Conformational features and ionization states of Lys side chains in a protein revealed by the stereo-array isotope labeling (SAIL) method

Mitsuhiro Takeda[1,2], Yohei Miyanoiri[1,3], Tsutomu Terauchi[4,5], and Masatsune Kainosho[1,5*]

[1]Structural Biology Research Center, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8602 Japan; [2]Department of Structural BioImaging, Faculty of Life Sciences, Kumamoto University, 5-1, Oe-honmachi, Chuo-ku, Kumamoto, 862-0973 Japan; [3]Research Center for State-of-the-Art Functional Protein Analysis, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka, 565-0871 Japan; [4]SAIL Technologies Co., Inc., 2008-2 Wada, Tama-city, Tokyo, 206-0001 Japan; [5]Graduate School of Science, Tokyo Metropolitan University, 1-1 Minami-ohsawa, Hachioji, Tokyo, 192-0397 Japan

[*]Correspondence should be addressed to:
Masatsune Kainosho, Ph.D.
Graduate School of Science, Tokyo Metropolitan University, 1-1 Minami-ohsawa, Hachioji, Tokyo 192-0397, Japan
E.mail: kainosho@tmu.ac.jp

Dedicated to Professor Robert Kaptein on the occasion of his 80th birthday.

Abstract

Although both the *hydrophobic* aliphatic chain and *hydrophilic* ζ-amino group of the Lys side chain presumably contribute to the structures and functions of proteins, the *dual* nature of the Lys residue has not been fully investigated by NMR spectroscopy, due to the lack of appropriate methods to acquire comprehensive information on its long consecutive methylene chain. We describe herein a robust strategy to address the current situation, using various isotope-aided NMR technologies. The feasibility of our approach is demonstrated for the Δ+PHS/V66K variant of *Staphylococcal* nuclease (SNase), which contains 21 Lys residues, including the engineered Lys-66 with an unusually low pK_a of ~5.6. All of the NMR signals for the 21 Lys residues were sequentially and stereo-specifically assigned by using the stereo-array isotope labeled Lys (SAIL-Lys), [U-^{13}C,^{15}N; β_2,γ_2,δ_2,ε_3-D_4]-Lys. The complete set of the assigned ^{1}H-, ^{13}C-, ^{15}N-NMR signals for the Lys sidechain moieties affords various structural information, for example, relative orientations of the Lys sidechains against nearby aromatic rings. The $^{13}C^{\varepsilon}$ and $^{15}N^{\zeta}$ chemical shifts of the SNase variant selectively labeled with either [ε-^{13}C; ε,ε-D_2]-Lys or SAIL-Lys dissolved in H_2O and D_2O showed that deuterium induced shifts for Lys-66 were substantially different from the rest of the Lys residues. Namely, the deuterium-induced shifts of the $^{13}C^{\varepsilon}$ and $^{15}N^{\zeta}$ signals depend on the ionization states of the ζ-amino group; i.e., -0.32 ppm for $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_3^+$-$N^{\zeta}H_3^+$] *vs.* -0.21 ppm for $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$], and -1.1 ppm for $\Delta\delta^{15}N^{\zeta}$[$N^{\zeta}D_3^+$-$N^{\zeta}H_3^+$] *vs.* -1.8 ppm for $\Delta\delta^{15}N^{\zeta}$[$N^{\zeta}D_2$-$N^{\zeta}H_2$]. Since the 1D-^{13}C NMR spectrum of a protein selectively labeled with [ε-^{13}C; ε,ε-D_2]-Lys shows extremely narrow (> 2 Hz) and well-dispersed ^{13}C signals, the deuterium-induced isotope shifts difference of 0.11 ppm for the protonated and deprotonated ζ-amino groups, which corresponds to 16.5 Hz at a field strength of 14 tesla (150 MHz for ^{13}C), will be a versatile index for searching the Lys residues having deprotonated ζ-amino groups at physiological pHs in larger proteins containing numerous Lys residues.

1 Introduction

Detailed studies on the structures and dynamics of the Lys residues in a protein have been severely hampered by the difficulty in gathering comprehensive NMR information on their side chain moieties. It is especially challenging to establish *unambiguous* stereo-specific assignments for the prochiral protons in the four consecutive methylene chain, which is the longest aliphatic chain among the 20 common amino acids. Given the lack of generally applicable strategies to overcome this obstacle, only a few systematic 1H-NMR studies using streospecifically assigned have probed the structural aspects of the Lys residues. The ionization states of the Lys ζ-amino groups also provide important information, as they are often involved in specific intra- and/or intermolecular molecular recognition processes, and thus play vital roles in protein functions. Therefore, the side chain moieties of Lys residues are considered to contribute to maintaining the structure and biological functions of a protein by these two elements: the *hydrophobic* methylene chain and the *hydrophilic* ζ-amino group. To investigate the *dual* nature of the Lys side chain, we have applied various isotope-aided NMR technologies, including the stereo-array isotope labeling (SAIL) method (Kainosho et al., 2006).

