Substrate binds in the S1 site of the F253A mutant of LeuT, a neurotransmitter sodium symporter homolog

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Review timeline:

Submission date: 09 April 2012
Editorial Decision: 02 May 2012
Correspondence: 09 May 2012
Revision received: 15 June 2012
Editorial Decision: 29 June 2012
Revision received: 03 July 2012
Accepted: 11 July 2012

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 02 May 2012

Thank you for the submission of your research manuscript to EMBO reports. It has been sent to three referees, and so far we have received reports from two of them, which I copy below. As both referees feel that the manuscript is interesting and recommend that you should be given a chance to revise it, I would like to ask you to begin revising your manuscript according to the referees' comments. Please note that this is a preliminary decision made in the interest of time, and that it is subject to change should the third referee offer very strong and convincing reasons for this.

In the current reports, referee 2 points out that the binding order of CMI and leucine to LeuT should be tested. The time course for leucine binding to both wild type and mutant LeuT should be shown under conditions when leucine is added first as well as when CMI is added first. The referee also indicates that it needs to be explained why leucine would not fit to the electron density observed near the S1 site, and that it needs to be examined whether selenomethionine could function as a substrate for LeuT. I also noticed that the figure legend for figure 1 says n=2 and at the same time experiments were repeated three times. If n=2 then no error bars can be shown. We strongly recommend that experiments should be repeated at least three times and that instead of a representative experiment, averages of at least three times repeated experiments should be depicted with error bars.
Given the present referee comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO reports

REFEREE REPORTS:

Referee #1:

Summary:
1. This manuscript reports a key finding accurately described in the title: "Substrate binds in the S1 site of the F253A mutant of LeuT, a neurotransmitter sodium symporter homolog".
2. This work is significant and further confirms past findings by the same authors and disproves conflicting models by another group.
3. It is of general interest to the molecular biology community since the basic question of the number and location of the substrate binding site in the NSS family is of importance.
4. The single major finding is robustly documented.

The manuscript, "Substrate binds in the S1 site of the F253A mutant of LeuT, a neurotransmitter sodium symporter homolog" by Wang and Gouaux addresses an open question regarding the number of high affinity substrate sites in LeuT. The Introduction describes the controversy in a fair manner and focuses the readers. The striking result of this manuscript is the x-ray structures of the F253A mutant with two independent ligands bind at the S1 site. Javitch and colleagues used this same mutant and surmised that LeuT substrates cannot bind at the S1 site. They accumulated much data, obtained by a variety of in solution methodologies, and attributed binding to the S2 site. This and other experimental differences for saturation binding assays that differ in some aspects between the groups are also discussed. The use of omit maps in many of the illustrations strengthens the claims made by the authors and are of a great importance for this publication.

The few minor revisions recommended are:
1. In the abstract S1 and S2 are mentioned without clarifying and it could be confusing for an uninformed reader.
2. More details are required in the method section. Maybe some technical data for PSA. It is only mentioned under the figure legend and under the methods section. How were the fits calculated? Which program was used for the calculation of the omit maps?

Referee #2:

This manuscript by Wang and Gouaux presents interesting new data about LeuT and its ability to bind substrates. It addresses an important aspect of an active controversy that has been pre-occupying those interested in neurotransmitter transporter structure and function. The work concerns a LeuT mutant, F253A, previously proposed to bind leucine at only one of two sites present in wild type. This S2 site was proposed to overlap with the antidepressant binding site previously identified.

The manuscript describes binding experiments interpreted to demonstrate a similar extent of leucine binding to wild type and mutant transporters, and similar insensitivity to CMI (an antidepressant)
displacement. Although these experiments provide data supporting the conclusion that CMI does not displace leucine, data with the F253A mutant is confusing and these experiments need to be developed further to be convincing.

The structures described in this manuscript also support the proposal that a single leucine binds to either wild type or mutant LeuT. However, some additional information needs to be provided for these structures to strongly argue for the proposal the authors put forward.

