Dear Colleagues/Editor, here are the comments of an anonymous referee #2 in red. My responses to the reasonable comments are in italics.

This is a largely exploratory manuscript investigating the potential to obtain (semi-)quantitative results on protein dynamics based on backbone amide 1H linewidths in 1H-15N HSQC or TROSY spectra. As Zuiderweg states himself, the underlying idea to glean dynamic information from the HSQC spectrum is commonly used by NMR spectroscopists in a qualitative sense by identifying particularly sharp or broadened cross-peaks. Here, Zuiderweg provides a more comprehensive analysis by attempting to account for all contributions to the 1HN linewidth from various relaxation mechanisms and other effects, such as unresolved J-couplings and B0 inhomogeneity. Naturally, the dominant part of the relaxation is due to dipole-dipole relaxation, which is estimated for each backbone amide based on the high-resolution crystal structure of the protein in question. The end result is that the calculated linewidths show only fair agreement with the experimental ones, but outliers appear to reliably identify backbone amides that are either undergoing large-amplitude fast internal dynamics (i.e., residues with low order parameters) or residues undergoing conformational exchange. Thus, we are left with the conclusion that 1HN linewidths cannot provide more detailed information than what is customarily obtained from a qualitative, first-glance interpretation of HSQC-type spectra. To this extent, the work clearly does not advance the field since it does not provide any substantial conclusions beyond current knowledge. Still, I appreciate the comprehensive, semi-quantiative analysis offered by Zuiderweg, which clearly shows the limitations of the proposed analysis. In essence, the work demonstrates that 1HN linewidths cannot be interpreted in terms of dynamics to any detailed extent. For these reasons, I believe that the study could be worth publishing.

I do agree with reviewer the work demonstrates that 1HN linewidths cannot be interpreted in terms of dynamics to any detailed extent. This is indeed <u>one</u> important outcome of the work: a "heads up" that there is still challenging (theoretical/computational/experimental) work to be done in solution structural biology.

But there is another part; the real question I am trying to address is: is a narrow line narrow because of a dilute dipolar environment, or because of fast motion? Is a broad line broad because of exchange broadening, or because of a very dense dipolar environment? Without taking the structure into account one cannot answer these questions. Thus it takes more than just looking at the spectra: and, one needs to correct for unresolved 3JHN-HA scalar coupling (a major factor in the spread of the line width distribution: the scalar coupling varies from 1 – 10 Hz; for this small protein, it spans the same range as the bulk of the reduced experimental line width; see Figure 4). Now one can compare the reduced linewidth with calculations. <u>Why calculations?</u> Take a look at Figure 4B. The yellow and black points are WITHIN the main distribution of experimental values. Without the calculation these points would not be identified as broadened. And take a look at Figure 8. The magenta points fall WITHIN the main distribution of experimental values. Without the calculations they would not be identified as motionally narrowed. Thus the work advances the field beyond current knowledge.

Minor points:

p. 4, Eq [1]: I do not follow this equation fully: the last two factors are not defined and it is not clear why they appear in the equation.

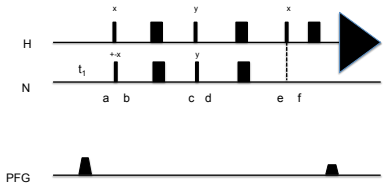
Indeed, there is an error. The equation should read

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

S/N is defined as peak height / noise. Peakheight is also proportional to the inverse linewidth.

The first exponential assumes that each step in the reverse polarization transfer (after t1) contributes equally to the linewidth, but this is not true for all pulse sequences (it depends on the details of the PEP scheme, etc).

I thought I had it right. Here is the PEP scheme.



Not paying attention to signs I get

$$\sigma_a = 2N_y H_z \cos(\omega_N t_1) + 2N_x H_z \sin(\omega_N t_1)$$
$$\sigma_b = 2N_z H_y \cos(\omega_N t_1) + 2N_x H_y \sin(\omega_N t_1)$$
$$\sigma_c = H_x \cos(\omega_N t_1) + 2N_x H_y \sin(\omega_N t_1)$$
$$\sigma_d = H_z \cos(\omega_N t_1) + 2N_z H_y \sin(\omega_N t_1)$$
$$\sigma_e = H_z \cos(\omega_N t_1) + H_x \sin(\omega_N t_1)$$
$$\sigma_f = H_y \cos(\omega_N t_1) + H_x \sin(\omega_N t_1)$$

*Between **b** and **c** both quad terms are transverse.*
If the protein was perdeuterated, the sine term would relax significantly slower than the cosine term.

*Between **d** and **e** the cosine term is in z, the sine term is transverse*
The cosine term relaxes more slowly.

By the gradient EA selection, one selects for an echo in which the cosine and sine components are of equal magnitude, thus, the smallest of both pathways. So effectively, in a PFG PEP scheme, R_2 ^{1}H relaxation determines the sensitivity during every "INEPT" step, and we have a total of 3 "INEPT" periods for the complete experiment in terms of relaxation (including the first one not shown).
Not using gradients results in quad images in f_1.

l. 94: aromatic ring flipping does not cause exchange linebroadening of amide protons (since the end states are identical).

Reviewer is correct. The ring protons themselves can be broadened, but not anything else.

Table 1: Please clarify what is listed in this table. By comparing with Fig. 4, I assume that the Sum of 1HN linewidths is taken over all residue pairs in the protein(?). This should be stated in the Table header (or footnote).

Indeed this could be clearer. I will also change it to average calculated 1HN linewidth.

Figure 6 legend: "open diamonds" should be 'open squares'.

Thanks

The text should be checked for typos, incorrect order of words, missing words, etc.