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Activation of initiation factor 2 by ligands and mutations for rapid docking of ribosomal subunits

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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.)

1st Editorial Decision 04 October 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below. You will see that the referees are generally positive about your work and that they would support its ultimate publication in The EMBO Journal after appropriate revision. I would thus like to invite you to prepare a revised manuscript in which you need to address the issues raised by the referees in an adequate manner.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This paper analyzes mutants of IF2 to show that some mutants can promote subunit association even with GDP and without formylation or even charging of tRNA. The authors conclude from this that the binding of GTP in association with charged tRNA switches the factor to a form active in subunit association and mutations can promote this switching even when not all of the components normally required are in place.

The paper as it stands is difficult to read, so although we get the feeling that the mutants are different in some important way, it is not clear exactly what is happening and whether models other than the one they propose could also fit the data. Moreover, the authors use several parameters that are not really standard for kinetic measurements. For example:

1. Why use kc, which is a non-standard parameter that is dependent on the concentration instead of measuring the association rate properly and quoting that value?

2. What are the actual affinities of GTP and GDP for the wild-type and mutant IF2s? How does this affect the analysis?

3. There is a structure of an IF2 ortholog, so perhaps a picture of a homology model would be useful to explain the nature of the conformational changes they discuss, and also to visually map where the mutations are, especially relative to the GTP binding site.

4. Some of the curves are biphasic, but others markedly not so. In those cases, is the first step really rate limiting? How were the authors able to separate the first part of the reaction scheme (1) on page 5 from the second part, which is really what is being measured? What is the error involved? Some discussion of this would be appropriate.

I believe that clarifying these issues will make the manuscript both more convincing and more accessible to readers.

Referee #2 (Remarks to the Author):

The authors have measured the kinetics of 50S ribosomal subunit docking onto the 30S preinitiation complex by using stopped-flow light scattering methodology. They reach the surprising and highly significant conclusion that the rate of docking is mostly influenced by the proportion of 30S-bound IF2 in its active conformation, a condition additively determined by features of the initiator tRNA and by guanine nucleotides. Thus GTP and fMet-tRNA together cause a remarkable 24,000-fold shift in the equilibrium between inactive and active IF2 conformations. Another surprising conclusion is that GDP also activates IF2, although not nearly as strongly as GTP. The results provide insights into the general mechanism of G-protein functions and should be of broad interest to the readership.
The experiments are well conceived, carefully executed and appropriately interpreted. The manuscript is clearly written and is suitable for publication in its present form.

Referee #3 (Remarks to the Author):

G-proteins perform crucial functions in most cellular processes, including translation. However, their mechanisms of action are not well understood, even at a superficial qualitative level.

In this manuscript, the authors present a rare insight into the mechanisms of action and regulation of the bacterial G-protein translation factor IF2. They analyze their results in the framework of a simple thermodynamic model (the theory of conditional switching of G-proteins), which

quantitatively accounts for their experimental data.

This is the best quantitative description of the mechanism of action of a G-protein as a molecular switch I have seen so far. It is also complemented with clear description of the concept and discussion of the evolutionary driving forces that have led to fine-tuning of the response of IF2 to GTP, GDP and fMet-tRNAfMet. It is thus likely to significantly advance our understanding of G-proteins.

Comments:

1. Where discussing the effects of GTP, GDP and fMet-tRNAfMet on the equilibrium between the active and inactive state of IF2, it would be useful if the authors also discuss (at least in qualitative terms) the effect of IF2 binding to the 30S on the equilibrium, by referring to previously published differences in affinity for GTP and GDP between free and 30S-bound IF2.

2. Since the A-type IF2 mutants shift the equilibrium toward the active state of IF2, it is possible that upon GTP hydrolysis, 70S-bound A-type IF2:GDP could have a greater propensity for the "active" GTP-like state with higher affinity of the 70S and show slower release rate (unless of course in the context of the 70S, the equilibrium is shifted so dramatically toward the "inactive" conformation that even the A-type mutants are almost exclusively in the "inactive" state).
Please comment on this possibility.

3. The authors show that in the case of IF2, GDP binding shifts the equilibrium toward the active state, albeit to a far smaller degree that GTP binding. It seems to me that this need not be true for all other G proteins (that GDP binding could have the opposite effect or no effect in other G proteins). It would be good to state this more clearly in the discusion (if the authors share this view, of course), in order to avoid any possible confusion in the field.

4. On p19. line 12, "domain IV" probably should be "domain II and the G domain", instead.

5. It is not immediately obvious to me why CTP is used in the experiment for comparison of GDP vs. no nucleotide (p 14 and Table II). Please explain in more detail.

