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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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I would like to contest the conclusions drawn in mr-2020-24. In a previous article in the new commercial OA journal Commun. Chem., the authors have shown that cold denaturation of the protein Yfh1 results in the disappearance of [15N,1H]-HSQC cross-peaks of backbone amides. The main conclusions of the present article are based on the observation that the NH cross-peaks of some of the residues in less ordered structural elements seem to disappear more slowly with decreasing temperature than those of buried residues. Does this necessarily imply (as the authors suggest) that these non-core residues are less prone to cold-denaturation than residues in the core of the protein? For example, Q63 appears to be in an unstructured part of the protein. How can it unfold any further? How can a residue in the RAD_0.1 group (i.e., as I

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understand, the most buried ones) be as solvent-exposed as the red ball in the top of the structure shown in Fig. 2a?

More evidence is needed to draw a conclusion that is this much at odds with common views of protein denaturation. Are there amide proton exchange data to back up the conclusions? Could the observations be explained simply as a reflection of chemical shifts that are more similar between the folded and unfolded state and, hence, less sensitive to exchange broadening? What are the exchange rates? Are the differences between residues really greater at the cold denaturation point than at the heat denaturation point? As the measurements were performed at pH 7.5, amide proton exchange with water would contribute significantly at 30 oC, i.e. cross-peaks could disappear regardless of the foldedness of the protein.

The figures and thermodynamic data of Table 1 are based on the assumption of a two-state equilibrium (folded and unfolded), but the indole resonances shown in Fig. S1 indicate the presence of more than two states. Does this not invalidate the two-state assumption?

MR strives to be a quality publication, i.e. sufficient information must be provided to validate the conclusions drawn by the authors. This implies that, even if details have been published previously, an article should be legible on its own. Which equation exactly was used to fit the amide cross-peak intensities as a function of temperature? How exactly is the fraction of folded protein derived from the cross-peak intensities? If the RAD calculations pertain to amide nitrogens, are the protein coordinates used with hydrogen atoms attached or not? Which residues exactly were determined to count as "average (RAD_0.1)"? What does RAD_0.1 actually stand for? How were the indole resonances of the unfolding intermediates assigned to W149 instead of W131? By mutation? Did the line widths of the cross-peaks vary with temperature and how did the Gaussian window used for resolution enhancement affect their intensities? Increased line widths due to chemical exchange would cause loss of 1H magnetisation during the INEPT delays of the HSQC experiment, compromising the integration of cross-

peak intensities. “The behaviour of the resonance of the exposed W131 side chain is instead fully consistent with that of RAD_0.1 and also with the original curve calculated from 1D NMR data (Pastore et al., 2007).” The curves of RAD_0.1 and the original curve look quite different in Fig. S2. What is similar about them?

There are too many instances, where basic standards of care have been violated, some of which in a rather obvious manner: The main text shows figures without any units displayed on the axes. The vertical axis of the first spectrum of Fig. S1 is improperly labelled. Full references are not given in the Supplement. The names of all parameters (Tm, Tc, DeltaH etc.) have not been spelled out. What is the vertical axis in Fig. 1? How is the reference curve determined? Where does the structure plotted in Fig. 2 come from? Fig. 4: which residues contribute to the average stability curve? The thermodynamic data of Table 1 are listed without explanation how they were obtained. Four significant digits suggest an accuracy that is hardly justified.

It is concerning that the primary data (i.e. peak volumes as a function of temperature) are not shown. The primary data hadn’t been shown in the authors’ previous article in Commun. Chem. either and there is no evidence that any new data have been recorded since.

It is suggested that special issues in MR are to fulfil the same criteria and maintain the same standard as regular articles.

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