

## ***Interactive comment on “Phosphoserine for the generation of lanthanide binding sites on proteins for paramagnetic NMR” by Sreelakshmi Mekkattu Tharayil et al.***

**Sreelakshmi Mekkattu Tharayil et al.**

gottfried.otting@anu.edu.au

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We are grateful for the insightful comments and identifying errors.

Line 100: A 100-fold excess of TEV was used, that seems an awful lot for an enzyme. How was it removed? Was second NTA column used?

Response: We appreciate detection of this error! TEV protease was added in 0.1 molar ratio to remove the His6 tag. In addition, a second 5 mL Ni-NTA column was used to remove the TEV protease from the protein. We propose to write in the revised manuscript: “the protein was dialysed into TEV protease buffer (50 mM Tris-HCl, pH

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8.0, 300 mM NaCl and 1 mM beta-mercaptoethanol) to remove the His6-tag by digestion with TEV protease overnight at 4 °C. His6-tagged TEV protease was added in 0.1 molar ratio. The protease and cleaved His6-tag were removed by running the sample again over a Ni-NTA column.”

Lines 108, 109: The Bruker line of consoles is called Avance, not Advance

Response: Thank you for pointing out this typo, which will be corrected in the revised manuscript.

Table 1: It would be useful to add the number of PCS used in each calculation in an extra column. Also, the tensors for Tm3+ are very low indeed compared to those for Tb3+. Other tags, such as CLaNP give very high values for Tm3+ ( $-55 \times 10^{-32}$ ). Do the authors know why these differ so much? The Q-values are very low, as mentioned, and all but one are 0.03, yet looking at the plot for Ubi E16Q/E18Sep(Tm3+) the spread looks clearly larger than for others (Fig. 2). How can that be?

Response: In the revised manuscript, we will replace Tables 1 and 2 in the main text as shown in the figures attached, with changes highlighted in yellow. All PCSs used for the tensor fits of the various ubiquitin and GB1 mutants are already listed in Tables S1 and S2, respectively.

Indeed, the difference in DeltaChi tensors obtained with Tm3+ and Tb3+ was larger than expected for the single-Sep mutants (but not for the GB1 mutant A24Sep/K28Sep). We observed previously that the ratio between the axial tensor components of these two ions can vary between different tags and even for the same tag at different sites of a protein (C.-T. Loh, B. Graham, E. H. Abdelkader, K. L. Tuck, G. Otting (2015) Generation of pseudocontact shifts in proteins with lanthanides using small “clickable” nitrilotriacetic acid and iminodiacetic acid tags Chem. Eur. J. 21, 5084-5092). These differences are not an artifact of fitting the tensors for Tm3+ and Tb3+ independently, as the fits yielded very similar coordinates for both metal ions. We do not understand the origin of these effects. It would help, if the effect of the ligand field

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could be predicted by quantum-mechanical calculations, but we were told by experts in the field that this is prohibitively difficult for lanthanide ions.

In the revised version, we propose to add the following paragraph in line 371: “The DeltaChi tensors obtained with Tm3+ instead of Tb3+ ions were unexpectedly low for the single-Sep mutants, but not for the GB1 mutant A24Sep/K28Sep. We observed previously that the ratio between the DeltaChi\_axial components of these two ions can vary between different tags and even for the same tag at different sites of a protein (Loh et al., 2015). These differences are not an artifact of fitting the tensors for Tm3+ and Tb3+ independently, as, with the exception of ubiquitin E18Sep, the fits converged to very similar metal positions (Tables 1 and 2). We do not understand the origin of different magnitudes of Chi-tensor anisotropies for Tm3+ and Tb3+ ions. In addition, much larger DeltaChi tensors have been reported for sterically rigid cyclen tags (Joss and Häussinger, 2019), suggesting that a rigid ligand field promotes large DeltaChi tensors.”

The quality factor for the DeltaChi-tensor fit of Tb3+ in ubiquitin E18Sep differed from that of ubiquitin E16Q/E18Sep in the second digit, which was not displayed. On re-inspection, we noted that the Q factors had been rounded incorrectly: the Q factor for worse-fitting data should have been reported as 0.04 instead of 0.03. This was fixed in Table 1 attached and will be fixed in the revised version of the manuscript. In the case of Tm3+, the back-calculated and experimental PCSs correlate similarly well in Fig. 2, and the Q factors were correspondingly similar.

