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Magn. Reson. Discuss., referee comment RC3
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Comment on mr-2021-54

Anonymous Referee #3

Referee comment on "Fluorine NMR study of proline-rich sequences using fluoroprolines" by Davy Sinnaeve et al., Magn. Reson. Discuss., https://doi.org/10.5194/mr-2021-54-RC3, 2021

In the manuscript 'Fluorine NMR study of proline-rich sequences using fluoroprolines', Sinnaeve et al. develop 19F NMR of (4R) and (4S)-fluoroproline as a tool to probe the conformation of proline residues within polyproline tracts, and the binding of such sequences to an SH3 domain. As noted by the authors, 19F NMR of fluoroproline is perhaps surprisingly undeveloped – although there are some precedents in the literature that have not been cited. As such, this work is a welcome contribution. However, it would be helpful to be more explicit about the conclusions the authors actually draw from the present study, beyond a mere demonstration of NMR prowess. The analysis of SH3 binding is also weak, and while additional experimental data may not be required, a more rigorous analysis of experimental uncertainties should be carried out. Lastly, the terminology of R/S fluoroprolines within RS/SR peptides, while logical, is certainly prone to confusion (at least for this reviewer!), and would benefit from a consistent colour coding and presentation within figures.

Major points:

- The authors state that 19F NMR of fluoroproline has not been explored. This is not completely accurate: Thomas et al. (2009, Chem Comm) report NMR of cis/trans isomerisation Ac-FPro-OMe, albeit without any apparent followup to larger peptides or proteins. Torbeev (2013), cited in the current paper, also present 19F NMR studies of cis/trans isomerisation of F2Pro-labelled ß2-microglobulin (e.g. Fig. S12). This was just the result of a very brief literature search, and may not be a complete list: the authors themselves should conduct a more careful survey and acknowledge prior work more fully.
- Figures: A consistent colour coding to distinguish R/S within RS/SR peptides would be extremely helpful. The relative left/right placement is also inconsistent, e.g. Fig. 1A/B vs Fig. 1D, Fig. 6A/B vs Fig. 7B/C, etc.
- 'Minor forms of prolines': minor peaks are discussed on many occasions, and are attributed to cis/trans isomerisation of neighbouring residues: what is the evidence for

this? Can impurities be ruled out, e.g. have independent samples been prepared and compared?

- P. 7, l. 156-158: I have no idea what the authors mean by 'dynamic frustration', but it seems like a very bold statement that should be explained and justified. As far as I can see, the authors simply observe that the endo/exo equilibrium is (a) different between 4R and 4S FPro, (b) unchanged within a polyproline peptide, and (c) has no effect upon the broader conformation of the peptide. Is this a fair summary? A more straightforward statement of conclusions would in general be welcome throughout this manuscript.
- P. 9, l. 173: provide a reference for the PPII destabilising nature of 4S-FPro. Can the authors quantify the energetics of this a little more carefully, e.g. what is the expected effect on cis/trans equilibrium, and its impact on the stability of the peptide structure?
- P. 11, l. 240-245: specify the CPMG frequency. Can the authors discuss the possible origins of the chemical exchange they identify?
- P. 11, l. 254-258: After an extensive section of method development, the authors report two correlation times for FPro residues, but provide no interpretation or discussion of these results. What was the point of this measurement, and what is the significance of the result?
- HSQC titration: provide results for the titration of non-fluorinated peptide. Provide concentrations/equivalents for data shown in Fig. 6 – assuming that the same concentrations are used for MpSR and MpRS titrations, the titration data look extremely similar, which is hard to reconcile with the reported three-fold difference in affinity. Provide axes for the inset figure panel. What HSQC pulse sequence was used for acquisition? Provide a table or plot comparing chemical shift perturbations between all three peptides and, if available, illustrate this on the structure of the SH3 domain. Where is Trp37 relative to the peptide binding site and expected location of the FPro residue? Trp37 is called a 'striking difference' but in reality the difference in bound chemical shifts appears to be extremely small.
- Have the authors considered 2D lineshape analysis of the HSQC titration to provide an independent assessment of binding kinetics? Can the authors comment on their decision to analyse 19F titration data in terms of CSPs and linewidths separately rather than directly via lineshape analysis, e.g. as performed by Stadmiller et al.?
- 19F titration: how are spectra in Fig. 6C/D normalised? The authors claim there is 'strong' exchange broadening, but from the data presented this seems exaggerated. What software was used to fit linewidths, and how were minor peaks handled during this fitting? R2 rates should have units of s-1 not Hz, and uncertainties should be reported. The authors consider more complex binding mechanisms on the basis of these linewidth measurements, but from inspection of the signal-to-noise in the spectra of Fig. 6C/D I'm not sure that this is entirely justified. In any case, a more careful analysis of uncertainties would resolve this issue.
- HSQC CSP analysis: I would suggest fitting (and plotting) data for individual residues and then averaging the results of the fit, rather than averaging the CSPs and performing a single fit. In performing a global analysis of HSQC and 19F CSPs, and 19F linewidths, how were uncertainties determined and the relative contributions of each measurement type weighted? Are fit results sensitive to this weighting?
- What is the basis for relating the magnitude of 19F chemical shift perturbations to the strength of binding?
- Based on the extensive literature of SH3-peptide interactions, can the authors model the structure of the bound peptide, and perhaps examine the relative placement of 19F atoms and the intermolecular contacts that might be made?
- In the discussion, the authors attempt to relate changes in 'conformational biases' with the effect on the binding equilibrium. However, their results indicate that the peptide conformations are in fact extremely similar - as gauged by near identical chemical shifts. The 'substantial shift' in binding affinity also corresponds to a very modest â□□â□□G of 0.6 kcal mol-1. In short, I struggle to understand the authors' interpretation of their results: more clarity is required.

Minor points:

- Fig. 2, caption: it would be helpful to note that this is the Cδ/Hδ region of the HSQC-NOESY rather than simply the Cδ region.
- P. 1, l. 25: within
- P. 9, l. 186: OMe
- Fig. 7: it's unclear what is being plotted in the RH panel of A. Chemical shifts should be provided in full on axes. What are the black/grey data in panel B? Are these fits to Eq. 1? The legend is unclear.
- P. 16, l. 375: Hz or s-1?