The binding results are puzzling. Based on previous results (Singh et al, Nature 2007) showing the relative locations of bound CMI and leucine, one would predict that leucine binding kinetics, shown in Fig. 1C and D, would be dramatically affected by the presence of bound CMI. As expected, CMI slowed down binding of leucine to wild type LeuT, although binding reached control levels after 15 hours. However in the F253A mutant, CMI appears to prevent binding for at least 20 hours. Part of the problem is the apparent difference in leucine affinity shown in Figure 1B. Especially if CMI affects binding kinetics, as it does for dissociation kinetics, there could be an anomalously low extent of leucine binding if CMI is added before or simultaneously with leucine. The decrease in Bmax is also difficult to swallow. If leucine and CMI can both bind simultaneously to LeuT F253A, the Bmax for leucine should be unchanged, although Kd might be affected. Is there some reason that LeuT F253A was not crystallized with both leucine and CMI bound? This would address the speculation regarding the origin of the apparent changes in leucine Kd and Bmax in this mutant.

A simple way to dissociate the CMI effect on binding kinetics from its effect on equilibrium binding would be to allow leucine to bind in the absence of CMI, and then to add CMI after equilibrium is achieved. If CMI affects only binding rate, there should be no effect on bound leucine, as expected from the structures with leucine and CMI bound. If there is a true effect on equilibrium, CMI addition should displace some of the bound leucine by increasing its dissociation constant. Binding order should be explicitly tested. Show the difference in leucine binding time course for both wild type and mutant when leucine is added first, allowed to come to equilibrium, and then CMI is added to see if any leucine is displaced. Additionally, CMI should be added first and allowed to come to equilibrium, and then the time course of leucine binding measured to the LeuT-CMI complex.

As an alternative, the analysis carried out for CMI in Singh et al (Nature, 2007) demonstrating noncompetitive inhibition in wild type LeuT would also reveal whether the inhibition is strictly competitive, although this might be impossible if F253A does not transport.

The crystallography would seem to be more straightforward and convincing, and it is in one sense. The structures show unequivocally that leucine and selenomethionine are bound at the central substrate site (S1) and that no additional selenium is bound in the region proposed for S2. However, there is the pesky sausage-shaped electron density near the S1 site but not quite in the S2 site in the leucine structure of F253A. I would like to believe the authors’ assertions that this is not another leucine molecule but rather something else. However, the case could be made better than it is in the current version. In particular, the authors should explain what they mean by not obtaining a satisfactory fit of leucine to the density. What criteria were used?

The selenomethionine structure more clearly shows binding of a single amino acid molecule. However, there is one issue that is not examined, and that is the ability of selenomethionine to function as a substrate. The original proposal by Javitch and coworkers was that substrate binding to the S2 site was required for release of S1-bound substrate to the cytoplasm. If selenomethionine is transported without binding to S2, it would provide an additional argument against that hypothesis. In either case, the manuscript should state whether selenomethionine has been tested as a substrate and if so, is it transported.

I think this is a typo, but the description at the top of page 6 ends with a reference to Supplementary Fig S3 when I think it should refer to Table II. This was very confusing.

In the first paragraph of the discussion, I suggest the following re-wording "...CMI affects only the kinetics of substrate..."

Details of the binding experiments, particularly the order of ligand addition and the times of incubation in Fig 1A&B need to be added.
We have finally received the third referee report on your manuscript that I paste below. Referee 3, while acknowledging the quality of the data, points out that the manuscript does not explain why the results reported in this manuscript are in direct contrast to the previous publication. For this reason, the referee does not support publication of the study in our journal. However, upon further consultation with referee 2, we agree with his/her opinion that a revised manuscript (along the lines suggested in my last email) addressing all raised concerns may to some extend elucidate the discrepancy. I therefore suggest that you continue to revise your study as suggested, and that we will decide on the suitability of your manuscript for our journal based on the referee reports from the second round of review.

Yours sincerely,

Editor
EMBO reports

REFEREE REPORT:

Referee #3:
The manuscript by Wang and Gouaux describes new data that contributes to a recent debate regarding the number of substrate binding sites required for transmembrane transport by proteins in the sodium-coupled neurotransmitter transporter family. This controversy relates to the possible role of a second (so-called) S2 site in the extracellular pathway of the amino acid transporter LeuT.

The present manuscript provides additional support, in particular from X-ray crystallographic structures of a LeuT mutant, for a mechanism involving only a single site. The crystallographic evidence is strong, and contradicts previous evidence that the LeuT mutant (at least in the available conformation) binds substrate in the S2 site. However, the data is focused on a single mutant, and does not address other data on other mutants. This lack of any explanation (convincing or otherwise) for the discrepancies between the two groups' work is a significant weakness that lowers the impact of the manuscript in my opinion. Moreover, new discrepancies are revealed in the current manuscript, in particular in the effect of an inhibitor compound CMI on the F253A mutant, which the authors admit are in direct contrast to previously-published data, but cannot explain why. Are there differences in conditions, such as the sodium concentration?

