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Interactive comment on "Towards resolving the complex paramagnetic NMR spectrum of small laccase: Assignments of resonances to residue specific nuclei" *by* Rubin Dasgupta et al.

Anonymous Referee #1

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The manuscript describes the assignment of the paramagnetic NMR spectrum of a small laccase protein, containing a trinuclear copper center. The spectrum has been previously reported, but, In this study, the authors perform the assignment of all hyperfine shifted resonances and succeed in rationalizing the patterns of signals due to chemical exchange processes and to the occurrence of two different redox states: the resting oxidized state and the intermediate state, each of them characterized by peculiar and distinctive features in terms of electron relaxation rates and contact chemical shift ranges. Finally, due to the chemical shift differences observed between the WT protein and Y108F variant, the authors propose the presence of a conformational exchange, involving hydrogen bonds of His 102, which would justify the observed chem-

ical exchange phenomena, and identify at the molecular level the nature of the previously evoked mobility of active site residues. The manuscript is well written, with a very high level of technical skills and a clear description of the results. I recommend its publication, provided the authors addressed a few comments listed below. Also, I would suggest to summarize the assignment of signals as well as the correlations arising from chemical exchange and from the two states, the NI state and the RO state, in a single Table. Comments: Abstract, line 21. Although it is explained in the introduction section, the expression "T2 histidines" in the abstract is unclear. Please replace it with "histidine residues belonging to the type-2 copper site". Abstract, lines 21-24. The last sentence of the abstract is not clear. Perhaps you might replace it with "This study demonstrates the utility of the approaches used for the sequence specific assignment of the paramagnetic NMR spectra of ligands in the TNC that ultimately may lead to a description of the underlying motions"

line 49, line 69, etc: replace "coppers" with "copper ions" [please spell check throughout the text]

lines 84-85: same as lines 21-24.

Line 96: The evolution period was shortened to 500 μs, balancing the time required for formation of antiphase magnetization and paramagnetic relaxation, to optimize S/N ratio for most of the resonances. Actually, previous works on this aspect (such as "I. Gelis, et al; A simple protocol to study blue copper proteins by NMR, Eur. J. Biochem. 270, 600–609, 2003" or "S. Ciofi-Baffoni et al; The IR-15N-HSQC-AP experiment: a new tool for NMR spectroscopy of paramagnetic molecules, Biomol NMR, 58:123-128, 2014") should be quoted here.

Lines 97-100: The identification and numbering of hyperfine shifted resonances of the spectrum have been reported in Dasgupta et al, 2020. Probably this should be clearly stated prior to the description of the current results. In the present version, the peak numbering appears unjustified (where are peaks 1 and 2?, why 10 is missing?...),

while a statement such as “The 1H NMR spectrum of SLAC-T1D has been previously recorded, 18 signals were identified between 60 and 15 ppm (REF)” would help the reader to follow the story. Furthermore, also the fact that the three pairs of resonances 3-5, 9-11, 13-12 arise from exchange process arise from Dasgupta et al (2020), is not explicitly mentioned here.

Line 107: “SLAC-T1D is predominantly in the NI state,” should be modified in “The relative intensities of signals in the range 60-22 ppm with those of the region 21-12 ppm shows that SLAC-T1D is predominantly in the NI state”

Line 108: replace “unpaired electrons” with “unpaired electron spin S”

Line 137: “..i.e. the chemical shift increases with an increase in temperature (Bertini et al., 2017, 1993; Bubacco et al., 2000; Tepper et al., 2006).” Here, the reference Bertini et. 2017 might be also replaced with “Banci, L., Bertini, I., and Luchinat, C., The 1H NMR parameters of magnetically coupled dimers -The Fe2S2 proteins as an example, Struct.Bonding, 72, 113-135, 1990”

Figure 2a: In the EXSY/NOESY shown in Figure 2a, some of the cross peaks are apparently quite asymmetric. This is clearly seen for upper- and lower-diagonal cross peaks e2/e1 but also peaks a2/a1 appear unequal. Is this due to the fact that the observed cross peaks (especially e2/e1) are barely detectable from the noise or there is a significant asymmetry in the relaxation properties of these peaks?

Line 178: please replace “in loss of copper or at least severe redistribution of unpaired electron density,” with “in loss of copper or at least in a severe redistribution of unpaired electron density,”

Lines 246 and 248: crystal structures 6S0o and 3cg8 should be better called according to the different organism and pdb number.

Lines 287-292: I would not recall Figures 1-4 in the conclusion section. Indeed, these Figures have been extensively discussed throughout the result section; in the final

recap there is no need to go back to the Figures.

Line 293: please replace “the first sequential assignment of the resonance” with “the first sequential assignment of the paramagnetically shifted resonances”

Lines 293-295: I would suggest to somehow smooth the last sentence. Indeed, the blind sphere around the polynuclear copper center might involve many residues besides those that are bound to the T1, T2 and T3 centers. I wonder whether mutagenesis of second shell residues would be enough to provide a full sequence specific assignment.

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