Dear Colleagues/Editor, here are the comments of an anonymous referee #3 in red. My responses to the reasonable comments are in italics.

The manuscript proposed by Erick Zuiderweg presents a valuable attempt to rationalize the intensities measured on protein 1H-15N correlation spectra in order to get a qualitative description of the backbone dynamics at different timescales. The different parameters affecting the 2D correlation peak's intensities are exhaustively listed and their values estimated from a structural model of the protein. The concept is tested on the BPTI protein and, as noted by the author, modelling the different known contributions to the signal intensities fails to reproduce the experimental measurements. The relevance of the approach is defended by the observation that large deviations from the modelled intensities do cluster in regions of BPTI where specific dynamical features where previously reported from 15N relaxation measurements. A graphic based clustering of these "model deviating" signals is therefore proposed as a fast approach to get qualitative insights on the protein dynamics. The approach is applied to a large protein (Hsc70) enabling some observations to be made on its dynamical properties.

As stated by the author in his introduction, such an approach would be very valuable in the field of protein NMR, as we do share the general feeling that the information content of 15N-HSQC or TROSY are under-exploited, and the author's attempt to address this task is interesting. However, my general opinion is that the current state of this development is far too preliminary and would desserve more work to be published. The general applicability of such a method should be assessed by probing the concept on different class of proteins that display distinct and well documented dynamical features (depending on their size, geometry, experimental conditions of the study T° pH ...). The "correlation" approach is indeed the only way to go when the model fails to reproduce the experiment. Anyhow, some statistical assessment is necessary for the applicability of the proposed approach on other systems: for instance, what criteria should be used to identify a residue with abnormal intensities ? A quantitative description of the deviating between the theoretical model and the experimental values is clearly missing here.

Indeed, the work is crying out for follow-ups. It is a "heads up" that there is still challenging (theoretical/computational/experimental) work to be done in solution structural biology. One first thing to do is to collect data for e.g. BPTI, Ubiquitin and GB3 at low temperature so that the relative contribution of the scalar coupling is reduced. I may invite colleagues to send me such data or collect it myself as soon as the pandemic is over. And yes, on the basis of better data, more quantitative characterizations of "outliers" can be formulated. Second is to explore if and how we can adapt (improve??) the structures to correspond better with the linewidths. I am already planning that with a colleague. I hope others will follow. For now, with the deadline of Festschrift, it will have to wait until this manuscript is published.

Some of the hypothesis made by the author are questionable. In particular, the assumption that intrinsic exchange rates are identical for all amide protons is probably not true since local chemical environment at the protein surface do modulate these exchanges with water. Such information may be obtained by simple 2D experiments such as the Het-SOFAST proposed by Paul Shanda and Bernhard Brutscher.

The unprotected amide proton mass exchange rate is calculated to be 1.15 s^{-1} exchange rate, giving rise to a broadening of ~ 0.3 Hz for <u>unprotected</u> amide proton resonances. Protected amides will exchange (much) slower. While reviewer is in principle correct that environment matters, it would in this case only affect differences in rates that are <u>slower</u> than 1 s-1, thus of no relevance to the (precision of) the current data.

Relaxation mechanisms different from the dipole-dipole interaction may also contribute for the amide transverse relaxation: can we fully discard scalar relaxation ?

This is an interesting point to keep in mind for later. The 3JHNHA will fluctuate when the dihedral angle phi fluctuates. Depending on the timescale, this may contribute to broadening. For now, however, we have left dynamics completely outside of the scope of this paper and we are exploring if we can calculate the linewidths on the basis of a static structure. The answer is that we cannot do that with any precision (but outliers are

valuable). As I stated on line 222: "At this point, we suggest that coordinate precision and dynamics are the main culprits for the lack of correlation."

Small points:

- Equation 1 contains some mistakes:

I guess the two factors in the denominator are line-width (delta_nu) and not frequencies (delta missing)

Yes, thanks.

