Reply to Prof. Otting observations

First of all, we wish to thank Prof. Otting for the time spent to carefully read not only the current manuscript but also our previous production. There are however a number of possible misunderstandings which we would like to correct/clarify.

1. **The possibility to interpret the data not in terms of cold denaturation**. First of all we would like to clarify that there can be no doubt that Yfh1 undergoes cold as well as heat denaturation as extensively proven quantitatively by CD and 1D NMR data, that yielded identical stability curves and the first evidence of cold denaturation under physiological conditions (Pastore et al., *J. Am. Chem. Soc.*, **129**, 5374-5375 (2007)). Our work relies on 12 years of studies on cold denaturation and on at least 12 papers published in more than respectable **peer reviewed** journals among which JACS (4 papers), JMB (1) and Nat. Comm. (1). Our results have been independently confirmed by other laboratories (Espinosa et al., 2016; Chatterjee et al., 2014; Bonetti et al., 2014; Aznauryan et al., 2013). Thus, our data might be interpreted in several different ways but anyway within the frame of knowing that the protein denatures at cold temperatures.

2. Two-state transitions or more. We agree with the reviewer that it is difficult to interpret the behaviour of all residues in terms of a simple two-state equilibrium, but this is not a surprise. Within the last 20 years and more, several studies have reported that the general assumption of a two-state transition breaks at the level of individual residues as for instance beautifully summarised in a recent paper by Grassein et al., 2020: "Thermal protein unfolding resembles a global (two-state) phase transition. At the local scale, protein unfolding is, however, heterogeneous and probe dependent." Both CD data and averaged NMR data can be authentically interpreted with a two-state transition, but probe dependence is precisely what we observe. The different behaviour of the side chains of W131 and W149 is a clear example: W131 is exposed and its behaviour is closer to a two-state transition. The buried W149 is instead trapped in heterogenous states. Accordingly, the curve of W149 has an impossible value of ΔCp.

3. About the main conclusions of the paper and on misinterpretations. We would like to respectfully disagree with the reviewer on that "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.". In our opinion this is not "the main conclusion". We believe that the main result is the proof that cold and heat denaturation have intrinsically different mechanisms at the single residue level. This is a very important aspect that was extensively discussed on theoretical bases by Prof. Privalov (P. Privalov, Cold denaturation of proteins. Crit ReV Biochem Mol Biol, 25: 281-305) but was not validated experimentally because of the obvious difficulties of cold denaturation studies. One of the corollaries of our results is that, surprisingly, "some of the residues in less ordered structural elements seem to disappear more slowly with decreasing temperature than those of buried residues". This observation can indeed seem strange as we mentioned ourselves in the manuscript. We interpreted it by remembering that the main driving

force of the heat denaturation mechanism is the increase of conformational entropy with temperature. This will automatically involve less ordered parts of the architecture in the unfolding process. On the contrary, cold denaturation occurs when entropy is *decreasing*, and the main driving force will 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 may happen that, while most of the (hydrophobic) core is destroyed, a few selected residues in less ordered parts keep some form of ordering. We are well aware that alternative interpretations are possible and are happy to discuss them if this can be done in a positive and constructive way (see also answer to referee 1).

4. On the possibility to use different approaches. We certainly value alternative approaches such as those alluded to by the reviewer (exchange data, chemical shift differences, exchange rates, etc…). We indeed discussed at length the interesting chemical shift differences observed between the cold and heat denatured states in Adrover et al., JMB 2012. However, in general these techniques pale with respect to accurate thermodynamic data calculated from the stability curves. The crucial point for studying cold denatured ensembles is the possibility to measure the full stability curve. This is possible whenever ΔCp can be measured directly. Using any experimental spectroscopic technique this is not possible if cold denaturation is not accessible. It is easy to prove that fitting high temperature dependence of denaturation data is completely insensitive to the value of ΔCp. When cold denaturation is accessible the value of ΔCp can be measured not just predicted, on the basis of protein composition.

5. On the technical quality of our manuscript. As for the inaccuracies in the manuscript, we ensure the reviewer that we are not new to publish and that the "unacceptable" quality of the figures comes simply from the inexcusable mistake of inserting in the final manuscript the only preliminary and incomplete versions of the figures. We have now replaced them with the final ones. We also agree that papers need to be largely (although not completely) self-explanatory but there are concepts, such as the definition of concepts such as ΔH that must be common to the whole readership of this journal. We anyway sincerely thank the reviewer for bringing to our attention some unnecessary short-cuts and have done our best to improve the text. We will provide independently a detailed point-to-point list of all the changes introduced in response of the reviewer's comments.

5. On the differences of this manuscript from former work. Finally, we wish to clarify that the stress of our previous Commun. Chem. article and the present work is completely different. We wanted to explore on the compatibility of CD and NMR spectroscopies in monitoring protein unfolding and were able to show that a judicious choice of buried residues and their averaging yields almost identical thermodynamic parameters as those obtained by CD, a technique in which signal averaging is intrinsic. NMR averaging should thus not be obtained from single residues, as it can be found in the literature. After publishing the article, it occurred to us that, rather than "throwing away" the residues that misbehave from the average, we could get detailed information on why they deviate. The expert

reviewer will certainly agree with us that one of the main strengths of NMR on other techniques is to provide information at the single residue level. This is the genesis of the present work.

We will submit a revised version of the manuscript as soon as allowed by the editor.