Reply to Anonymous referee 1 (answer in bold face for clarity):
The work of Puglisi et al. deals with the observation of protein thermal denaturation processes occurring at low and high temperatures with the very interesting model of Yfh1. The authors stress the merits of 2D HSQC spectra in addressing the denaturation processes at the single-residue resolution level. This approach can surely shed light into the characteristics of the unfolding/folding transitions that may be more complex than the general all-or-none model.

We wish to thank the reviewer for these positive observations.
Indeed, as we already mentioned in our reply to the comments of Prof. Otting, our study is not isolated and addresses a problem that has been considered for more than 30 years: whether it is possible to extract sequence-specific information about the process of unfolding as recently spelled out by Grassein et al., J Phys Chem B 2020, 124:4391-4398: "Thermal protein unfolding resembles a global (two-state) phase transition. At the local scale, protein unfolding is, however, heterogeneous and probe dependent."

However, the main point the authors stress, i.e. the bipartite behavior of locally structured and unstructured residues of the protein with respect to the denaturation transitions, appears really paradoxical, as the same authors point out. The intensity or volume change of the amide resonances with temperature may well indicate an unfolding transition, but may also report different processes. It may be conceivable that flexible regions of the protein could locally anticipate the unfolding transition obtained by heating the protein, thereby providing evidence in favor of a redefinition of the all-or-none model.
However, it is difficult to imagine a protein exoskeleton of flexible or even locally unstructured residues that undergo the cold denaturation transition at lower temperatures with respect to the collapse of the main core. Which would be the driving forces for this "resilience", as the authors define the scenario? The authors do not provide any independent evidence supporting their interpretation. In my opinion, the lower temperature of the flexible or unstructured residue "transitions" could be interpreted as progressively slowing-down local exchange processes that eventually reach the intermediate exchange regime. These processes seem quite uncorrelated if one considers the spread of the curves in Figure 1d. The authors should at least rule out the possibility of local conformational exchange taking place in the statistically-disordered unfolded state that is achieved at Tc. The manuscript should be profoundly modified to be accepted for publication.

We agree by and large with the referee that it is in general difficult to deal with parts of a protein with different flexibility. Our way of reasoning was the following: precisely as we cannot simply think in terms of two-state cooperative transitions when we consider thermal unfolding, high and low temperatures are not governed by the same rules. We ourselves demonstrated that the unfolded states at low temperature are different from those at high temperature (Adrover et al., Understanding cold denaturation: the case study of Yfh1. *J Am Chem Soc.* 132, 16240-16246. (2010); Adrover et al., The role of hydration in protein stability: comparison of the cold and heat unfolded states of Yfh1. *J. Mol. Biol.* 417(5):413-24 (2012). Alfano et al., An optimized strategy to measure protein stability highlights differences between cold and hot unfolded states. *Nat. Commun.* 8,15428 (2017)).
Here, we show that the process of unfolding itself is quite different and in full agreement with the theory published by Prof. Privalov (1990). According to this theory, the driving force of heat denaturation is the increase of conformational entropy with temperature. This will automatically disfavour less ordered parts of the architecture since they were disordered to start with. They will be those less changing. On the contrary, cold

denaturation occurs when entropy is *decreasing*. In this case, the driving force of unfolding would be the sudden solvation of the hydrophobic residues of the core (P. Privalov, Cold denaturation of proteins. Crit ReV Biochem Mol Biol, 25: 281-305). As a consequence, it can happen that, while most of the (hydrophobic) core is destroyed, a few selected residues in less ordered parts are the last to change.

In support to this hypothesis is what we observed in Adrover et al., 2010: the amide protons of the cold denatured state are ALL shifted downfield as compared to the heat denatured state. This was interpreted, as also fully supported by extensive molecular dynamics calculations, as the consequence of a more dominant effect of hydrogen bonding. Since at low temperature, hydrophobic forces are weaker, hydrogen bonds with the solvent will eventually dominate over the intramolecular hydrogen bonding. The effect that we observe in the present paper, with exposed residues undergoing cold denaturation of a lower temperature could thus reflect the fact that they are already exposed and hydrogen bonded with the solvent in the folded state. As a consequence, their volumes change less readily than resonances in the hydrophobic core that experience a more rapid all-or-none mechanism.

The reviewer very helpfully suggests an alternative explanation: "the lower temperature of the flexible or unstructured residue "transitions" could be interpreted as progressively slowing-down local exchange processes that eventually reach the intermediate exchange regime." This is certainly possible, but we wonder whether we are not saying the same thing with different words. Slowing-down local exchange processes is in fact what one would expect from a decrease of entropy and the effect of hydrogen bonding involves an exchange. Please, let us know we can agree on this point. We can easily admit that the reviewer's formulation provides a more accurate description of the phenomenon in NMR terms which could be more appropriate for the audience of this journal. We would thus be happy, if the reviewer agreed, to mention both possibilities suggesting that the two formulations might result in a different description of the same phenomenon (this is not unusual when thermodynamics concepts, that are for their very nature statistics, are described at the molecular level).

Finally, the reviewer noticed that "These processes seem quite uncorrelated if one considers the spread of the curves in Figure 1d." Indeed, this is what we would expect for a process mediated by the local exchange properties of each residue.