Reply to Dmitry Korzhnev

The manuscript of Puglisi et al. describes the analysis of site-specific stability of Yfh1 based on peak integrals of the amide resonances in 1H-15N HSQC spectra. Yfh1 is a marginally stable small protein, which undergoes cold and heat denaturation at temperatures above 0C and thus represents an excellent model system to study protein folding. Building on the results of their extensive previous studies, in this paper the authors report significant differences in per-residue stability curves derived from 2D NMR for secondary structure elements and flexible loop regions.

We are very grateful to this reviewer for his encouraging words on the thermodynamic explanation offered for per-residue stability curves.

The authors offer a sound thermodynamic explanation for this behavior, which I'm tempted to believe provided they can demonstrate that variations in site-specific stability curves derived from peak integrals cannot be explained by magnetization losses during transfer periods in 1H15N HSQC experiment due to exchange line broadening, intrinsic relaxation and exchange with the solvent.

We took gladly on board the helpful reviewer's comments because we realise that, even though having compensated for non-linear effects in the 2D NMR experiments, other important factors could in principle bias our data. The main challenge of the analysis is that peak intensities are affected by many factors, and these are expected to be different in the folded and in the unfolded states. Bitterly we notice that previous thermodynamic studies using HSQC, published in very high IF journals, did not address any of these concerns and no precaution was taken either for the non-linearity or for the exchange. But this is of course another matter.
On the plus side, however, changes in transverse relaxation during the t1 and t2 periods should not affect our conclusions, since these changes should not affect peak volumes; it is the transfer periods that matter, and perhaps the possibility that the starting magnetization might be a function of temperature if the spectra were not acquired under fully relaxed conditions (they were not).
The spectra in Figure S1 clearly prove that the folded and the unfolded forms are in a slow exchange regime on the NMR time scale, where peak intensities are approximately proportional to the underlying population. We have now estimated, as the reviewer suggested, the magnetisation loss during the INEPT periods during the non-enhanced ^{15}N-^{1}H HSQC experiments in collaboration with Prof. Hansen of University College London. We assumed in our simulations a two-site exchange, kex from Bonetti et al., 2015, individual populations and delta omega from the assignment of folded state and random coil. We found that the difference of populations calculated from a numerical integration of the modified Bloch-McConnell equations at low temperature is overall small. The difference is somewhat bigger at high temperature but these data are also affected by larger error because of instrumental limitations. These estimates suggest that the 'uncertainties' caused by disregarding exchange during the INEPT period is likely smaller than the spread in Fig 1c and 1d. Therefore, our conclusions about per-residue stability curves hold.

We are certainly aware that some of these arguments should be further backed up experimentally, but a complete NMR analysis is outside the scope of the present work. Given the undeniable complexity of the multi-site system we are dealing with, our results may be the starting point for an important scientific debate which may allow the whole NMR and protein folding community to extract site-specific information about unfolding from 2D NMR spectra.