From: Michael Sattler <michael.sattler@tum.de>
Sent: 06 Dec 2020 20:16
To: Copernicus Publications Editorial Support <editorial@copernicus.org>
Cc: Mark Bostock <mark.bostock@tum.de>
Subject: Re: mr-2020-26 (referee) - final response

…
Please find my comments enclosed, with help from Dr. Mark Bostock, a postdoc in my group.

Regards, Michael

The manuscript by Tharayil et al. demonstrates a new strategy for the use of the phosphoserine introduced via genetic code expansion using recombinant protein expression to incorporate lanthanide binding sites into proteins to enable the of paramagnetic data, i.e. pseudo-contact shifts. The authors propose that this provides an measurement efficient strategy to establish lanthanide binding sites and avoids chemical coupling with LBTs. The model systems ubiquitin and GB1 are used to assess the potential for measuring PCSs in such systems, with very low Q scores obtained, indicating excellent correlation between the experimental and back-calculated data and hence successful immobilisation of the lanthanide metal. The structural features allowing successful expression of folded protein and lanthanide binding are assessed using a range of different proteins and mutants. The authors suggest empirical guidelines for incorporation of Ln^{3+} binding sites using this method, although it remains likely that there will be a considerable amount of trial and error required in this approach.
The manuscript is carefully written, the data and figures well-presented and the strengths, limitations and possible structural interpretations of the results clearly discussed. Whilst it seems likely that this technique will not be easy to implement in other systems, the manuscript provides useful information for other researchers and establishes an alternative approach for PCS determination in systems not amenable to other approaches.

Specific comments
- Was it always possible to achieve saturation of the various proteins with the lanthanide metals? For example, in Figure 1 (a) the complex is in slow exchange and a fraction of the peaks still appear at the (assumed) unbound position. This is also observed for other spectra in this paper e.g. Figure 3, GB1. For many applications e.g. observation of PCSs on binding partners, complete saturation with metal ions is necessary to accurately interpret the PCSs. Is this affected by the choice of metal?
- The authors say that the proteins were titrated with paramagnetic lanthanide metals. In some cases they mention that a 1:1 ratio of lanthanide:protein was used. Was this used in all cases? How did the authors avoid free Ln3+ in solution potentially creating non-specific bleaching due to the PRE component of the lanthanides ("solvent PRE"). It would be useful to comment on this.
- An impressive range of different proteins and mutants is tested in this manuscript. It would be helpful to include a supplementary table comparing all the different mutants studied in terms of number of phosphoserines, other mutations, expression level, metal binding etc.
- The rather low binding affinity for the lanthanides has potential disadvantages and would for example prohibit the use in combination with nucleic acids as free lanthanide ions will bind and potentially cleave the nucleic acid. But free lanthanides may also interfere with other regions of a given protein. The authors may want to comment and discuss this.

Technical comments
Line 62: “only *a* few” (“a” missing).
Line 112 (Methods): Were the lanthanide stock solutions for NMR titration also prepared in NMR buffer as for the ITC experiments?
Line 245: “The difficulties to express most of the double-phosphoserine mutants was not due to expression into insoluble inclusion bodies, as we did not find the proteins in the insoluble fraction after cell lysis.” à The difficulties *in* expressing most of the double-phosphoserine mutants *were* not …. Also in line 250.
NMR spectra: The ^{15}N axis label looks like it is divided by ppm.
Figure 2: It would be useful to provide a key to the colours on the graphs or maybe to choose different colours – blue and red could be confused with the blue and red lobes of the PCS tensors shown on the right hand side. This is true in some of the other figures too.
Figure 5: It would be helpful to mark the distances to the lanthanide metal – in particular in (a) Glu26 does not appear to be close to the metal position, whilst in (b) the proximity is evident.
Figure 6: It would be useful to mark the proposed interactions as discussed in the text.

On 03.12.2020 22:20, editorial@copernicus.org wrote:

Dear Michael Sattler,

We are pleased to inform you that the open discussion of the following MR manuscript was closed:

Title: Phosphoserine for the generation of lanthanide binding sites on
proteins for paramagnetic NMR
Author(s): Sreelakshmi Mekkattu Tharayil et al.
MS No.: mr-2020-26
MS type: Research article
Special Issue: Robert Kaptein Festschrift

No more referee comments and short comments will be accepted, but the authors are encouraged to post final author comments on behalf of all co-authors (final response phase). We will inform you as soon as an author comment has been posted in the discussion forum at: https://mr.copernicus.org/preprints/mr-2020-26/#discussion

If you have prepared a review but could not meet the discussion deadline, you are kindly asked to send it directly to me at:
editorial@copernicus.org

Please log in with your Copernicus Office user ID to monitor the processing of the manuscript via your MS overview at:
https://editor.copernicus.org/MR/my_manuscript_overview

Thank you very much for your support and we look forward to a future cooperation. In case any questions arise, please do not hesitate to contact me.

Kind regards,

The editorial support team
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editorial@copernicus.org