

In response to the reviewers' comments, we have made the changes detailed below. For some of the points, more detailed responses have been given during the discussion phase of the preprint.

In response to Ad Bax's comments:

1. I'm a bit confused about the gamma gauche effect when talking about two monofluorinated methyl groups in Leu. Both ^{19}F nuclei can simultaneously be trans relative to C_{γ} , so it's not clear that this helps with stereo assignment, even though the correlation between JHF and ^{19}F shift clearly shows it to be correct. Is it conceivable that C_{α} - C_{δ} gamma gauche effects contribute to the ^{19}F chemical shift (as they do for $^{13}\text{C}_{\delta}$)?

Response: Obviously, the preservation of high-field and low-field shifts between singly and doubly fluorinated leucines is a purely empirical observation prone to exceptions. Nonetheless, it is unlikely that CH_2F groups populate the same rotamers in monofluoro- and difluoro-leucine residues, as the conformations with two parallel C-F bonds are energetically disadvantaged (Marstokk and Møllendal, 1997).

Fluorination seems to override the C_{α} - C_{δ} γ -gauche effect for the CH_2F groups in GB1-1 and GB1-2: Figure S7 shows that the ^{13}C chemical shift of the $\text{C}^{\delta 1}\text{H}_2\text{F}$ group of residue 12 is greater than that of the $\text{C}^{\delta 2}\text{H}_2\text{F}$ group, although (according to the solution structure 3GB1) the latter carbon is trans to the α -carbon in the wild-type protein. The C_{α} - C_{δ} γ -gauche effect and the reference <https://doi.org/10.1007/BF00202043> (MacKenzie et al., 1996) are now cited in line 365.

2. For the HOESY measurements, I suspect the NOE effect to be very sensitive to internal motion due to the closeness of w_H and w_F . Heteronuclear ^1H - ^{19}F NOE can be negative or positive, depending on applicable spectral densities and some comments may be helpful.

Response: Internal motions of CH_2F groups are more restricted than for methyl groups. In 1-fluoropropane the intrinsic energy barrier between staggered rotamers has been calculated to be 4-5 kcal/mol (Feeney et al., 1996), which is comparable to the energy barrier for rotation about the central C-C bond in n-butane. Therefore, ^1H - ^1H NOEs with CH_2F groups are not intrinsically different from other ^1H - ^1H NOEs. Experimentally, we did not observe negative ^1H - ^{19}F cross-peaks with the CH_2F groups apart from NOEs with water, which we now point out in a sentence on line 267.

3. ^{19}F line widths are reported to be 7-15 Hz, which corresponds to R_2 values of 20-40/s, but this seems fast considering the 60-ms TOCSY mixing times used. Could incomplete decoupling or isotope effects contribute to these line widths? Perhaps a $R_{1\rho}$ number would be helpful.

Response: After double-checking, we corrected the upper limit to 18 Hz. We now report $R_{1\rho}(^{19}\text{F})$ values of GB1-d and GB1-d-D (which is the same as GB1-d except for

having been made with deuterated diFLeu) in the new Table S3. The slower $R_{1\rho}(^{19}\text{F})$ relaxation rates observed for GB1-d-D suggest that dipolar relaxation by the nearest protons contributes significantly to the ^{19}F relaxation. This is now reported in two sentences on lines 132 to 136.

As the solvent contained 90% H_2O /10% D_2O , deuterated amides could indeed contribute to isotope effects. Measurements in the presence of less D_2O (2%), however, did not result in narrower signals.

4. Line 146-147: “faster rotation of the CH_2F group about the $\text{C}_{\gamma}\text{-C}_{\delta}$ bond results in slower transverse relaxation”. This may well be true, but the magnitude of this effect seems larger than expected considering the modest chemical shift difference of $\sim 10\text{ppm}$. Could crank-shaft sidechain motions, previously suggested to be responsible for different C_{δ} ^{13}C relaxation rates, play a role?

Response: We find it hard to picture a crankshaft motion about the $\text{C}_{\gamma}\text{-C}_{\delta}$ bond that would be equally benign in terms of structural conservation as a simple rotation of the CH_2F group, at least for motions changing the conformation to a similar degree. We imagine that the slopes of the potential wells between different staggered rotamers are steeper for CH_2F groups than CH_3 groups because of the greater energy barrier between them. Our sentence (now line 171) *suggests*, rather than claims, that the line width is governed by the speed of rotation of the CH_2F groups.

