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Reply on RC3

Davy Sinnaeve et al.

Author 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-AC3, 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.

The conclusion has been modified to highlight the specific results brought by our study on the interaction between the Vinexin b SH3 and proline rich binding motifs as well as to better explicit the relevance of such approach for the study of larger interaction networks involving SH3 domains.

We have revised the analysis of the SH3 binding according to the specific comments made by the referee, in particular concerning the analysis of the uncertainties (see below).

We understand that the MpRS and MpSR terminology may be confusing. It highlights the subtle stereoisomeric difference between the two peptides and provides a non-ambiguous designation of the peptides. Thanks to the referee's comment, we believe that the source of confusion has been reduced by checking the consistency of the color coding throughout the manuscript. The figure 7 was indeed particularly confusing in the first version of the manuscript and has been revised.

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.

We were familiar with the works discovered by the referee. Our statement aimed at pointing out the limited use of fluoroprolines in large peptides and proteins, not in single amino-acid molecules (such as Thomas et al.): "Surprisingly, despite the well-established use of FPro residues in chemical biology, they have so far not found any application as 19F NMR reporters in protein studies,.. ". With this statement, we were aiming for similar applications such as those known for the widely used fluorinated aromatic amino acids, such as interaction studies, or dynamics. We note that a similar conclusion was drawn recently in a review paper on fluorinated prolines (Verhoork et al., "Fluorinated Prolines as Conformational Tools and Reporters for Peptide and Protein Chemistry", Biochemistry, 2018). However, it is true that there are studies that have used 19F NMR simply to confirm the individual conformational state of the residue, such as Torbeev et al. We are also aware of one paper (Dietz et al., ChemBioChem, 2015) that used 19F NMR to monitor the folding/unfolding of a foldon peptide. To avoid misinterpretation, we have modified the statement to:

" Surprisingly, despite the well-established use of FPro residues in chemical biology, they have so far found very limited at-tention as 19F NMR reporters in protein studies, in contrast to aromatic amino acids (Verhoork et al., 2018). In the limited protein or peptide studies that have used 19F NMR, it was mainly used to confirm the local conformational state of the fluoroproline residue (Torbeev et al., 2013, Verhoork et al., 2018). To the best of our knowledge, only one study went fur-ther and exploited 19F NMR of a foldon domain peptide containing (4R)-FPro and (4S)-FPro residues to monitor the fold-ing/unfolding process as a function of temperature (Dietz et al., 2015)."

- 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.

The figures 1D has been revised to match a consistent placement of 4R and 4S fluoroprolines respectively at the left and right sides. We added (4R)- and (4S)-FPro labels to figure 6 to ease its reading and figure 7 has been changed for consistent placement of RS and SR peptides in the left and right sides, respectively.

- '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?

Indeed, by checking the purity of MpRS sample that showed 35.5% of second form for 4R-FPro residue we identified it as a by-product (with distinct retention time and mass increase of 14 Da). The text and legend to figure 4 have been updated:

"By analyzing this particular sample by analytical HPLC and mass-spectrometry we identified this species as an impurity (with mass increase of 14 Da that is localized to Pro1 residue based on tandem MS2 experiment). Other minor peaks can correspond to minor forms of the peptide where a single proline or fluoroproline is in the cis-form. Thus, additional peaks could be conformers and impurities."

- 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.

This summary is correct, although the conformational endo-exo biais of (4R)- and (4S)-fluoroprolines was known before and does not result from our study. The modified conclusion is now including our conclusions on the conformational preferences of FPro within a polyproline peptide.

Dynamic frustration can be defined as non-native dynamics resulting from distinct equilibrium dihedral angles and non-native intramolecular contacts. It is a more extended concept than conformational frustration and includes modified rates of interconversion between different conformations in the altered conformational ensemble. In our study, the introduction of different fluoroprolines results in an altered equilibrium of conformations compared to the wild-type peptide, resulting in different binding affinities to the SH3 domain.

- 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?

The effect of 4S-FPro on destabilizing the PPII conformation in collagen was studied by Raines and colleagues and destabilization due to incorporation of this residue was shown (e.g., Bretscher et al. J. Am. Chem. Soc. 2001, 123, 777 and Horng et al. Protein Science 2006 15, 74). Dissecting the energetic contributions of a single residue is more challenging, however and was not subject of the present study. In the mentioned sentence, we use the word “destabilizing” in qualitative rather than quantitative manner.

- P. 11, l. 240-245: specify the CPMG frequency. Can the authors discuss the possible origins of the chemical exchange they identify?

The half-echo delay is now mentioned in the main text. We have added one sentence to discuss the possible origin of the exchange contribution:

" The origin of the exchange contribution is unclear, but possibly may arise from transient interactions between the polyproline segment and the flanking sequence (RVYK). Further studies will be required to investigate this unexpected finding."

- 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?

The likely cause of the different dynamical behavior is the differing nature of the flanking residues on both sides of the two homopolymer segments. Indeed, it has recently been shown for other homopolymers (notably polyglutamine) that the flanking regions greatly determine the conformational preference and dynamics. It is likely that something similar occurs here, though further investigation is required. We have added modified the text to add this point. Here, the intention was to demonstrate the potential of FPro residues and 19F to reveal this.

- 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.

