Responses to RC1, RC2 and RC3

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.

Technical corrections:

- *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.

To RC2:

I can join the gerenal comments by referee 1. My main concern is the one-bond deuterium isotope effect on ^{15}N chemical shifts of Lys66. This is probably not fully protonated juged from the isotope effects (see below). Most interactions will lead to a decrease compared to the free lysines (Williamson, Chem.Commun. 49, 9824, 2013). On the other hand it is too large to be -ND_2 as the effets of amines are of the order or 0.7 ppm (Lycka, 23, 973, 1985). As the experiments are done a a Shigimi tube and from what I can tell no special precautions are taken to take into account the difference in pK_a values in H_2O and D_2O, one could fear that part of the large effect is caused by a change in the equilibrium due to deuteriation, as the pH is in the vicinity ot the pKa value. Therefore, I strongly recommend that the measurements are repeated in a single tube and with varying amounts of D_2O to obtain the isotope effects.

Response: The ζ-amino group of Lys66 in the RNase variant, which is known to have a pKa of 5.7 as indicated in lines 87-88 (García-Moreno et al., 1997; Fitch et al., 2002), is *not at all protonated but completely deprotonated* at pH/pD 8.0, where all of the NMR data were obtained. The deuterium isotope shifts, $^{1}\Delta^{15}$N ppm, for the $^{15}N^{\zeta}$ signals in barnase (Williamson et al., Chem. Commun., **49**, 9824-9826, 2013; we cite this paper), correspond to the differences between $\Delta\delta[\zeta\text{-}^{15}NH_3^+\text{-}\zeta\text{-}^{15}NH_2D^+]$ and $\Delta\delta[\zeta\text{-}^{15}NH_2D^+\text{-}\zeta\text{-}^{15}NHD_2^+]$. Therefore, the previously reported averaged isotope shifts, 0.357 ppm, should be tripled (i.e., 1.071 ppm) for comparison to the value of 1.1 +/- 0.1 ppm shown in Table 2 in the manuscript (*page 17*). Obviously the two values are almost identical within the errors, even though their data were obtained at pH 4.8. The deuterium isotope shifts reported by Lyčka and Hansen (Magn. Reson. Chem., **23**, 973-976, 1985) are actually not appropriate model compounds. However, as mentioned above, the ζ-amino group of Lys66 at pH/pD 8.0, which is 2.3 pH units higher than the pKa value, should be completely deprotonated as clearly demonstrated by Takayama et al. (J. Am. Chem. Soc., 130, 6714-6715, 2008). Therefore, the isotope shift for the ζ-amino group of Lys66 should correspond to $\Delta\delta[\zeta\text{-}^{15}ND_2\text{-}\zeta\text{-}^{15}NH_2]$. Since the ζ-^{15}N deuterium shifts for all 21 Lys residues measured in H_2O-D_2O (1:1) appeared exactly in the middle of the spectra obtained in 100% H_2O (no D_2O contamination since we used a dual-tube) and 100% D_2O, the fractional factors for the isotopomers are within statistically random distributions (*lines 394-401*).

To RC3:

Takeda, Kainosho and co-workers present an in-depth investigation of lysine residues in a variant of SNase that has been engineered to harbor a lysine residue in its core, of which the pKa constants i shifted by about 5 units to below physiological pH. They make elegant use of their SAIL technology for stereo-specific protiation/deuteriation of the side chain, and demonstrate several approaches for the investigation of side chain conformation and charge state. The work displays a high degreee of technical rigor and is well-documented. The paper is also examplary when it comes to scholarly presentation and quality of illustrations. At the same time it is unclear to what extent the presented methods are going to alter the ways in which Lys pKa constants are studied, and the suggestion that isotope shifts on the 13Cε might be used as a proxy for Lys charge state is not yet sufficiently substantiated. It is clear from published data, as well as from results in the report that the chemical shifts of various side chain 13C and 15N atoms emerge as very powerful reporters. This has previously convincingly been demonstrated by André et al (Andre et al. JACS 2007; https://doi.org/10.1021/ja0721824), and also by the group of R.E. London (Gao et al. JACS 2006; doi: 10.1021/ja061473u) and it would appear that these methods would remain those of choice? The current work does, however, clearly point a path forward. As the Kainosho group has clearly demonstrated, access to partially deuteriated side chains displays significant improvements for higher molecular weight proteins, where the methods mentioned before will probably fail. There, utilization of SAIL Lys can present significant advances. Gauging charge state of amino acids based on chemical shifts alone presents a small uncertainty, but it is unclear at this point that isotope shifts would prove more reliable. Ultimately,either (a) the method put forward by McIntosh and co-workers (Poon et al. JACS 2006; https://doi: 10.1021/ja065766z) where the multiplet pattern of the amino groups is observed, or (b) observing a titration in the 13C or 15N shifts in the HECENZ NMR experiment would remain the unambiguous approaches. Therefore, I think that the title might do better justice when "revealed" would be replaced by "studied" or "investigated". In similar vein, the abstract might be overly optimistic to state that the isotope shift "will" be a powerful tool - possibly it "might" (Indeed on p18, the authors use a more cautious formulation). Also the 13C 1D spectrum of ε-[13C,d2]-Lys is not as dispersed as one would wish, and for larger proteins than SNase may present severe shortcomings when compared with 2D NMR. It is unfortunate that the authors were not more successful with HECENZ experiments using SAIL-Lys (as judged from Table 1, where several 15Nζ shifts are missing).