5. Line 199: “different” from what? Perhaps use “multiple”?

Response: We now use the term “multiple” (now line 220).

6. Line 201: “greater conformational freedom than suggested by 3GB1”. This is a bit of a philosophical issue, but the width of an “NMR bundle” does not reflect motional freedom but the certainty at which the structure that agrees best with NMR restraints can be determined. If not, measuring fewer restraints would make the protein more dynamic.

Response: We now point out on line 223 that the NMR structure was determined with the aim of presenting the single best approximation to the average structure.

7. Line 322: “the third example of the gamma-gauche effect”. Probably correct, but perhaps useful to remind the reader of how commonly this is used in ^{13}C analysis, including proteins (e.g. <https://link.springer.com/article/10.1007/BF00202043>)

Response: We meant the γ -gauche effect only with regard to CH_2F groups (now clarified on line 366). We also cite the reference of MacKenzie et al. (1996) on line 365.

8. There is a considerable amount of older literature on TS-JFF couplings, with an empirically determined very steep distance dependence. See e.g. Bakhmutov and references therein. Perhaps including some reference to this historic work would be helpful, e.g. <https://doi.org/10.1002/mrc.1260231117>

Response: We now refer the reader to the comprehensive review by Hierso (2014) on line 35 and cite a 1978 article for a large $^{\text{TS}}J_{\text{FF}}$ coupling.

9. Lines 358-362, c=chi; d=delta

Response: Typos now fixed.

10. Please include the RF field strength and mixing scheme (DIPSI?) used for the TOCSY spectrum.

Response: This information is now provided in line 205.

11. Can the authors provide approximate TS-JHH values based on the cross/diagonal peak ratios?

Response: We measured the ratio of cross-peaks versus diagonal peaks in FF-TOCSY experiments recorded with increasing mixing times. The new Figure S5 shows curves fitted to three prominent cross-peaks, which indicate that the through-space J_{FF} couplings in GB1-d are up to 2-3 Hz. The results are described in the new section 3.5.

12. Trivialities: Spell out CFPS upon first use; Juszewski is really Kuszewski (3 times).

Response: We corrected the spelling.

In response to reviewer 2:

1. The authors observe 'through-space' J-couplings between $^{19}\text{F}^{\delta}$ atoms of the leucines, which are supposedly in spatial contact. Due to the Fermi contact mechanism, the J-couplings show that the respective s-electrons are correlated in their motion. How do the authors imagine this electronic polarization transfer? Given that the fluorines have strong negative partial charges, would they interact directly in an attractive or repulsive way? Could it also be that the hydrogens in the CFH_2 group acquire partial positive charges and are then attracted to the fluorine of the neighboring leucine, such that the transfer is achieved as $\text{F}\cdots\text{H}-\text{C}-\text{F}$? Maybe this is unlikely, since Teflon is very hydrophobic. An educated discussion would be very helpful.

Response: The quantum mechanical underpinning of through-space ^{19}F - ^{19}F J -couplings is complicated. We now refer to the 2013 review by Hierso in Chem. Rev.

Small through-space couplings between ^{19}F and ^1H would be difficult to measure given the relaxation rates of CH_2F groups. We would expect a F..H-C-F interaction between ^{19}F spins to be weaker than a direct F-F interaction. Furthermore, C-F groups are known not to be good H-bond acceptors and the protons of CH_2F groups are barely acidic. Instead of speculating we cite the finding by Alexeev et al. (2003) that fluorine atoms of CH_2F groups are not specifically attracted to each other (line 395).

2. Can the authors estimate the size of the J_{FF} -couplings from the intensities of the TOCSY-spectra?

Response: We have done this. The results are reported in the new section 3.5.

3. The spectra in Figure 3 were recorded at different pH and buffer for the different GB1 analogues (pH 6.5 MES vs pH7.5 HEPES). Is there a reason? How much would the pH affect the chemical shifts? This should be discussed in the text. Also, the Figures of other spectra do not mention the pH and buffer conditions. They should be indicated throughout.