- We added a supplementary figure 4 to show the fit with the non-fluorinated peptide.

- A factor of three in the binding strength is indeed not dramatically affecting a titration curve from the point of view of the receptor (the titrated molecule) while a stronger difference is observed for the titrating molecule. We provide now a Jupyter notebook showing that a three-fold difference in affinity has a great impact on the fluorine chemical shift evolution during the titration of the protein by the peptide while the impact on the protein chemical shifts is limited. This is due to the fact that the affinity has a pronounced effect on the peptide bound fraction at low concentrations of the peptide.

- The stoichiometry is now shown in Figure 7 and axes are shown for the inset of Figure 6.

- Details of the HSQC pulse sequence are now provided in the Material and method section.

- A supplementary table 2 is showing the chemical shift perturbation for the 3 peptides.

- The residues used for the binding study are shown on the 3D model built by homology modelling shown in Supplementary Figure 1. This figure clearly shows the implication of Trp37 in the polyproline binding site. The difference in the trajectories of Trp37 side-chain Hε-Nε resonance is indeed very small but consistently above the precision of the frequency measurements. We find that this is a notable difference, considering the known importance of this residue in the interaction and the remote location of the fluorine atom that excludes a direct effect of the fluorine atom on the tryptophane resonances. The supplementary figure 4 is showing the similarity between the Trp37 Hε-Nε cross peak observed for the titration with MpSR and the non-fluorinated peptide.

- 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.?

We added a supplementary figure 5 to show that the lineshape of the fluorine signals are lorentzian. We therefore performed the analysis within the frame of a fast on and off binding kinetics. The challenge in interpreting the MpRS data was to find a unique model able to explain our observations prior the determination of the parameters of this model. This proved to be difficult due to the large number of possible models as compared to the modest number of observables we currently have.

- 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.

- The peaks intensities shown in Figure 6 Peak were normalized to account for the

difference in peptide concentrations and number of scans used to record the spectrum. This is now specified in the legend of figure 6.

- We replaced 'strong' by 'significant' in the sentence. The reader can now get an insight on the quality of the line fits: we have added a supplementary figure 5 to show the goodness of fit for the first titration point, where the signal/noise ratio is the lowest. We also provide as supplementary material the Jupyter notebook that was used to fit the fluorine resonance lines. The units have been changed to s-1. The uncertainties in the R2 derived from the line widths are now shown in figure 7.

- While it is true that we initially considered more complex binding schemes, with two possible sites, all our analysis relies on a binding site: " Based on the goodness of fit reported as the reduced χ^2, the ternary complex turned out to be unnecessary to explain the data, thus implying that only one SH3 binds to the peptide."

- 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?

- We have added a Supplementary table 1 that provides the dissociation equilibrium constants for every single residue that was considered for the average. As expected, we find a Kd value that is consistent with the one derived from fitting the sum of all composite chemical shifts.

- Incorporating fluorine chemical shifts into the fit led to a slight increase of the Kd value from 74 µM to 96 µM for the high affinity MpSR peptide. The rmsd on the fluorine and proton chemical shifts were 0.0027 and 0.0125 ppm for the fluorine and proton resonances, respectively while a rmsd value of 0.009 ppm was obtained when only the proton chemical shifts were considered. No scaling factor was used to weight the contributions of the fluorine and proton in the target function. The uncertainties derived from the sole fit are largely underestimated due to the uncertainty on the concentrations of interacting species, which may be difficult to assess. A reference carefully addressing this issue is provided (Koehler et al, Methods Mol. Biol. 1286 (2015) 279–296). This is why we report uncertainties on equilibrium constants that are larger than those resulting from the fit. Nevertheless, the factor of three between the two affinities is far above the measurement uncertainties and is highly significant. This is now better explained in the text.

- What is the basis for relating the magnitude of 19F chemical shift perturbations to the strength of binding?

This is addressed in point 8 and in the associated Jupyter notebook.

- 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?

Supplementary figure 1 shows a homology model of the complex together with the location of observed chemical shift perturbations.

- 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

âˆ†âˆ†G of 0.6 kcal mol-1. In short, I struggle to understand the authors' interpretation of their results: more clarity is required.

We referred to the conformational bias of the ring conformation of fluorinated prolines. It is true that the effect of this local bias on the remaining part of the polyproline peptide is very limited, nevertheless it results into a weaker binding. In eukaryotic protein sequences, proline rich motifs are often clustered and minor changes in the binding affinity of a domain to a single site upon a chemical modification such as phosphorylation may lead to important biological effects if this change is correlated with other interactions. We believe that fluoroprolines represent an elegant tool to study such phenomenons. We have revised the conclusion to state more explicitly this point.

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.

The legend of figure 2 has been revised accordingly:

"1H-13C HSQC-NOESY (mixing time: 80 ms) with a narrow 13C window focussing on the 13Cδ/Hδ correlations regions of both MpRS and MpSR peptides, recorded at 298 K and 700 MHz."

- P. 1, l. 25: within
- P. 9, l. 186: OMe

The two typos have been corrected.

- 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.

The figure 7 has been revised as well as the legend that presents explicitly the meaning of the solid lines in the panel C. These lines are now shown in black for both fluoroprolines to avoid confusion.

- P. 16, l. 375: Hz or s-1?

kon rate constants are reported in M-1 S-1.