The Lys ζ-amino groups, which usually have pKa values around 10.5, are protonated (NH_3^+) at around neutral pH. However, certain proteins have Lys residues with deprotonated ζ-amino groups even at neutral or acidic pH (Harris and Turner, 2002). In such cases, the pK_a values of the Lys ζ-amino groups are substantially lowered due to their particular local environments. Since the Lys ζ-NH_2 groups are endowed with significantly different physical chemical properties, as compared to the ζ-NH_3^+, they can perform specific functions such as Schiff base formation through nucleophilic attacks on various substrates (Highbarger et al., 1996; Barbas et al., 1997). Although the ionization states of Lys ζ-amino groups in a protein have been characterized by X-ray crystallography, they may not always be identical to those in solution. The NH_3^+ and NH_2 states of Lys residues in solution could also be identified by the cross peak patterns in the ^1H-^{15}N correlation NMR spectra, if the hydrogen exchange rates are sufficiently slow, or by the $^{15}N^{\zeta}$ and/or $^1H^{\zeta}$ chemical shifts (Poon et al., 2006; Iwahara et al., 2007; Takayama et al., 2008). Under physiological conditions, however, the observations of ^1H-^{15}N cross peaks are often hampered due

to the rapid hydrogen exchange rates of the Lys ζ-amino groups (Liepinsh et al., 1992; Liepinsh and Otting, 1996; Otting and Wüthrich, 1989; Otting et al., 1991; Segawa et al., 2008). The ionization states can also be identified by the pH titration profiles for the $^{13}C^{\varepsilon}$ and $^{15}N^{\zeta}$ signals of individual Lys residues (Kesvatera et al., 1996; Damblon et al., 1996; Farmer and Venters, 1996; Poon et al., 2006; Gao et al., 2006; André et al., 2007). Unfortunately, long-term experiments such as pH titration are hampered by the stability and solubility issues of a protein over the wide pH range. Therefore, straightforward and robust alternative methods to identify Lys residues with distinct ionization states for the ζ-amino groups are highly desired.

We used a variant of *Staphylococcal* nuclease, Δ+PHS/V66K SNase (denoted as the SNase variant, hereafter), as the model protein (Stites et al., 1991). This variant was engineered to add the following three features to the wild-type SNase: (i) introduction of three stabilizing mutations, P117G, H124L and S128A (*PHS*); (ii) deletion of amino acids 44-49 and introduction of two mutations, G50F and V51N (*Δ*); and (iii) substitution of Val66 with Lys (*V66K*). With these three modifications, the *Δ+PHS/V66K* SNase variant becomes thermally stable, even with the ζ-amino group of Lys-66 entrapped within the hydrophobic cavity originally occupied by the Val-66 side chain in the wild-type SNase. As a result, the ζ-amino group of Lys-66 in the SNase variant exhibits an unusually low pK_a value of 5.7 (García-Moreno et al., 1997; Fitch et al., 2002).

Although the SNase variant contains 21 Lys residues (Fig. A1), including the engineered Lys-66, the ^{13}C, ^{1}H and ^{15}N NMR signals for the Lys side chains were unambiguously observed and assigned by using the SNase variant selectively labeled with SAIL-Lys; i.e., [U-^{13}C,^{15}N; β_2,γ_2,δ_2,ε_3-D_4]-Lys (Kainosho et al., 2006; Terauchi et al., 2011). In this article, we examine some of the structural features inferred from the comprehensive chemical shift data and the deuterium-induced isotope shifts on the $^{13}C^{\varepsilon}$ and $^{15}N^{\zeta}$ of the Lys residues in the SNase variant, and show that the side chain NMR signals can serve as powerful probes to investigate the *dual* nature of a Lys side chain in a protein.

2 Material and methods

2.1 Sample preparation

The Δ+PHS/V66K SNase variants selectively labeled with either L-[U-^{13}C,^{15}N]-Lys, L-[U-^{13}C,^{15}N; β_2,γ_2,δ_2,ε_3-D_4]-Lys (SAIL-Lys), or L-[ε-^{13}C;ε,ε-D_2]-Lys, which were synthesized in house, were prepared using the *E. coli* BL21 (DE3) strain transformed with a pET3 vector (Novagen), encoding the Δ+PHS/V66K SNase gene fused with an N-terminal His-tag. The transformed *E. coli* cells were cultured at 37 ºC in 500 mL of M9 medium, containing anhydrous Na_2HPO_4 (3.4 g/L), anhydrous KH_2PO_4 (0.5 g/L), NaCl (0.25 g/L), D-glucose (5 g/L), NH_4Cl (0.5 g/L), thiamine (0.5 mg/L), $FeCl_3$ (0.03 mM), $MnCl_2$ (0.05 mM), $CaCl_2$ (0.1 mM), and $MgSO_4$ (1 mM), with 10 mg/L of the mono-hydrochloride salts of either [U-^{13}C,^{15}N]-Lys, SAIL-Lys, or [ε-^{13}C;ε,ε-D_2]-Lys. Each culture was maintained at 37 ºC. An additional 20 mg/L of each isotope-labeled Lys was supplemented when the OD_{600} reached 0.5, and then protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. At 4-5 h after the induction, the cells were collected by centrifugation and the SNase variant proteins were purified on a Ni-NTA column according to the standard protocol. The enrichment levels for Lys were ~70%, as measured by mass spectrometry. The purified proteins were dissolved in 20 mM sodium phosphate buffers containing 100 mM KCl (pH 8.0), prepared with either H_2O, D_2O or H_2O:D_2O (1:1).