In summary, although I feel that the data is robust and informative, its reach is unfortunately somewhat limited and would therefore be more suited to a specialized journal.

Minor comments:
- page 3 "deemed the S1 site", would perhaps be better written as "termed"
- on page 4, it would be useful to include a brief description of the evidence than CMI binds in the extracellular vestibule only.
- page 5; a comment on the results in Figure 1D for F253A would be useful, as would a more descriptive explanation of the kinetic effects.
- page 7; "does not profoundly disrupt"; I would say "does not abolish", since Figure 1D shows a significant effect.
- page 7: "rates constants" -> rate constants.

Additional comments from Referee 2 after cross-sending the referee reports:
I would agree with Reviewer 3 that the work at present is incomplete and leaves an
My feeling is that if the binding data were improved, in particular to specifically test the order of ligand addition, then the story could be more logical and there could be a rational explanation for why the two labs get different results. I suspect that CMI dissociates slower or binds tighter to the mutant relative to wild type.

My recommendation would be to ask for a revised version with new binding experiments that explain all the phenomena presented in Figure 1 and provide an explanation for the difference between the Javitch and Gouaux labs binding results.

Referee #1:
1. **Comment:** In the abstract S1 and S2 are mentioned without clarifying and it could be confusing for an uninformed reader.
   
   **Reply:** We have defined the S1 and S2 sites in the abstract.

2. **Comment:** More details are required in the method section. Maybe some technical data for SPA. It is only mentioned under the figure legend and under the methods section. How were the fits calculated? Which program was used for the calculation of the omit maps?
   
   **Reply:** We have provided the requested experimental details in the Methods section of the revised manuscript.

Referee #2:
1. **Comment:** However in the F253A mutant, CMI appears to prevent binding for at least 20 hours. Part of the problem is the apparent difference in leucine affinity shown in Figure 1B. Especially if CMI affects binding kinetics, as it does for dissociation kinetics, there could be an anomalously low extent of leucine binding if CMI is added before or simultaneously with leucine.
   
   **Reply:** We appreciate this comment yet we believe that the binding reaction in the F253A mutant actually comes to equilibrium faster than that observed for the WT transporter, likely because the affinity of the mutant transporter for leucine is substantially diminished and the off-rate of leucine is increased. The low extent of leucine binding to the mutant transporter in C12M is in part due to the fact we used subsaturating concentrations of leucine and also because CMI tends to reduce the concentration of LeuT in solution, based on our FSEC experiments (see Fig S3). Thus we suggest there are multiple factors that conspire to give the impression that there is an anomalously low extent of leucine binding to the mutant.

   Most importantly, in our revised manuscript, we show that the detergent MNG-3 better stabilizes the apo form of the F253A mutant and that inclusion of CMI at any stage of the binding reaction does not substantially decrease the binding of leucine, as we describe in more detail below.

2. **Comment:** The decrease in $B_{\text{max}}$ is also difficult to swallow. If leucine and CMI can both bind simultaneously to LeuT F253A, the $B_{\text{max}}$ for leucine should be unchanged, although $K_d$ might be affected.
**Reply:** We appreciate this comment and have gone back and carried out binding assays with the new and more stabilizing detergent, MNG-3 (Chae et al, Nature Methods, 2010, 7, 1003-1008). Using WT LeuT and the F253A mutant solubilized in MNG-3 we see little decrease in B$_{\text{max}}$ upon inclusion of CMI in the binding assay (see Fig 1 and Fig S2). We go on to show that incubation of LeuT with CMI leads to a decrease in protein concentration, as estimated by size-exclusion chromatography (see Fig S3). Thus we conclude that CMI tends to reduce the solubility of LeuT and this phenomenon provides one possible explanation for why the Javitch group observes a dramatic reduction of $^3$H leucine binding to the F253A mutant in the presence of CMI. At present we don’t know the mechanism by which CMI leads to a decrease in the concentration of LeuT in solution. We emphasize that under all conditions explored in our binding assays, we observe robust binding of $^3$H leucine to the F253A mutant of LeuT.

