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Interactive comment on “Anatomy of unfolding: The site-specific fold stability of Yfh1 measured by 2D NMR” *by* Rita Puglisi et al.

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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. 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

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1H-15N HSQC experiment due to exchange line broadening, intrinsic relaxation and exchange with the solvent.

One likely explanation of variations in temperature dependencies of peak integrals in HSQC spectra is contribution to transverse relaxation Rex due to exchange between native (N) and denatured (D) states leading to magnetization losses during transfer periods. The authors previously reported that the rate of exchange between N and D state (kex) range from 10 to 1000 1/s in the temperature range between Tc and Tm (Bonetti et al, Phys. Chem. Chem. Phys., 2014, 16, 6391-97). Considering that protein folding is accompanied by large changes in 1HN and 15N chemical shifts (dwH and dwN) and that population of the D state (pD) of Yfh1 range from 30% at the room temperature to 50% at the midpoints of transition, exchange between N and D states may result in considerable Rex even at kex of 100 1/s or slower. Due to large dwH and dwN, magnetization losses are expected to be more pronounced for NH groups from regular secondary structure elements, leading to disappearance of their signals when approaching midpoints of transition and resulting in narrower Tm-Tc range. The only rigorous way to show these effects of N-D exchange can be neglected is to estimate magnetization losses during transfer periods for each residue by numerical simulation of magnetization evolution in HSQC sequence. The authors are well equipped to do such simulations, as they already know the temperature dependences of kex and populations of N and D states, and can estimate dwH and dwN from the differences between peak positions in HSQC spectra and predicted random-coil chemical shifts.

Magnetization losses due to intrinsic 1HN and 15N spin relaxation is another factor that has to be taken into account when considering variations in temperature dependences of peak intensities in HSQC spectra. First, relaxation losses are expected to increase with decreasing the temperature due to slowing down molecular overall rotation. Second, relaxation losses are expected to be less pronounced for the flexible loop regions having slower 1HN and 15N transverse relaxation rates. Finally, amplitudes and/or time scales of internal dynamics may change with temperature and thus contribute to

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temperature dependence of magnetization losses.

In addition to N-D exchange and intrinsic relaxation, HN peak intensities in the flexible protein regions at pH 7.5 may be affected by rapid amide proton exchange with water, which is expected to slow down with decreasing the temperature.

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