Response: Thanks for the comments. According to the related comments by RC1 on the HECENZ experiment, we added the spectrum (Fig. A4) in the Appendices. As one can see from the spectrum (Fig. A4a), we could actually determine the $^{15}\mathrm{N}^{\zeta}$ chemical shifts for all of the 21 Lys residues in the SNase variant, although some of them are nearly identical. It should be noted that the $^{1}\mathrm{H}^{\varepsilon 2}$-$^{15}\mathrm{N}^{\zeta}$

correlation signals in the HECENZ spectrum for the residues that showed isolated signals in both the 1D $^{13}C^{\varepsilon}$ spectrum (Fig. 4a) and 2D $^{1}H^{\varepsilon 2}$-$^{13}C^{\varepsilon}$ spectrum (Fig. A4b); i.e., K66, 78, 136, 133 and 9, are clearly separated from the congested spectral region. As stated in the revised abstract, those residues are likely to exist in an unusual local environment and might have peculiar ionization states. The deuterium-induced isotope shifts for $^{13}C^{\varepsilon}$ and $^{15}N^{\zeta}$ of these residues could be accurately estimated, as compared to the other residues. We didn't include the $^{15}N^{\zeta}$ chemical shifts for K5, 6, 63, 70 and 97 in the previous Table 1, since their sequential assignments were not firmly established due to the incidental signal overlapping of their $^{1}H^{\varepsilon 2}$-$^{13}C^{\varepsilon}$ signals. The isotope shifts for the $^{15}N^{\zeta}$ data of these five residues were excluded in Table 2, since the uncertainties were larger than those for the other residues.

Although the manuscript is very clearly written, I have a few textual comments:

p5 l111 - when referring to "standard protocol", a reference should be given

Response: We cited the paper by Isom et al., describing the expression and purification protocol for SNase variants.

p5 l111 - labelling "rates"; do the authors refer to incorporation level?

Response: Yes, but we replaced "labeling rates" by "enrichment levels".

Methods section - the chemical shift referencing procedure is missing. Was it IUPAC (Markley et al) or Bruker?

Response: We described the chemical shift referencing procedure in section 2.1 and 2.2.

p6 l196 - "outrageous" probably means "outlier"? I urge the authors to be more transparent about how this was done

Response: According in BMRB deposition, “outliers” are defined as those with > 8 σ from the mean of the chemical distribution histograms. Therefore, we deleted this misleading expression and recalculated the averaged chemical shift values in Table 1 by excluding those for Lys-66.

p12 Could the ring currents that are discussed possibly be predicted from the structure, and utilized?

Response: The discussion about the aromatic ring current shifts for the Lys sidechain NMR signals is not quantitative. We just compared the high-field shifted signals to the crystal structure of the SNase variant. We are hoping to measure the NOEs between the Lys-chain and aromatic ring proton signals using SNase simultaneously labeled with SAIL-Lys and SAIL-Tyr/Trp. With such *quantitative* data, the comparison between the aromatic ring current shifts predicted by the crystalline structure and the NMR data would be interesting.

p12 l257 - rather than being "quite useful" is would appear that 15Nζ shifts would be "decisive" or "unambiguous"?

Response: We fully agree with RC3 that the $^{15}N^{\zeta}$ shifts, if accurately measured and sequentially assigned, would be "decisive" information for the ionization states of the Lys ζ-amino groups. Nevertheless, the present method using $^{13}C^{\varepsilon}$ isotope shifts would still serve as a useful initial step to characterize the residues showing distinctive $^{13}C^{\varepsilon}$ signals for an [ε; ε,ε-D_2]-Lys labeled protein, which may have deprotonated Lys residues under certain physiological conditions, possibly due to an unusual local environment stabilizing the deprotonated ζ-amino group.