3. **Comment:** Is there some reason that LeuT F253A was not crystallized with both leucine and CMI bound? This would address the speculation regarding the origin of the apparent changes in leucine K$_d$ and B$_{\text{max}}$ in this mutant.

**Reply:** We appreciate this suggestion and have carried out multiple rounds of crystallization and X-ray diffraction and have not yet defined conditions that yield well diffracting crystals. We emphasize that the focus of the present work is to examine the binding of leucine to the F253A mutant, by both direct binding measurements and X-ray crystal analysis, and that in-depth characterization of the complex with CMI is beyond the scope of the present work.

4. **Comment:** A simple way to dissociate the CMI effect on binding kinetics from its effect on equilibrium binding would be to allow leucine to bind in the absence of CMI, and then to add CMI after equilibrium is achieved. If CMI affects only binding rate, there should be no effect on bound leucine, as expected from the structures with leucine and CMI bound. If there is a true effect on equilibrium, CMI addition should displace some of the bound leucine by increasing its dissociation constant. Binding order should be explicitly tested. Show the difference in leucine binding time course for both wild type and mutant when leucine is added first, allowed to come to equilibrium, and then CMI is added to see if any leucine is displaced. Additionally, CMI should be added first and allowed to come to equilibrium, and then the time course of leucine binding measured to the LeuT-CMI complex.

**Reply:** We appreciate these insightful suggestions and carried out additional experiments (see Fig 1 and Fig S1), focusing first on WT LeuT and the F253A mutant prepared in the detergent MNG-3. Under these conditions, we see little effect of the order of addition of CMI on $^3$H leucine binding. However, when we use C$_{12}$M and preincubate the protein with CMI, we do see a decrease in $^3$H leucine binding by 20%, and as described above we believe that this is due, at least in part, to the experimental fact that CMI tends to reduce the concentration of LeuT in solution (see Fig S3).

We also want to note that the explanation for the lower counts observed upon preincubation of the F253A mutant with CMI in C$_{12}$M (see Fig. S1, panel D) is not due to the binding reaction not being at equilibrium, but rather there are fewer sites occupied by $^3$H leucine. This is likely attributed
to two factors: firstly, 100 nM $^3$H leucine was used in the reaction, a concentration not at the top of dose/response curve; secondly, CMI tends to reduce the concentration of LeuT in solution, as we show in Fig.S3. Indeed, because the F253A mutant has a reduced affinity for leucine, we suggest that the off-rate of substrate is faster than in the WT transporter and thus the binding reaction comes to equilibrium faster than that observed for the WT transporter.

5. Comment: As an alternative, the analysis carried out for CMI in Singh et al (Nature, 2007) demonstrating noncompetitive inhibition in wild type LeuT would also reveal whether the inhibition is strictly competitive, although this might be impossible if F253A does not transport. 
Reply: Unfortunately the LeuT F253A mutant is not active in substrate transport (Zhao, et al, Nature 474, 109–113, 2011) and thus we cannot perform the suggested steady-state kinetic experiments.

6. Comment: The crystallography would seem to be more straightforward and convincing, and it is in one sense. The structures show unequivocally that leucine and selenomethionine are bound at the central substrate site (S1) and that no additional selenium is bound in the region proposed for S2. However, there is the pesky sausage-shaped electron density near the S1 site but not quite in the S2 site in the leucine structure of F253A. I would like to believe the authors’ assertions that this is not another leucine molecule but rather something else. However, the case could be made better than it is in the current version. In particular, the authors should explain what they mean by not obtaining a satisfactory fit of leucine to the density. What criteria were used? 
Reply: We have expended substantial effort in modeling the aforementioned electron density, examining multiple, different orientations of a leucine molecule in the density. No orientations of leucine in the density yielded satisfactory results and, after refinement, all orientations gave rise to difference electron density maps with strong residual density, indicating that a leucine molecule provides a poor fit to electron density (see Fig S7 for details).

