

Magn. Reson. Discuss., author comment AC1  
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## Reply on RC1

Christopher A. Waudby and John Christodoulou

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Author comment on "Analysis of conformational exchange processes using methyl-TROSY-based Hahn echo measurements of quadruple-quantum relaxation" by Christopher A. Waudby and John Christodoulou, Magn. Reson. Discuss.,  
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We are grateful to the reviewer for their careful reading of our manuscript, and for the helpful comments they have provided. We provide a point-by-point response to these below, to be incorporated into a revised manuscript.

1. We assume that conformational exchange processes can be well represented by chemical exchange between discrete states, described by relative populations and forwards and backwards rate constants, and that each methyl within a state can be associated with defined  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts. Exchange between two states is therefore associated with both  $^1\text{H}$  and  $^{13}\text{C}$  chemical shift differences, both of which are perfectly correlated. However, we certainly do not exclude the possibility of additional states, associated with distinct conformational fluctuations. Indeed, we already point out (l. 311–314) that  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts have differential sensitivity to effects such as rotamer changes and aromatic ring currents. Where different exchange processes affect different regions of a molecule, measurements of the timescale of exchange or population of the minor state using CPMG or similar experiments can be used to identify and distinguish them (as in the present example). Where a single methyl undergoes multiple exchange processes, we also demonstrate, at least theoretically, how the analysis of field-dependent MQ HE measurements may be used to identify this (l. 253–256 and Fig. S5). However, we are happy to clarify this point in the revised manuscript, particularly in relation to the referenced work on T4 lysozyme.

2. We are happy to rephrase this in the revised manuscript – we are not trying to suggest that the present work provides an approach to determine the absolute sign.

3. We are happy to mention this in the revised manuscript.

4. We are grateful to the reviewer for spotting this error – the text should refer to the four-spin double quantum transition (DQ') for L701CD1.

5. The relative intensity of inner and outer lines depends on  $J(0)$ , while the relative intensity of upfield and downfield lines depends on the product of  $J(0)$  with the  $^{13}\text{C}$  CSA. Because we are performing a parametric estimation (pseudo-3D lineshape analysis), the strong decay of outer lines even with no relaxation delay provides a strong constraint on  $J(0)$ , which we have previously validated up to correlation times of ca. 100 ns (Waudby et al., 2021, J Magn Reson). The slowly relaxing inner lines are still readily observed for such

cases, allowing access to the  $^{13}\text{C}$  CSA. Because peak positions can already be determined from a decoupled 2D spectrum, and  $^1\text{JCH}$  scalar couplings are more or less uniform, the issue of overlap is also not as severe a problem as might be expected: in a manuscript under preparation, we have successfully applied a similar analysis to a 45 kDa protein. However, it is true that this approach will undoubtedly reach a limit, at which point the pseudo-4D approach may be helpful to resolve overlap. Alternatively,  $J(0)$  could be measured using a separate experiment and held constant during lineshape fitting to determine the  $^{13}\text{C}$  CSA. We are happy to include a more detailed discussion of this in the revised manuscript.

6. We are grateful to the reviewer for spotting these typographical errors, which have been corrected in the revised manuscript.

7. We will label the figure as suggested.

8. The absolute sign has not been determined, only the relative sign of  $^1\text{H}$  and  $^{13}\text{C}$  chemical shift differences. We are grateful to the reviewer for pointing this out and have added a note to the legend to clarify this important point.

9. We are happy to clarify this: we have indeed used a non-stereospecific  $^{13}\text{CH}_3/^{12}\text{CD}_3$  labelling scheme for Leu/Val residues.

10. The fitted slope is  $0.861 \pm 0.096$ , such that the deviation of the slope from 1 is  $0.139 \pm 0.096$  (a tension of 1.4 sigma). Similarly, the fitted offset is  $0.93 \pm 0.98$  (a tension of 0.95 sigma). There is also a strong negative covariance between estimates of the slope and offset. Therefore, we do not believe there is evidence of significant systematic deviations between the two methods. However, we are happy to include a discussion of this point in the revised manuscript.