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170 **Table 1.**  $\Delta\chi$ -tensor parameters of the ubiquitin mutants E18Sep, E16Q/E18Sep and T22Sep/N25D/K29Q and the GB1 mutant K10D/T11Sep complexed with Tb<sup>3+</sup> and Tm<sup>3+</sup> ions.<sup>a</sup>

| Protein                                   | N <sup>b</sup> | $\Delta\chi_{xx}^c$<br>(10 <sup>-32</sup> m <sup>3</sup> ) | $\Delta\chi_{yy}^c$<br>(10 <sup>-32</sup> m <sup>3</sup> ) | x<br>(Å) | y<br>(Å) | z<br>(Å) | $\alpha$<br>(°) | $\beta$<br>(°) | $\gamma$<br>(°) | Q <sup>d</sup> |
|---|----------------|--|--|----------|----------|----------|-----------------|----------------|-----------------|----------------|
| ubiquitin E18Sep (Tb <sup>3+</sup> )      | 20             | 17.1 (0.6)   | 2.8 (0.3)  | 10.095   | -1.846   | -11.711  | 170             | 138            | 50              | 0.03           |
| ubiquitin E18Sep (Tm <sup>3+</sup> )      | 27             | -2.7 (0.1)   | -1.0 (0.1)   | 9.463    | -0.674   | -12.207  | 168             | 129            | 49              | 0.03           |
| ubiquitin E16Q/E18Sep (Tb <sup>3+</sup> ) | 27             | 15.9 (0.6)   | 3.4 (0.8)  | 9.695    | -1.754   | -11.833  | 162             | 135            | 37              | 0.04           |
| ubiquitin E16Q/E18Sep (Tm <sup>3+</sup> ) | 28             | -4.5 (0.1)   | -2.1 (0.1)   | 9.441    | -1.902   | -11.918  | 164             | 131            | 59              | 0.03           |
| GB1 K10D/T11Sep (Tb <sup>3+</sup> )       | 26             | 7.3 (0.1)  | 1.6 (0.1)  | 3.513    | 14.367   | 0.093    | 35              | 116            | 174             | 0.01           |
| ubi. T22Sep/N25D/K29Q (Tb <sup>3+</sup> ) | 20             | 3.5 (0.1)  | 1.3 (0.1)  | 5.505    | 1.144    | -8.867   | 150             | 104            | 9               | 0.03           |

<sup>a</sup> The  $\Delta\chi$ -tensor fits used PCSs measured with Tb<sup>3+</sup> and Tm<sup>3+</sup>, using Y<sup>3+</sup> as the diamagnetic reference. The metal coordinates and tensor parameters for the ubiquitin and GB1 mutants are reported relative to the NMR ensemble structure of ubiquitin (PDB ID: 2K0X; Fenwick et al., 2011) and the crystal structure of GB1 (PDB ID: 1PGA; Gallagher et al., 1994), respectively.

<sup>b</sup> N: number of PCSs used in the fit.

<sup>c</sup> Uncertainties (in brackets) were determined from fits obtained by randomly omitting 10 % of the PCS data.

<sup>d</sup> The quality factor was calculated as the root-mean-square deviation between experimental and back-calculated PCSs divided by the root-mean-square of the experimental PCSs.

180 **Fig. 1.** revised Table 1

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**Table 2.**  $\Delta\chi$ -tensor parameters of the GB1 mutants K10Sep/T11Sep and A24Sep/K28Sep.<sup>a</sup>

| Mutant                             | $N$ | $\Delta\chi_{xx}$<br>( $10^{-32} \text{ m}^3$ ) | $\Delta\chi_{yy}$<br>( $10^{-32} \text{ m}^3$ ) | x<br>(Å) | y<br>(Å) | z<br>(Å) | $\alpha$<br>(°) | $\beta$<br>(°) | $\gamma$<br>(°) | $Q$  |
|------------------------------------|-----|---|---|----------|----------|----------|-----------------|----------------|-----------------|------|
| K10Sep/T11Sep ( $\text{Tb}^{3+}$ ) | 31  | -14.5 (0.1)                                     | -3.2 (0.1)                                      | 27.455   | 13.449   | 12.675   | 88              | 13             | 155             | 0.01 |
| A24Sep/K28Sep ( $\text{Tb}^{3+}$ ) | 34  | 34.7 (0.6)                                      | 5.3 (0.1)                                       | 17.628   | 34.049   | 21.869   | 178             | 46             | 69              | 0.02 |
| A24Sep/K28Sep ( $\text{Tm}^{3+}$ ) | 31  | -15.5 (0.4)                                     | -2.5 (0.1)                                      | 17.666   | 34.141   | 21.937   | 178             | 46             | 47              | 0.03 |

230 <sup>a</sup> The  $\Delta\chi$ -tensor fits used the crystal structure 1PGA (Gallagher et al., 1994) and the PCSs measured with  $\text{Tb}^{3+}$  (or  $\text{Tm}^{3+}$ ) and  $\text{Y}^{3+}$ . See footnotes b-d of Table 1 for further details.

**Fig. 2.** revised Table 2