7. Comment: The selenomethionine structure more clearly shows binding of a single amino acid molecule. However, there is one issue that is not examined, and that is the ability of selenomethionine to function as a substrate. The original proposal by Javitch and coworkers was that substrate binding to the S2 site was required for release of S1-bound substrate to the cytoplasm. If selenomethionine is transported without binding to S2, it would provide an additional argument against that hypothesis. In either case, the manuscript should state whether selenomethionine has been tested as a substrate and if so, is it transported.
Reply: We surely appreciate this comment. Unfortunately radio-labeled SeMet is not available and custom synthesis would cost approximately $10K, a cost that we cannot justify given the explanations provided below. As we point out in our previous paper (Wang H, et al Nat Struct Mol Biol. 2012, 19: 212-219), SeMet inhibits $^3$H Ala uptake by LeuT to the same extent as Met. We further note that in a previous publication from this lab on LeuT we showed, in detail, that Met is a substrate for LeuT (Singh SK, et al. Science. 2008, 322, 655-661) and we also know that SeMet is
an isosteric analog of Met and can replace Met in a broad range of contexts (see Hendrickson WA et al. EMBO J. 1990, 9, 1665-1672). Taken together, we suggest that the overwhelming weight of data is consistent with the conclusion that SeMet is likely a substrate for LeuT and thus, like Leu, should label the S2 site.

8. Comment: I think this is a typo, but the description at the top of page 6 ends with a reference to Supplementary Fig S3 when I think it should refer to Table II. This was very confusing.
Reply: We refer to Table II as suggested.

9. Comment: In the first paragraph of the discussion, I suggest the following re-wording "...CMI affects only the kinetics of substrate..."
Reply: We have changed the wording as suggested.

10. Comment: Details of the binding experiments, particularly the order of ligand addition and the times of incubation in Fig 1A&B need to be added.
Reply: We have included these experimental details in the methods section of the revised manuscript.

Referee #3:
1. Comment: However, the data is focused on a single mutant, and does not address other data on other mutants. This lack of any explanation (convincing or otherwise) for the discrepancies between the two groups' work is a significant weakness that lowers the impact of the manuscript in my opinion.
Reply: We have focused on the F253A mutant using both binding assays and crystallographic analysis because work from the Javitch lab suggested that substrate only bound to the S2 site of the mutant and that CMI entirely ablated binding of substrate to the S2 site. This mutant, therefore, provided a clean and direct test of the S2 site model for substrate binding to LeuT. Moreover, in a previous paper from our lab (Piscitelli CL et al Nature, 2010, 468, 1129–1132), other mutants such as Y108F, L400C and L400A have been examined and provide further evidence that this is only a single high affinity substrate binding site in LeuT. We believe that further examination of these mutants would not be a productive use of time or resources.

2. Comment: Moreover, new discrepancies are revealed in the current manuscript, in particular in the effect of an inhibitor compound CMI on the F253A mutant, which the authors admit are in direct contrast to previously-published data, but cannot explain why. Are there differences in conditions, such as the sodium concentration?
Reply: An important element of the present work is to carefully examine the effect of CMI on the F253A mutant and we went to great pains to determine why our results differ from those obtained in the Javitch lab. Thus, the conditions used in the binding assays are the same. However, because we want to understand why our results differ from those of the Javitch lab, we examined a new
detergent, MNG-3, that better stabilizes LeuT to loss of activity, and we also showed that incubation of LeuT (WT or mutant) with CMI results in a loss of LeuT from solution. Thus, in the revised manuscript, we provide multiple pieces of strong evidence from completely different experimental techniques supporting the conclusion that substrate binds in the S1 site of the F253A mutant and that CMI does not interfere with substrate binding to the F253A mutant. Furthermore, we provide plausible explanations for why the Javitch lab does not see binding of $^3$H leucine in the presence of CMI.

3. **Comment**: page 3 "deemed the S1 site", would perhaps be better written as "termed"

**Reply**: Thanks, we changed the wording accordingly.

4. **Comment**: on page 4, it would be useful to include a brief description of the evidence that CMI binds in the extracellular vestibule only.

**Reply**: We have elaborated on this point as suggested.

5. **Comment**: page 5; a comment on the results in Figure 1D for F253A would be useful, as would a more descriptive explanation of the kinetic effects.

**Reply**: Please see our replies to the comments of referee 2. In addition, we have augmented our comments on Fig 1D in the revised version of the manuscript.

6. **Comment**: page 7; "does not profoundly disrupt"; I would say "does not abolish", since Figure 1D shows a significant effect.

**Reply**: We have altered our description of the effects of CMI on substrate binding accordingly.

7. **Comment**: page 7: "rates constants" -> rate constants.