1st Revision - authors' response 04 November 2010

Referee #1:

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General:
The paper as it stands is difficult to read, so although we get the feeling that the mutants are different in some important way, it is not clear exactly what is happening and whether models other than the one they propose could also fit the data.

Our response: We have tried to improve the readability of the manuscript. The two-state model of the 30S pre-initiation complex (PIC) implemented by our kinetic Scheme (1) provides the simplest explanation of the whole experimental data set in Tables I and II. The model is a special case of G-protein activation as theoretically defined (Hauryliuk et al., 2008a) and used in other contexts (Hauryliuk et al., 2008b; Hauryliuk et al., 2009). The revised version of the manuscript contains a validation of the model by comparing its predictions of equilibrium constants for GDP and GTP binding to IF2 in complex with the 30S subunit and tRNA ligands with existing literature data (see below).

Comment 1: *Why use kc, which is a non-standard parameter that is dependent on the concentration instead of measuring the association rate properly and quoting that value?*

Our response: The kinetics of subunit association is generally bi-phasic, involving the transition from a docking-inactive to a docking-active state of 30S-bound IF2. Hence, the kinetics cannot be described in terms of a simple association rate constant. The rate constant k_c was introduced to illustrate the kinetics in a simplistic manner for all cases described in the manuscript. As clarified in Materials and Methods k_c relates in a simple way to the association rate constant k_a (namely, $k_a=k_c/[50S]$) in the special case when the equilibrium fraction of docking-active 30S subunits is close to one, as now emphasized in the manuscript. However, a complete description of the docking kinetics requires the use of a model as in Scheme 1. The use of k_c is a compromise to facilitate reading of the paper, but over-interpretation of k_c in terms of a single association rate constant would be misleading and, hence, we would like to keep the k_c-notation.

Comment 2: *What are the actual affinities of GTP and GDP for the wild-type and mutant IF2s? How does this affect the analysis?*

Our response: Affinities of GTP and GDP to IF2 are now thoroughly discussed in the revised version of the manuscript in the new section "Effect of IF2 activation on its guanine nucleotide binding affinity" in the Discussion. The available data show that IF2 is always saturated by guanine nucleotides which makes the absolute affinities of guanine nucleotides redundant and justifies the use of Eq. 2 in the manuscript in our analysis. Furthermore, we compare our predictions of guanine nucleotide affinities with already existing literature data obtained under similar conditions and discuss how our model can be further validated by comparing the predicted affinities with the yet unknown affinities for mutant IF2s.

Comment 3: *There is a structure of an IF2 ortholog, so perhaps a picture of a homology model would be useful to explain the nature of the conformational changes they discuss, and also to visually map where the mutations are, especially relative to the GTP binding site.*

Our response: Such structural information (Zorzet et al., 2010) is now provided as a supplementary figure of the revised manuscript.

Comment 4: *Some of the curves are biphasic, but others markedly not so. In those cases, is the first step really rate limiting? How were the authors able to separate the first part of the reaction scheme (1) on page 5 from the second part, which is really what is being measured? What is the error involved? Some discussion of this would be appropriate.*

Our response: We now discuss the model more explicitly in the text stressing the point that 50S subunits are added to an equilibrated mixture of active and inactive 30S states. In cases when the scattering curves are close to mono-phasic, as for the A-type IF2 mutants with GTP and fMet-tRNA$_i$ on the 30S subunit, the equilibrium is so much shifted towards the active state that the first step is effectively by-passed and cannot be rate limiting. In other cases, like when the 30S PIC contains WT IF2 and deacylated tRNA$_i$, the major fraction of the 30S PICs is in the inactive state and then the first step in Scheme (1) is rate limiting. The light scattering curve contains information about the rates of both steps of Scheme (1), not just about the second step. It also contains information about the initial conditions (the relative amounts of $30S_A$ and $30S_I$). It is quite analogous to a standard two-step reaction, where the kinetics of final product accumulation contains information about the rates of both steps. Formally speaking, what we do is standard procedure: we just solve numerically the differential equations for Scheme (1) for particular values of k_1, q_1 and k_a with initial conditions (the relative amounts of $30S_A$ and $30S_I$) defined by the values of k_1 and q_1. Then we use the Marquardt algorithm to find k_1, q_1 and k_a producing the best fit of the experimental scattering curve. The algorithm also gives errors and cross-correlations of the parameters. Tables S1 and S2 in the Supplementary Information contain the values of these optimal parameters and the errors involved. We have now added a clarification of the procedure in the supplementary information.

