Responses to RC1

To RC1:

Specific comments:

• *line 34* and the first introductory paragraph - Although the SAIL approach certainly facilitates the characterization of lysine residues, I do not agree that the information "could not be obtained by other methods" and that there only been a "only a few systematic NMR studies" on lysines. Indeed, an inspection of the BMBR (https://bmrb.io/ref_info/csstats.php) shows extensive chemical shift assignments for lysine ^{1}H, ^{13}C and ^{15}N nuclei. Also, a simple Web of Science search for "lysine and NMR" yields 4486 results.

Response: Thanks for your comments on this critical issue. What we emphasized in the abstract, as well as in the first introductory paragraph, was that the comprehensive ^{1}H, ^{13}C and ^{15}N chemical shift information for Lys sidechain moieties in a protein with unambiguous sequential (for the ^{1}H, ^{13}C and ^{15}N-signals) and stereospecific (*prochiral* methylene ^{1}H-signals) assignments for the sidechain, which could not be efficiently established without using stereospecific deuteration, gives us a unique opportunity to characterize both their conformational features and ionization states. Although there are indeed numerous ^{1}H-NMR chemical shifts of Lys sidechain methylenes in BMRB depositions, as the RC1 correctly pointed out, most of them are classified by Ambiguity Index Value (AIV) “2”, which means their stereospecific ^{1}H-signal assignments are provisional. Only a few methylene proton chemical shifts are classified by AIV “1”, which indicates that the chemical shifts of the methylene protons are either incidentally identical or tentatively assigned by the grid search approach, including automatic NOESY assignment algorithms such as CYANA. Stereospecific deuteration removes all of the ambiguities associated with the stereospecific assignment of prochiral groups. The structural information obtained from the streospecifically assigned single methylene proton (*CHD) can lead to unambiguous structural information, even in cases where the (CH_2) protons would have had equivalent chemical shifts. Therefore, at this moment, the comprehensive stereospecific deuteration of Lys methylene chains, implemented by the SAIL method, is the only practical approach to establish the unambiguous stereospecific ^{1}H-signal assignments for all Lys side chains. Although we believe that the unbiased stereospecific assignments for the prochiral methylene protons are absolutely prerequisites for precise characterizations of Lys sidechain moieties, we probably overemphasized the benefit of the SAIL approach. We therefore have toned down the expression related to this issue.

• *lines 74, 256 and 263* - It would be worth emphasizing (rather than unnecessarily downplaying) that the $^{15}N^{\zeta}$ and $^{13}C^{\delta}$ (but not the $^{13}C^{\varepsilon}$) actually serve as an excellent reporter nuclei for determining the ionization states and, when monitored as a function of pH, the pKa values of lysine residues in proteins (Gao et al. J.A.C.S. 128:8104; reviewed in Platzer et al. J. Biomol. NMR 60:109). This can

be seen for Lys66 in Table 1.

Response: We certainly have no intention at all to downplay the previous approaches cited at lines 74, 256, and 263. Therefore, in the abstract we included our deuterium shift data for the $^{15}N^{\zeta}$, together with the $^{13}C^{\varepsilon}$ data, in order to emphasize their values for characterizing the ionization states of Lys ζ-amino groups. To avoid statements that seem to unintentionally downplay the previous work, we deleted lines 258-260 and lines 267-273 from the original manuscript.

• l*ine 100* - Could the authors comment on the use of an E. coli lysine auxotroph (lysA) to improve labeling efficiency (Waugh J. Biomol. NMR 8:184)?

Response: We have not tried to use the *auxotrophic E. coli* strains, since the current level of isotope enrichment (~70 %), which could be obtained by using the *standard E. coli* expression system, was sufficient for the present work.

• *Figure 1* - Given the importance of the $^{15}N^{\zeta}$ signals, it might be useful to also include the HECENZ spectrum of SNase in this figure, or as a supplemental figure.

Response: We added Figure A4 in the Appendices.

• *line 223 and elsewhere* - Although often referred to as "hydrophobic interactions" (i.e., a non-specific exclusion from water), the specific packing of the lysine sidechains within SNase is better described as resulting from "van der Waals" interactions.

Response: Although we meant *specific* hydrophobic interactions, we replaced “hydrophobic interactions” with “van der Waals interactions”.

• *Line 307* - The pH-dependent deuterium isotope shifts of lysines were investigated by Led and Petersen J. Mag. Res. 33:603 (see also Led et al. J. Mag. Res. 20:530, Tomlinson et al. J.A.C.S. 131:4674, and reviewed in Platzer et al. J. Biomol. NMR 60:109).

Response: We have cited these references.

• *abstract, section 3.3*., and lines 411 to 413 - Certainly an interesting and most emphasized result from this study is that deuterium shifts of the lysine $^{13}C^{\varepsilon}$ signals appear to depend on the ionization states of the ζ-amino group (~ -0.3 ppm for $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_3^+$-$N^{\zeta}H_3^+$] versus ~ -0.2 ppm for $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$]). Although consistent with the results reported by Led and Petersen J. Mag. Res. 33:603, it is not clear than such small isotope shifts can provide unambiguous evidence for the ionization states of lysines in proteins. The shifts were only measured at a single sample pH/pD value (where all lysines in SNase are positively charged, except for the sole Lys66 being neutral). To draw this conclusion, the isotope shifts should be monitored over the course of pH/pD titrations. Furthermore, the value of ~ -0.2 ppm for $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$] was derived from only Lys66, which has a very perturbed pKa value of 5.7 due to its burial within SNase. An unusual environment may well cause an unusual isotope shift. Indeed, for the protonated amines, the $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_3^+$-$N^{\zeta}H_3^+$] values in Table 2 span from -0.39 ppm