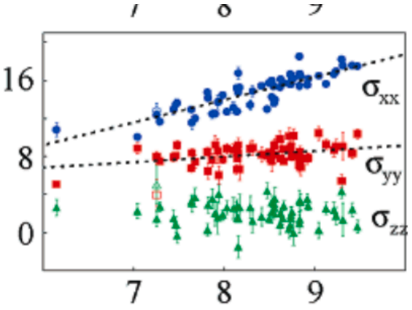
$$S / N \sim \left\{\exp\left(-\pi\delta\upsilon_{1/2}^{HN} / \left(2 \times {}^{1}J_{HN}\right)\right)\right\}^{M} \times \exp\left(-\left\langle t_1 \right\rangle \delta\upsilon_{1/2}^{N}\right) \times \frac{1}{\delta\upsilon_{1/2}^{N}} \times \frac{1}{\delta\upsilon_{1/2}^{HN}}$$

The term describing the nitrogen relaxation doesn't make sense to me: why is the average <t1> considered ? The amplitude of the peak on the F1 (15N) dimension depends on the magnetisation's level at the end of the t1 increment.

Of course. But when t1 is short the transfer will be large, and when t1 is long, the transfer will be smaller, and some average determines the overall transfer efficiency.

-140 Please describe how the amide value of CSA is derived ? Is it reasonable to assume the same CSA for all amides ? could this not be one major reason of the observed deviations, since the amide may be engaged within a hydrogen bond modulating the distribution of electrons.

I have taken the amide CSA as measured for Ubiquitin in Figure 3, right column, second panel by Loth et all.



As one sees, the individual tensor principal values vary quite a bit.

For R2 CSA relaxation (rhombic tensor) one has

$$\frac{1}{T_2^{CSA-non-axial}} = \frac{\left(\omega_I \Delta\sigma\right)^2}{18}\left\{1 + \frac{\Delta\eta^2}{3}\right\} \times \left\{4 j_0(0) + 3 j_{-1}\left(\omega_I\right)\right\}$$

where, with $\sigma_{11} \geq \sigma_{22} \geq \sigma_{33}$

$$\Delta\sigma = \sigma_{11} - \frac{\sigma_{22} + \sigma_{33}}{2}$$

$$\Delta\eta = \frac{\sigma_{22} - \sigma_{33}}{\sigma_{11} - \sigma_{iso}}$$

$$\sigma_{iso} = \frac{\sigma_{11} + \sigma_{22} + \sigma_{33}}{3}$$

I calculate from the values in Figure above a variation 0.4 Hz to 0.08 Hz in 1H CSA contribution to line width for tc=3.5 ns, on a 500 MHz spectrometer. Yes, it varies a bit, but the values are really small.

I will change the statement

"We estimate that the ^{15}N-^{1}HN dipolar interaction accounts for 3 Hz, that the ^{1}H CSA contributes 1 Hz at 500 MHz, while field inhomogeneity typically is limited to 1 Hz. What the exact values are may be disputed, but they are small and approximately constant for all amides, or partially cancel in the TROSY version of the HSQC."

To

"We estimate that the ^{15}N-^{1}HN dipolar interaction accounts for 4 Hz, and should be constant for all amides. The ^{1}H CSA varies a bit , but contributes less than 1 Hz to the 1HN linewidth at 500 MHz for this small molecule, while field inhomogeneity typically is limited to 1 Hz. "

145 The author should mention the model they used to derive the linewidths from Sparky (Lorentzian fitting ? gaussian ?)

For the BPTI HSQC I found that Lorentzian was best, while for the Hsc70 TROSY Gaussian was best. I will add this to the legends.

- Figure 4: There are some discrepancies between the text and the figure:

  - orange points are below 11 Hz for Reduced Experimental Line width

Would the following be clearer? " The orange points all have a reduced experimental line width of less than 11 Hz"

  - What is meant by "at opposite side of the diagonal ?" I suggest using "Upper triangle" and lower triangle regions

Yes, I like that suggestion.

- Figure 6:

  - Legend : plain circle and squares

Agree. Should read "open squares"

- Figure 9 : Labelling the different domains of Hsc70 would be helpful to follow the dynamical description.

Agree. Will do.