I appreciate the comments of Dr. Bodenhausen, but I think he is too pessimistic about what can be learned from the HSQC. He states:

"The problems are summarized in the conclusions: "The theory of 1HN R2 for proteins is not iron-clad; issues such as "like" and "unlike" R2, "in-phase/antiphase" relaxation, "selective” and “unselective” R1 rates and cross-correlated R2 relaxation all play roles in these issues. “I cannot agree more. The main problem is that these issues cannot be resolved by establishing clear boundaries."

Generally true of course, and dealing with R2 relaxation for all protons would be too difficult. But if one focuses just on the amide protons in a HSQC, I think some boundaries are automatic. Also, the value of the approach depends which precision of analysis / correlation with experiment one is comfortable with. More about that later.

It is clear that amides and aliphatics are always un-like. Amides themselves can be like if they have the same chemical shift within the linewidth. Since we know the structure, and shifts, we can take that into account in the calculations (it will only make a real difference when they are also close in space).

In-phase / anti-phase ratios are also knowable since we can calculate the scalar coupling—but I must admit that I only thought about the length of the acquisition time, and not about the R2 itself; this will make the influence of the anti-phase term only smaller.

Reviewer states about selective/non selective R1:

"The distinction between “selective” and “unselective” R1 rates of neighbouring scalar-coupled protons (that contribute to transverse relaxation of antiphase terms) depends on the degree of saturation, the breadth of the rf irradiation (the statement “hence the 5 kHz r.f. field “hits” those HA whereas the 500Hz r.f. field does not“ does not leave any room
Reviewer is referring to the T1rho experiment. I think the r.f. argument is not relevant to the HSQC. In addition, an offset-dependent grey area for the T1rho falls, luckily, between the amide and aliphatic resonances. I am happy that reviewer seems otherwise content with the T1rho approach.

What may be an issue of concern, is what the state of the HA’s is during the data acquisition in the HSQC. Certainly I did not expect them to be uniformly in +Z, and I reasoned that they would become quickly saturated through J(0) with the other (saturated) aliphatics. Coming to think of it, it is likely better to run the HSQC with a purge pulse in the first INEPT, then everything not 15N-labeled will be saturated.

He also states:

*it is tricky to extend consideration of cross-correlated fluctuations to a manifold of densely packed neighboring protons.*

Sure, I actually started the entire project with this, because I thought it would be a real problem I could solve. I spent lots of calculations on this issue, but could show that the deviations caused by cross-correlations are not a major issue at this level of precision (Appendix).

Still, if one could be experimentally and computationally perfect, I do not believe that one will ever get a super correlation between the HSQC experiment and the calculations. After all, proteins are not static – the inter-proton vectors will fluctuate in length and direction. And small distance changes make for big relaxation changes. Despite that, I *do* get an overall agreement, with a RMSD of a ~ 2 Hz. And with that, one can define “outliers”, and the outliers are to my interest: they tell us something about the protein dynamics.

This is a philosophical argument: does one require real precision – as is the hallmark of Reviewer’s science, or is one content with progress over what was previously done (the latter being: hey, this HSQC line is narrow, it must belong to a dynamic area in the protein, and the converse). For me the progress is most important – and I show with the analyses of the HSQC of BPTI that one can indeed identify areas of conformational dynamics that correspond to what was found before with $^{15}$N relaxation methods. The latter swayed me towards accepting the imprecision, and to enjoy the progress. Reviewer does not comment on the BPTI part, which for me is the most important.
So, I hope that this may sway Dr. Bodenhausen, and that he could go along with publishing despite our philosophical differences about scientific progress (small changes of course to be made)

For me, I will continue with the T1rho, since that is the better defined experiment, and with perdeuterated proteins once I can make them again, for another publication – the timespan for the Festschrift and corona rules do not allow me to do that for this manuscript anymore.