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Reply on RC2

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-AC2, 2021

- The authors present a detailed NMR investigation of two SH3 class II binding peptides, containing each a (4R)- and (4S)-fluoroproline but at different location. They first present the NMR assignment of the peptides, and exploit a high resolution NOESY-HSQC spectrum to assign all proline resonances.

In this aspect, I am suprised why they only give a single value for the Hδ protons, knowing that the two Hδ's can be distinguished, and actually give infomation on the flexibility of the residues (see for example Ahuja et al. JMB 2016).

We thank Guy Lippens for pointing this point to our attention. Both Hδ chemical shifts are provided in the table enabling the comparison of their differences to be performed as suggested in the reference. Beside P1 and fluorinated prolines, the profile of these chemical shift differences is very similar suggesting a similar local dynamics. This profile is shown in the supplementary figure 2 and a sentence has been added in the text to mention this with a link to the suggested reference:

" The dynamics of the non-fluorinated prolines are also not impacted by the insertion of either (4S)- or (4R)-FPro, as measured from the difference between the diastereotopic Hδ chemical shifts (Ahuja et al., 2016) (Supplementary Fig.3).

I also wonder whether the larger dispersion of the Pro Cδ carbons in the MpRS peptide compared to that of the MpSR peptide (Figure 2) has a meaning ? When I compare the Cδ spread of the two prolines flanking the 4R-FP in the MpRS peptide (ΔCδ(3-5) = -0.3ppm) with the same value in the MpSR peptide (ΔCδ(7-9) = +0.27ppm), I again wonder whether the chemical shift contains structural information.

The difference in chemical shift dispersion observed in the two peptides is indeed striking and has not escaped our attention, it is explicitly mentioned in line 180. We refer to a possible change of the psi dihedral angle of the two prolines preceeding the FPro (3 and 7) that display the largest difference but we refrained to further any structural interpretation

due to the lack of structural data on FPro containing polypeptides.

Not only the ring pucker but also the backbone conformation of the proline is influenced by the fluorine incorporation, with the (4S)-FP favoring the cis conformation. Here, I am somewhat confused. If the fluorine spectrum of the (4R)-FP4 in MpRS shows a major and minor peak in a 1:3 ratio, what do they represent ? A major trans form and a minor cis form of this floroproline? But these should then also show up in the 1H-13C HSQC spectra, and the cis form should be characterized by a H□□-H□□ cross peak ? And is the situation different for the (4R)-FP8 in the MpSR peptide ? What about the (4S)-FP in both peptides ? Are they in the cis conformation ? Elucidating these points seems important for the interested reader.

The analysis of NOEs measured in D_2O unambiguously shows that Pro-(4S)-FPro and Pro-(4R)-FPro peptide bonds are in the trans conformation in both peptides as indicated by the similar intensities of the Hα(i-1) - Hδ(i) NOEs observed in the four cases and the lack of Hα(i-1)-Hα(i) cross peak. The significant amount of a minor peak observed for the (4R)-FP in the MpRS peptide is therefore not related to a local change of conformation.

By performing analysis of the sample used in NMR measurements, we detected that 35% peak for (4R)-FPro observed in ^{19}F NMR spectrum of MpRS peptide corresponds to by-product with distinct retention time in reverse-phase HPLC trace and mass increase of 14 Da. MS-MS analysis enabled to localize this modification to the Pro1 residue in the sequence. Hydrolytic and/or oxidative modification could take place over long time during NMR measurements. The manuscript has been updated to mention the origin of the peak heterogeneity.

In order to characterize the movements of the FP rings, they turn to relaxation measurements. These are not easy to interpret, knowing the multiple dipolar terms and the important csa contribution. With the help of Spinach simulations, they obtain reasonable estimates for the different rates as a function of correlation time. The experimental data are then presented in a Table form, but I would suggest the authors indicate them by lines on the theoretical curves to allow easier interpretation by the reader.