Response: It was an accident that different buffers were used for different preparations. Fortunately, the ^{19}F -NMR spectra did not vary significantly with the buffer (now stated in line 112; see also the spectra in the supplement to our response to reviewer 2 in the discussion phase). On double-checking, the samples made with diFLeu consistently were in MES buffer, those with FLeu2 consistently were in HEPES buffer, those with FLeu1 mostly in HEPES buffer unless reported otherwise. This is now clarified in section 2.6. As GB1 contains no histidine in the structured part of the protein, also the ^1H chemical shifts were conserved between the pH values of the different buffers (6.5 and 7.5).

4. The authors mention ^{19}F T_1 relaxation times of 0.3 s (line 121). Can they estimate the T_2 s? They indicate line widths of 7–15 Hz. Would this be 20–50 ms? Do they have information from spin-echos?

Response: Transverse relaxation rates are now reported in Table S3.

5. Figure 9: a plot of the $^3J_{\text{HF}}$ constants vs. the ^{19}F shifts would be very helpful to visualize and quantify the γ -gauche effect.

Response: The plot is now shown in Figure S8.

6. Apparently the GB1 construct contains an additional MASMG sequence at the N-terminus. Although no effect is expected, it would be good to mention this in the main text.

Response: This is now spelled out in line 62.

7. Figure S3: CD melting. Is there a reason for the larger (absolute) ellipticity of wild-type GB1? Please mention/discuss.

Response: We attribute the differences to different sample concentrations. We now state in the legend of Figure S3 that the sample concentrations were *about* 0.3 mg/mL. The ^1H NMR data leave no doubt about the structural integrity of the fluorinated samples.

8. Figure S5: is this a natural-abundance ^{13}C HSQC? This should be mentioned in the main text.

Response: Yes, natural abundance, which is now stated in the legends of Figures S5 and S6.

Presentation:

The accessibility of the manuscript could be improved by considering the following:

1. The nomenclature is hard to follow. (2S,4S)-5-fluoroleucine, (2S,4R)-5-fluoroleucine and 5,5'-difluoro-L-leucine are FLeu1, FLeu2 and diFLeu. Apparently FLeu1, FLeu2 and diFLeu are leucines labeled in the $\delta 1$, $\delta 2$ or $\delta 1+\delta 2$ respectively (if I am not mistaken). This should be clearly indicated in the drawing of Figure 1 (not in the legend). A standard IUPAC NMR nomenclature of all leucine atoms would be also very helpful in this Figure. Could FLeu1, FLeu2 and diFLeu be replaced by something like $\text{F}^{\delta 1}\text{Leu}$, $\text{F}^{\delta 2}\text{Leu}$, $\text{F}^{\delta 1\delta 2}\text{Leu}$ throughout the text?

Response: We changed Figure 1 to show the $\delta 1$ and $\delta 2$ labels. In general, we prefer not to relegate the stereospecificity information to a small superscript.

2. Throughout the figures: the authors are very terse with labeling in the figure graphics. While this emphasizes the data, it makes them hard to grasp. In particular:

2.1. Figure 2: indicating the $\delta 1$, $\delta 2$ color code directly in the graphic would be very helpful.

2.1. All 1D spectra: the stereospecific assignments would be better indicated by $\delta 1$, $\delta 2$ instead of a red dot only for $\delta 1$.

2.3. All 2D spectra: individual peaks should be labeled with 2D, stereospecific assignment information whenever possible.

2.5. Figure S5: please provide color code in graphic.

Response: We now provide a colour code in Figure 2.

In the 1D NMR spectra, we used red dots instead of $\delta 1$ labels to make it graphically obvious that the ^{19}F chemical shift of the $\delta 1$ group can be both high-field and low-field of the $\delta 2$ signal. In addition, some of the red dots come close to each other, leaving insufficient space for labelling with characters.

We believe that labelling every cross-peak in the 2D spectra would lead to unhelpful clutter.

The colour code has been added in Figure S5.

1. line 286: please indicate the size of the 'different 3JHF for residue 5'.

Response: We now quote the couplings of the most unexpected outlier and refer to Table S4 (line 298).

2. lines 358–360: the Greek characters were lost.

Response: We fixed the typos.