to -0.28 ppm, i.e. a range of 0.11 ppm. The causes for such a range might be worth commenting upon. However, if $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$] spanned a similar range, then the deuterium isotope shifts for protonated versus neutral lysines would overlap and could not be used to unambiguously determine their ionization states. Accordingly, it would be very useful if the authors could measure the $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$] values for SNase at pH/pD > 12.5. These could be extracted from two 1D spectra of [ε-^{13}C; ε,ε-D_2]-Lys labeled SNase as 2 assignments would not be necessary. The goal would be to determine the range of $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$] values exhibited for neutral lysines in a protein. Admittedly, this could be challenging as the stability of SNase falls off rapidly at pH > 10 (Garcia-Moreno et al. Biophys. Chem. 64:211). Alternatively, numerous variants of SNase with buried lysines having pKa values in the accessible range of 5 - 9 could be investigated (Isom et al. P.N.A.S. 108:5260). Of course, this may be beyond the scope of this paper. Nevertheless, the authors should discuss the concerns outlined above when effectively proposing that small deuterium isotope shifts are more reliable and easily measured than very diagnostic $^{15}N^{\zeta}$ and $^{13}C^{\delta}$ chemical shifts for determining lysine charge states. In the end, these approaches (and others, such as measuring $^{15}N^{\zeta}$-$^{1}H^{\zeta}$ scalar couplings) provide complementary insights that collectively re-enforce a more complete view of the structural, dynamic and electrostatic properties of lysines in proteins.

Response: The comprehensive pH-dependent measurements are, indeed, beyond the scope of this study. The isotope shifts of all but K66 are close to that expected for a charged amino group. As a matter of fact, the averaged deuterium isotope shift values for the Lys $^{13}C^{\varepsilon}$ attached to protonated and deprotonated ζ-amino groups at 150 MHz (^{1}H: 600 MHz), which were -0.32 +/- 0.02 ppm (- 48 +/- 3 Hz) and -0.21 ppm (-31.5 Hz), were -0.11 ppm (16.5 Hz) and substantially larger than the experimental errors. The chemical shifts for the outlying, and thus well-separated, residues; i.e., K78, 136, 133 and 9 shown in Figure 5, can be accurately determined as compared to the other residues in the severely crowded region, for which $\Delta\delta^{13}C^{\varepsilon}$[$N^{\zeta}D_3^{+}$-$N^{\zeta}H_3^{+}$] was in the range of -0.28-0.30 ppm (Table 2). The averaged deuterium shift, -0.29 +/- 0.01 ppm (-44 +/- 1.5 Hz), is still substantially larger than the -0.21 ppm (-32 Hz) $\Delta\delta^{13}C^{\varepsilon}$[$N^{\zeta}D_2$-$N^{\zeta}H_2$] value observed for K66. Although there is only one residue, K66, with a deprotonated ζ-amino group at pH/pD 8.0 in the SNase variant, we believe that the deuterium-induced $^{13}C^{\varepsilon}$ chemical shifts provide a useful auxiliary index to $^{15}N^{\zeta}$; i.e., -1.1 ppm vs. -1.8 ppm (Table 2), for protonated and deprotonated ζ-amino groups. Although there are only a few Lys residues with deprotonated ζ-amino groups at physiological pH values, they are likely to have specific biological functions. Even though we do not have additional experimental data at this moment, one can easily identify the Lys $^{13}C^{\varepsilon}$ just by comparing the 1D ^{13}C-NMR spectra of a protein selectively labeled with [ε-^{13}C; ε, ε-D_2]-Lys, dissolved in H_2O and D_2O. Therefore, the deuterium shift for the Lys-$^{13}C^{\varepsilon}$ signals will serve as a versatile screening probe for detecting such unusual Lys residues, even in larger proteins.

<u>Technical corrections:</u>

• *lines 29 and 89* - "contains as many as 21 Lys resides" could be replaced with "contains 21 Lys residues".

Response: We deleted “as many as”.

• *line 66* - It would useful to provide references for cases where the "ionization states of Lys ζ-amino groups in a protein have been characterized by X-ray crystallography." Except with neutron or ultra-high resolution X-ray crystallography, the ionization states of lysines are generally inferred from physical arguments.

Response: What we meant by this phrase is that X-ray crystal data cannot discriminate the NH_3^+ and NH_2 ionization states experimentally. It is obvious that these states are inferred from the distances and angles of the related heavy atoms, rather than from the hydrogen atoms bound to the ζ-amino nitrogen.

• *lines 99 and 100* - It would help to include the reference (Terauchi et al., 2011) for the source of the SAIL-Lys, which presumably the authors prepared in-house (or is it commercially available?). Similarly, what were the sources of the [U-^{13}C,^{15}N]-Lys and [ε-13C; ε,ε-D2]-Lys? Also, were these pure L-enantiomers or DL-racemic mixtures?

Response: We included the reference here, as well as in section 2.1, and specified that all of the labeled Lys are pure enantiomers.

• *line 111* - "labeling rates" could be replaced by "enrichment levels". • line 196 - "outrageous" could be replace by "outlying".

Response: We followed the suggestions.

• *line 258* - "Lys residues, given that ..." could be replaced by "Lys residues. However, the ..." • line 391 - "exchange rate constant (kex)", rather than "exchange rate (Kex)"

Response: We followed the suggestions.

• *line 393* - s-1, rather than S-1 • Table A1 - Dihedral angles should be rounded off to reflect realistic precision (i.e., not to 0.1o).

Response: We agree with the comments.