Figure 5 has been modified to display the range of experimental relaxation parameters.

The heteronuclear NOE values indicate the surprising finding that the position in the peptide rather than being a (4R)- or (4S)-proline dictates the dynamic behaviour ? This is puzzling, and so is the large exchange contribution to the R2 rates. The delay between pulses is 400µs, so this implies movements on the millisecond time scale ? Finally, for the MpRS peptide, do both lines for the (4R)-FP have similar relaxation parameters ?

The reviewer is correct: the heteronuclear NOE points to a different dynamical behaviour for both segments of the peptide, irrespective of the FPro identity. Also ms time scale dynamics are present, mostly on the second polyproline stretch. We speculate that the specific flanking amino acid sequences, which differ for both polyproline stretches, may have an impact on the conformational ensemble of the polyproline segments, in a similar way as has been observed recently for other homopolymeric sequences. More investigation is required to investigate this. We have updated the text to mention the time scale and comment on its possible origin:

" This revealed about double values throughout, revealing exchange contributions on the ms time scale at both sites for both MpRS and MpSR peptides. As residual exchange contributions cannot be excluded in the CPMG experiment, an interpretation of transverse relaxation rates would also be unreliable. 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."

The minor form of the (4R)-FPro residue in MpRS turns out to be an unexpected impurity from the synthesis. The longitudinal relaxation parameters of this signal (R1: 2.18 s-1, rho: 1.95 s-1 and sigma: -0.13 s-1) are very similar to the major form (R1: 2.23 s-1, rho: 1.85 s-1 and sigma: -0.12 s-1). The transverse relaxation measured with the CPMG experiment is however significantly different (6.65 s-1 versus 8.5 s-1).

They finally look into the binding to the SH3 domain by a titration experiment, and measure both a protein 1H-15N spectrum and a direct 19F spectrum. Both peptides interact, but with a threefold different affinity and apparently different mode. The amplitude of the 19F CSPs in the MpSR peptide are different from those in the MpRS peptide, even for the residue in position 4 that should not interact (lines 114-115) ? I would have expected the red spectrum in Figure 6C to be identical to the ones of the free peptides (Figure 4), is there a referencing issue ? Finally, can the authors distinguish the major and minor 19F signals in the interacting spectra, or is line broadening too important ?

The observation of comparable CSP at position 4 and 8 was indeed unexpected and contradicts our initial expectations when the peptide were designed according the notion of Small Linear Motifs introduced by Toby Gibson (Tompa P, Davey NE, Gibson TJ, Babu MM (2014). Mol Cell 55(2):161–169.). In lines 114-115, we present the rational of the peptide design "position 4 falls outside the expected PXXPX+ binding motif". We also provide now a 3D model (Supplementary figure 1) of the complex to visualize the relative positions of the two fluorine atoms within the peptide. These comparable CSP may be either due to a specific geometry of the two polyproline segments induced by the serine residue that may bend the PPII helix positioning FP 4 close to the SH3 surface and/or a dynamic averaging of CSP values due to one-dimensional diffusion of the SH3 on the peptide. This interpretation has been added to the text.

It is true that the frequencies of fluorine lines measured for the final titration point do not match those measured for the free peptides (in figure 4). This is mainly due to the fact that the solvent conditions are different, while the spectra of free peptides were recorded

in water, the titration was performed in a buffer suitable for the SH3 domain (40 mM phosphate pH 7). The measurement conditions are now explicitly mentioned in the legend of figure 4.

The line broadening of the minor forms are indeed to large preventing their specific measurement during titration experiments.

In conclusion, this is a thorough study of the influence of a fluorinated proline in a peptide motif, that should lay the basis for further use of this residue in advanced protein studies.

Minor remarks

Line 126 strong H□(i) to H□(i-1) NOE Line 549 Acknowledgments

The modification has been implemented and we thank Guy Lippens for his careful reading and discussion.