribosome, I can accept that the authors are not allowed to reveal Rodnina's results, but it is rather unfortunate that we cannot get an answer to this question in this paper. Particularly so, since the new Table 3 indicates that the M+ effect is actually not huge on the intrinsic rate. In terms of transition state stabilization free energy, we are only talking about 1.2-1.3 kcal/mol here, so the entire story weakens a bit in my opinion.

2nd Revision - authors' response

01 August 2014

Response to the editorial points

-> All coordinates and structure factors have been deposited in the PDB and are now listed in the manuscript (page 25): PDB-IDs 4TMW, 4TMV, 4TMZ, 4TMT, 4TMX, and 4TN1.

<http://www.rcsb.org/pdb/search/structidSearch.do?structureId=4TMW>

<http://www.rcsb.org/pdb/search/structidSearch.do?structureId=4TMV>

<http://www.rcsb.org/pdb/search/structidSearch.do?structureId=4TMZ>

<http://www.rcsb.org/pdb/search/structidSearch.do?structureId=4TMT>

<http://www.rcsb.org/pdb/search/structidSearch.do?structureId=4TMX>

<http://www.rcsb.org/pdb/search/structidSearch.do?structureId=4TN1>

-> The Discussion part has been restructured:

1. We were able to shorten the overall discussion by nearly 15% to emphasize the main conclusions of the work.
2. The section 'Concluding remarks' has now been removed and replaced by a short paragraph entitled 'implications for the evolution of trGTPases' which does not contain the summarizing passages of the original 'Concluding remarks' but instead focuses entirely on the conceptual implications of our findings.
3. In order to shorten the discussion and improve the stringency of the argumentation, we fused the second and third sections of the original discussion into one section entitled 'Universality of M^+ -dependent conformational switching among trGTPases'

-> We expanded the Materials and Methods section of the main article. The main article now contains more detailed information about the conditions under which protein crystallization was performed as well as how the ITC and GTPase experiments were carried out.