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The authors have measured the kinetics of 50S ribosomal subunit docking onto the 30S preinitiation complex by using stopped-flow light scattering methodology. They reach the surprising and highly significant conclusion that the rate of docking is mostly influenced by the proportion of 30S-bound IF2 in its active conformation, a condition additively determined by features of the initiator tRNA and by guanine nucleotides. Thus GTP and fMet-tRNA together cause a remarkable 24,000-fold shift in the equilibrium between inactive and active IF2 conformations. Another surprising conclusion is that GDP also activates IF2, although not nearly as strongly as GTP. The results provide insights into the general mechanism of G-protein functions and should be of broad interest to the readership.

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This is the best quantitative description of the mechanism of action of a G-protein as a molecular switch I have seen so far. It is also complemented with clear description of the concept and discussion of the evolutionary driving forces that have led to fine-tuning of the response of IF2 to GTP, GDP and fMet-tRNAfMet. It is thus likely to significantly advance our understanding of G-proteins.

Comment 1. *Where discussing the effects of GTP, GDP and fMet-tRNAfMet on the equilibrium between the active and inactive state of IF2, it would be useful if the authors also discuss (at least in qualitative terms) the effect of IF2 binding to the 30S on the equilibrium, by referring to previously published differences in affinity for GTP and GDP between free and 30S-bound IF2.*

Our response: The revised manuscript contains an extensive discussion of these affinity issues (see section “Effect of IF2 activation on its guanine nucleotide binding affinity” in the Discussion and our response to referee 1).

Comment 2. *Since the A-type IF2 mutants shift the equilibrium toward the active state of IF2, it is possible that upon GTP hydrolysis, 70S-bound A-type IF2:GDP could have a greater propensity for the "active" GTP-like state with higher affinity of the 70S and show slower release rate (unless of course in the context of the 70S, the equilibrium is shifted so dramatically toward the "inactive" conformation that even the A-type mutants are almost exclusively in the "inactive" state).*
Please comment on this possibility.

Our response: We comment in the revised manuscript in the last section of the Discussion that “GDP-activation of A-type IF2 may preserve its active conformation in the 70S complex after GTP-hydrolysis, thereby delaying dissociation of IF2 and subsequent peptide bond formation.” We further speculate that this scenario could provide yet another fitness cost of the A-type mutations.

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Comment 3. *The authors show that in the case of IF2, GDP binding shifts the equilibrium toward the active state, albeit to a far smaller degree that GTP binding. It seems to me that this need not be true for all other G proteins (that GDP binding could have the opposite effect or no effect in other G proteins). It would be good to state this more clearly in the discussion (if the authors share this view, of course), in order to avoid any possible confusion in the field.*

Our response: We fully agree with the referee that the GDP-activation could be idiosyncratic to IF2 and have now stated this explicitly.

Comment 4. *On p19. line 12, "domain IV" probably should be "domain II and the G domain", instead.*

Our response: This has been corrected.

Comment 5. *It is not immediately obvious to me why CTP is used in the experiment for comparison of GDP vs. no nucleotide (p 14 and Table II). Please explain in more detail.*

Our response: The rationale here was to adjust the free Mg^{2+} concentration to that in the GTP case by adding another, Mg^{2+} chelating, nucleotide. We have now modified Table II to avoid misunderstanding.

References

Hauryliuk, V., Hansson, S. and Ehrenberg, M. (2008a) Co-factor dependent conformational switching of GTPases. *Biophys J.*

Hauryliuk, V., Mitkevich, V.A., Draycheva, A., Tankov, S., Shyp, V., Ermakov, A., Kulikova, A.A., Makarov, A.A. and Ehrenberg, M. (2009) Thermodynamics of GTP and GDP binding to bacterial initiation factor 2 suggests two types of structural transitions. *J Mol Biol*, 394, 621-626.

Hauryliuk, V., Mitkevich, V.A., Eliseeva, N.A., Petrushanko, I.Y., Ehrenberg, M. and Makarov, A.A. (2008b) The pretranslocation ribosome is targeted by GTP-bound EF-G in partially activated form. *Proc Natl Acad Sci U S A*, 105, 15678-15683.

Zorzet, A., Pavlov, M.Y., Nilsson, A.I., Ehrenberg, M. and Andersson, D.I. (2010) Error-prone initiation factor 2 mutations reduce the fitness cost of antibiotic resistance. *Mol Microbiol*, 75, 1299-1313.

2nd Editorial Decision 16 November 2010

Thank you for sending us your revised manuscript. Our original referee 3 has now seen it again, and you will be pleased to learn that in his/her view you have addressed all criticisms in a satisfactory manner. The paper will now be publishable in The EMBO Journal and you will receive a formal acceptance letter shortly.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #3 (Remarks to the Author):

The authors have addressed all questions and concerns from the initial submission.