**Reply**: Change made.

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2nd Editorial Decision 29 June 2012

Thank you for the submission of your revised manuscript to our offices. Since my colleague Esther Schnapp who initially handled your manuscript is currently out of the office I am writing to you to avoid unnecessary delays. We have now received the enclosed report from the referee that was asked to assess it. The reviewer still has minor suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

Please upload the final version of the manuscript via our website when it is ready.

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

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORT:

Referee #2:

The addition of a more thorough investigation of binding adds a lot to this work. My comments at this point are limited to minor wording issues.

In the introduction, "ortholog" is misused. It refers to proteins serving the same function in different species. "Homolog" would be suitable here.

It's curious that CMI increased the KD for leucine in MNG-3 but not DDM. Almost as if the KD in DDM was anomalously low in the absence of CMI. The data is presented in the discussion, but not discussed. There may not be any data that address this issue, but it is unexpected and an attempt to explain it should be made, especially since earlier structures gave no indication of how CMI would decrease leucine affinity. There is no overlap between the sites and one might expect CMI to actually increase leucine affinity by increasing its occlusion (dehydrating the R30-D404 salt bridge).

Page 6, end of first complete paragraph: I suggest making this statement more specific by adding "equilibrium": "...the aforementioned equilibrium binding assays".

In the first paragraph of the discussion, it would be appropriate to mention that the CMI effect on the leucine binding time course is likely to explain the apparently non-competitive binding reported by Wang and Reith.

Page 9: The conclusion "that MNG-3 is a superior detergent" has more to do with the loss of protein in DDM than the difference in leucine binding affinity that precedes it in the discussion. Perhaps "This last result suggests that...MNG-3 is a superior detergent" rather than "These results".

The 30% decrease in LeuT F253A concentration in DDM and CMI is unlikely to completely ablate leucine binding as reported by the Javitch lab. The suggestion that this alone could account for the loss of binding seems strained. It might be appropriate at least to add that the decreased affinity for leucine in the presence of CMI may have also contributed.

Last paragraph of discussion: "Taken together"

2nd Revision - authors' response 03 July 2012

1. **Comment:** In the introduction, "ortholog" is misused. It refers to proteins serving the same function in different species. "Homolog" would be suitable here.

**Reply:** The suggested change has been made.

2. **Comment:** It's curious that CMI increased the KD for leucine in MNG-3 but not DDM. Almost as if the KD in DDM was anomalously low in the absence of CMI. The data is presented in the discussion, but not discussed. There may not be any data that address this issue, but it is unexpected and an attempt to explain it should be made, especially since earlier structures gave no indication of how CMI would decrease leucine affinity. There is no overlap between the sites and one might expect CMI to actually increase leucine affinity by increasing its occlusion (dehydrating the R30-D404 salt bridge).

**Reply:** Actually, for the F253A mutant, our results suggest that CMI increases the KD for leucine in DDM but not MNG-3. Without structural data on the leucine/CMI complex for the F253A mutant it is difficult to reliably speculate on explanations underpinning the effect of CMI on leucine Kd.
3. **Comment:** Page 6, end of first complete paragraph: I suggest making this statement more specific by adding "equilibrium". "...the aforementioned equilibrium binding assays".

**Reply:** The change in wording has been made.

4. **Comment:** In the first paragraph of the discussion, it would be appropriate to mention that the CMI effect on the leucine binding time course is likely to explain the apparently non-competitive binding reported by Wang and Reith.

**Reply:** We have made the suggested change.

5. **Comment:** Page 9: The conclusion "that MNG-3 is a superior detergent" has more to do with the loss of protein in DDM than the difference in leucine binding affinity that precedes it in the discussion. Perhaps "This last result suggests that...MNG-3 is a superior detergent" rather than "These results".

**Reply:** The suggested change has been made.

6. **Comment:** The 30% decrease in LeuT F253A concentration in DDM and CMI is unlikely to completely ablate leucine binding as reported by the Javitch lab. The suggestion that this alone could account for the loss of binding seems strained. It might be appropriate at least to add that the decreased affinity for leucine in the presence of CMI may have also contributed.

**Reply:** We appreciate this comment and have edited the text accordingly.

7. **Comment:** Last paragraph of discussion: "Taken together"

**Reply:** The suggested change has been made.

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I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports