Dear Colleagues/Editor, here are the comments of an anonymous referee in red. My responses are in italics. As you will see, I disagree with reviewer's critiques at virtually every point.

Reviewer: The article tries, and fails, to correlate measured line widths with line widths calculated from crystal structures, assuming dipolar relaxation as the dominant relaxation mechanism. The ultimate conclusion is that amides with narrower line widths than average are mobile, whereas amides with broader line widths reflect us – ms dynamics. This is not at all new and the conclusion in line 215 is trivial.

This is not trivial at all. One cannot just look at the spectrum and come to that conclusion (except for very extreme outliers). 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.
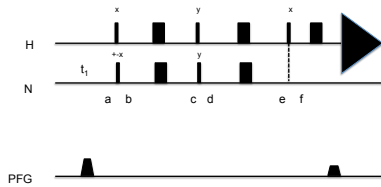
Furthermore, quite a bit of hurdles must be taken: one needs to exclude amide hydrogen mass exchange, wonder about anisotropic diffusion and relaxation cross correlation. Especially the latter is not trivial at all. Without that all being considered, one can not even attempt to calculate the line widths. With the literature data I used, one needs to also correct for unresolved $^3J_{HN\text{-}HA}$ 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). Only if all of this is taken into account, one can attempt to make a calculation based on the coordinates.

Obviously, I had also hoped that I would find a nice correlation between experiment and calculation for such a sturdy protein as BPTI, which also has a high-resolution crystal structure. But I am not getting that; it is what it is. Even if we disagree on the equations used (but see below), other equations cannot generate a correlation either – and what is suggested by reviewer would make it even worse. Most likely, coordinate precision, dynamics and conformational exchange are the cause of the lack of correlation. It is not at all clear if all of these factors can ever be disentangled. This is a message into itself to the expert NMR community, and possibly an encouragement for new research / theory.

But the real value of this manuscript lies in the fact that I show that one can extract some dynamical information from the outliers in a simple HSQC. It does provide new and useful information, which can also be used by less-than experts that do not have access to sophisticated relaxation experiments.   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.

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 ^1H 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.

This is a very important point. I did think about this deeply; I did include much of the following in an earlier version of the manuscript, but I was recommended to simplify matters and left it out. No problem from my side to put it back in as an Appendix.

The R_2 relaxation rate for "unlike" spins such as ^{1}H-^{15}N is given by (Goldman, 1988)

$$R_2^{N(H)} = \frac{1}{20}\left(\frac{\mu_0}{4\pi}\frac{\gamma_N\gamma_H\hbar}{r_{NH}^3}\right)^2$$

$$\times\left\{4\tau_c + \frac{\tau_c}{1+\left(\omega_H-\omega_N\right)^2\tau_c^2} + \frac{3\tau_c}{1+\omega_N^2\tau_c^2} + \frac{6\tau_c}{1+\omega_H^2\tau_c^2} + \frac{6\tau_c}{1+\left(\omega_H+\omega_N\right)^2\tau_c^2}\right\}$$

(1)

where μ_0 is the permittivity of space, γ are the gyromagnetic ratios, $\hbar$ Planck's constant divided by 2π, ω the resonance frequencies, and τ_c the rotational correlation time.

However, the R_2 relaxation for "like" spins, such as ^{1}H-^{1}H is more complicated:

One may start by replacing the ω_N terms with ω_H in Eq (1) and obtain

$$\rho_2^{H(H)} = \frac{1}{20}\left(\frac{\mu_0}{4\pi}\frac{\gamma_H\gamma_H\hbar}{r_{HH}^3}\right)^2 \times \left\{5\tau_c + \frac{9\tau_c}{1+\omega_H^2\tau_c^2} + \frac{6\tau_c}{1+\left(2\omega_H\right)^2\tau_c^2}\right\}$$

(2)

However, when the chemical shifts of the two ^{1}H resonances are very close, one cannot neglect a "ROESY" cross-relaxation leakage effect,

$$\sigma_{ROE} = \frac{1}{20}\left(\frac{\mu_0}{4\pi}\frac{\gamma_H\gamma_H\hbar}{r_{HH}^3}\right)^2 \times \left\{4\tau_c + \frac{6\tau_c}{1+\omega_H^2\tau_c^2}\right\}$$

(3)

Thus, in the literature, one adds the two terms together, just like for the Solomon R_1 NOE equations, and one arrives at the classical "like spins" R_2 equation (Goldman, 1988):

$$R_2^{H(H)} = \rho_2^{H(H)} + \sigma_{ROE} = \frac{1}{20}\left(\frac{\mu_0}{4\pi}\frac{\gamma_H\gamma_H\hbar}{r_{HH}^3}\right)^2 \times \left\{9\tau_c + \frac{15\tau_c}{1+\omega_H^2\tau_c^2} + \frac{6\tau_c}{1+\left(2\omega_H\right)^2\tau_c^2}\right\}$$

(4)

From a solution-NMR approach, one asks the question how close the ^{1}H frequencies have to be in order to count in the ROE effect? If they are too far apart, the coherences will become out of phase within the R_2 timespan, and the ROE effect will be "decoupled" away, leaving an equation (2) as correct. Indeed, by applying spin-lock the coherences will be synchronized and enable the ROE (Camelspin) to be visualized.

But, I am not sure if this solution intuition is the right approach. Originally, the R_2 relaxation rates were defined as proportional to the second-moment of the dipolar / CSA powder pattern. In solid-state NMR, the "ROE effect", or the flip-flop term is expressed as the second term of the dipolar Hamiltonian (Slichter, 1992):

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$$H_{DD}^{like} = \left(\frac{\mu_0}{4\pi} \frac{\gamma_i \gamma_j \hbar}{r_{ij}^3} \right) \times \left(I_{iz} I_{jz} + \frac{1}{4} \left(I_{i+} I_{j-} + I_{i-} I_{j+} \right) \right) \qquad (5)$$

It cannot be neglected, because (Slichter says) the dipolar interaction is much larger than the ^1H chemical shift differences (certainly true when that was written).
But is the dipolar coupling really that much larger? I use the leading term of Eq (5) as a measure of the "power term".

$$Dip_{1H-1H}^{like} = \frac{1}{4} \left(\frac{\mu_0}{8\pi^2} \frac{\gamma_H \gamma_H \hbar}{r_{ij}^3} \right) \quad (Hz) \qquad (6)$$

From this equation I calculate that the dipolar coupling for the canonical ^1HN-^1HA proton-pair at 2.2 A distance in a beta sheet is (+/-) 22 KHz.
The chemical shift difference between ^1HN and ^1HA is ~2000 Hz on a 500 MHz instrument. So for solid state NMR, the dipolar interaction greatly supersedes the shift difference, and therefore these are "like" spins. But for an ^1H and a ^1HA at 6A distance the dipolar coupling is just 1100 Hz . These are then more-or-less "unlike" spins for which that second term in the Hamiltonian can be neglected.
However, spins at > 6A doe not contribute much to relaxation (see Table 1 in the manuscript). So I tend to think that the "like spins" equation (4) is appropriate for those dipolar interactions due to close interactions which dominate the linewidth.
Besides from theory, I also think the proof is in the pudding. When using the "unlike" equation (2) the calculated relaxation rates would all be 5/9 of what I calculated before, and all points in Figure 4B would lie below the main diagonal. Yes, one may argue that maybe the rotational correlation time for BPTI is 6.12 ns instead of 3.4 ns, which would bring the median of the calculated distribution back to the median of experimental distribution. But this is inconsistent with at least three independent literature reports on the the rotational correlation time of BPTI using different techniques (quoted in the manuscript). Similarly, the vast majority of points in Figure 8 would lie below the main diagonal. That cannot be right either.

So, may be reviewer may be shocked that I disagree with esteemed Prof. Axel Bothner-By, not in that one would need a spinlock field to visualize the rotating frame NOE, but by the argumentation and calculations referred to above. And, as far as I can see, Abragam actually never defines what limits there are what one may call "like" spins.

As admitted by the author, Figure 4 shows no correlation. Figure 6 doesn't show a significant correlation either, again as admitted by the author. This indicates failure of the entire concept. If coordinate precision is a major problem (line 222), the concept cannot be salvaged.

No, the value of this manuscript lies in thinking about what the outliers tell us.

Line 167: The line width of the amide proton of Asp3 could very well be due to enhanced H-exchange, as the N-terminus is positively charged and the exchange base-catalyzed.

I suggested that too, on lines 205-206.

Line 187: Much more is known about the dynamics in BPTI than suggested by the manuscript, see, e.g., Grey et al., JACS 125, 14324 (2003).

It is not my purpose to review the BPTI literature.

Line 225: water molecules on protein surfaces are not known to contribute to dipolar relaxation.

Known? What if some of them reside for a few ns? Or if many stay within a few Angstroms and form a (diverging??) dipolar field? Literature, coming from different experimental techniques, is completely at odds about these points. I know – Dave Case and I tried to publish something about this using MD and got ourselves into a hornet's nest. So, I agree, it is better to take that line out. But not because it is "known".

Line 235: detailed dynamical data have been obtained for large proteins from methyl relaxation.

Sure, I mentioned this at the beginning. I can mention it again. But as reviewer is undoubtedly aware, it is hard to obtain the methyl assignments (expensive labeling – many more 3D/4D experiments that are not in standard libraries) and do the methyl dynamics measurements (not in libraries either, with good reason). <u>These difficulties are exactly the problem that keeps the dynamics insights from taking hold in the general biochemical/medical community dealing with larger proteins.</u> My manuscript suggest a way how, without these difficulties, one can obtain at least SOME insight.

Line 280: independent movement of a domain relative to the rest of the protein produces narrower lines. This is not novel: there are countless examples, such as calmodulin, trigger factor etc.

The domain reorientation for one of the subdomains the 42 kDa Nucleotide-binding domain of Hsc70 was obtained using RDC experiments. Whether the reorientation is static or dynamic was not known (S/N was not good enough to obtain the magnitude of the alignment tensors with sufficient precision). The data obtained in Fig 8 shows with its magenta points that there is a dynamic component to that reorientation. This was not known. And to repeat the point I made before, the magenta points fall WITHIN the main distribution of experimental values. Without the calculations they would not be identified as motionally narrowed. And, I want to stress again, that these simple computations provide a way to obtain some dynamical information for large molecules the biochemical/biomedical world is interested in, for which relaxation experiments or RDC experiments are not practical, or lie beyond the expertise level of the research group.

Figure 3 does not specify the acquisition times used in the spectrum - too short acquisition times would cause artificial line broadening to a variable degree for amide protons with different intrinsic line width.

Agree – that should be mentioned. The t2 acquisition time was 136 ms. Not optimal. But it does allow a 10 Hz line (the most narrow one considered in the figure below) to decay to 1.4% of its initial value $I = I_0 \exp(-10 \times \pi \times 0.136)$. So it is OK. I need to mention that in the past Covid-year I did not have the opportunity to collect better data myself (longer acquisition time, lower temperature so that the linewidth is more dominated by the dipolar coupling than the scalar coupling, and/or using selective pulses to eliminate the JHNHA).

Legend of Fig. 1: EM and cosine not explained

Agree; it should read: processed with a 1 Hz exponential window in t2, a cosine window in t1.
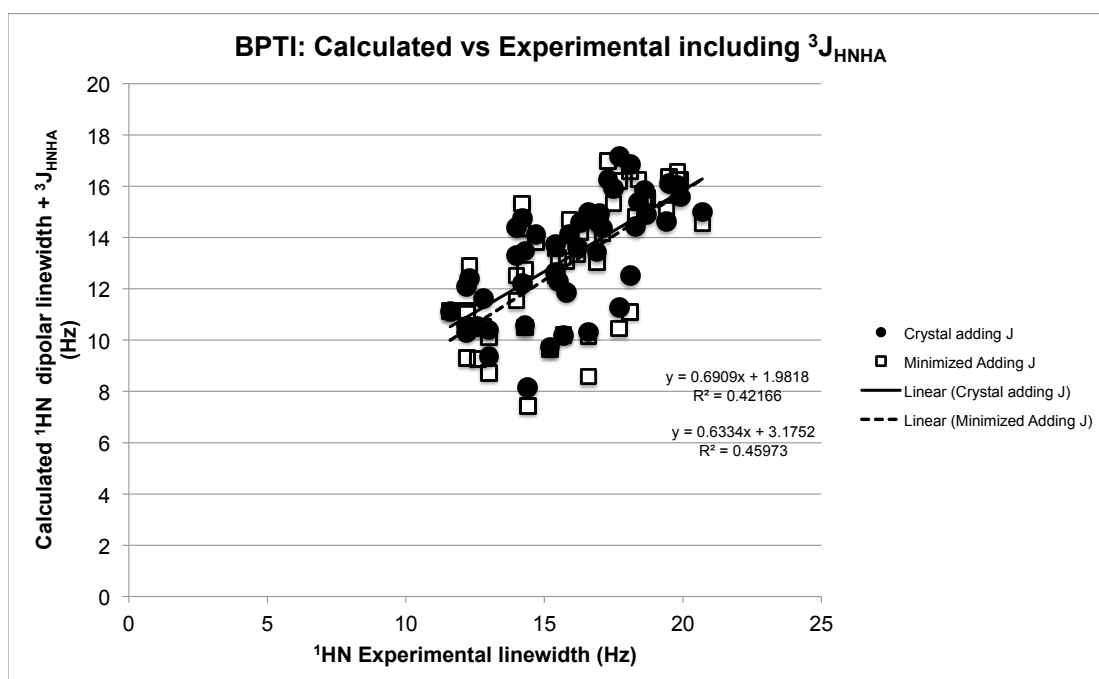
Line 57: equal to what?

Agree, it was not clear. Equal from residue to residue.

Line 94: us is not a unit.

These are micro seconds. Making sure they do not get misprinted.

Line 155: what is meant by flattered comparison?

Good point; this should have been explained. If I add the calculated scalar coupling to the calculated dipolar linewidth I obtain:

This is as one can see not too bad; but since it is dominated by the scalar coupling, we learn nothing with respect to dynamic line narrowing or line broadening.

Line 269: what is meant by ATP conformation?

Hsc70 exists in two conformations; one stabilized by ADP and one by ATP. The conformations are very close in free energy so that the "ATP" conformation is also present when ADP is bound. It is described in the reference given and the literature referenced therein.

In the opinion of this reviewer, the manuscript falls far short of the criteria of Magnetic Resonance in terms of scientific impact (absence of a clear advance) or scientific quality (wrong equations used, data mostly not provided and, hence, the conclusions of the manuscript cannot be reproduced independently). It would be unexpected if it were to pass the review process of any NMR journal.

As documented above, I disagree with reviewer in almost every respect. There is a clear advance – (some) dynamical data may become available for many more proteins. And not only the small ones most NMR spectroscopists like to work on. We disagree on the PEP scheme, but I show that reviewer is not correct. The value of the R_2 relaxation equations used is at discussion – I show here – part theory – part proof-in-the-pudding that my approach to the R_2 equations is defendable. I would love to put these arguments back into the manuscript as an Appendix. And, what does reviewer mean with "data mostly not provided "? Everything is available on the internet. The BPTI spectrum is available at BMRB, the crystal structure at the PDB, and the program, simple as it is, after I figured out that it does not have to be more complicated than that, can be provided by me. I guess very few would be interested to take up Hsc70.