2.2 NMR spectroscopy

The 600 MHz 2D ^{1}H-^{13}C constant-time HSQC spectra of the SNase variant, selectively labeled with either [U-^{13}C,^{15}N]-Lys or SAIL-Lys, were measured in D_2O at 30 ºC on a Bruker Avance spectrometer equipped with a TXI cryogenic probe. For the latter sample, additional deuterium decoupling was applied during the t_1 period. The data sizes and spectral widths were 1,024 (t_1) × 2,048 (t_2) points and 12,000 Hz (ω_1, ^{13}C) × 8,700 Hz (ω_2, ^{1}H), respectively. Each set of 32 scans/FID with a 1.5 s repetition time was collected, using the ^{13}C carrier frequency at 38 ppm. The 600 MHz 3D HCCH-TOCSY spectrum was measured in D_2O at 30 ºC for the SNase variant labeled with SAIL-Lys (Clore et al., 1990; Cavanagh et al., 2007). The data size and spectral width were 1,024 (t_1) × 32 (t_2) × 2,048 (t_3) points and 6,000 Hz (ω_1, ^{1}H) Hz × 9,100 Hz (ω_2, ^{13}C) × 9,000 Hz (ω_3, ^{1}H), respectively. Each set of 16 scans/FID with a 1.5 s repetition time was collected, using the ^{13}C carrier frequency at 40 ppm.

The Lys ζ-^{15}N signals of the SAIL-Lys labeled SNase variant dissolved in D_2O at 30 ºC were

assigned using the HECENZ pulse sequence, utilizing the out-and-back magnetization transfer from $^{1}H^{\varepsilon 2}$ to $^{15}N^{\zeta}$ via $^{13}C^{\varepsilon}$. The correlations between the $^{1}H^{\varepsilon 2}$ and $^{15}N^{\zeta}$ signals for most of the 21 Lys residues were firmly established by the pulse sequence, which was basically the same as the H2CN pulse sequence developed by Andre et al. (Andre et al., 2007). The data size and the spectral width were 512 (t_1) × 1024 (t_2) points and 1,200 Hz (ω_1, ^{15}N) Hz × 9,600 Hz (ω_2, ^{1}H), respectively, and deuterium decoupling was applied during the t_1 period. The carrier frequencies were 38 ppm and 28 ppm for ^{13}C and ^{15}N, respectively, and 128 scans/FID with a 2 s repetition time were accumulated.

The 125.7 MHz 1D ^{13}C NMR spectra of the SNase variant proteins selectively labeled with either [U-^{13}C,^{15}N]-Lys or [ε-^{13}C;ε,ε-D_2]-Lys were measured in D_2O, H_2O, and H_2O-D_2O (1:1), at 25 ºC on a Bruker Avance 500 spectrometer equipped with a DCH cryogenic probe under the proton and, for the latter sample, simultaneous deuterium decoupling using the WALTZ16 scheme. The spectral width and repetition time were 6,300 Hz and 5 s, respectively. In the experiment in H_2O solution, a 4.1 mm o.d. Shigemi tube containing the protein solution was inserted into a 5 mm o.d. outer tube containing pure D_2O for the internal lock signal. By taking advantage of the selective deuteration on the ε-^{13}C in [ε-^{13}C;ε,ε-D_2]-Lys (~98 atom %), the background ^{13}C signals due to the naturally abundant, and therefore protonated, ^{13}C nuclei were readily filtered out by using the pulse scheme shown in Fig. A2.

3 Results and discussion

3.1 Complete assignment of the Lys side chain NMR signals in the SNase variant selectively labeled with SAIL-Lys

Although the chemical shifts with sequential assignments for the backbone ^{1}H, ^{13}C and ^{15}N signals of SNase are available in the BMRB (Entry #16123; Chimenti et al., 2011), we reconfirmed them by the HNCA experiment for the [U-^{13}C, ^{15}N]-SNase variant, since the solution conditions were slightly different. The complete side chain assignment for all 21 Lys residues was not trivial, even for the SNase variant residue-selectively labeled with [U-^{13}C, ^{15}N]-Lys, due to the extensive signal overlap as illustrated in the *F1-F3* projection of the 3D HCCH TOCSY spectrum (Fig. 1a). On the other hand, a markedly improved 3D HCCH TOCSY spectrum was obtained, under the

simultaneous deuterium decoupling, for the SNase variant residue-selectively labeled with SAIL-Lys (Fig. 1b), enabling us to firmly establish the full connectivity for the side chain ^{1}H, ^{13}C and ^{15}N NMR signals of the 21 Lys residues. To illustrate the improved spectral quality obtained with the SAIL-Lys in lieu of [U-^{13}C,^{15}N]-Lys, a panel obtained for the *F1-F3* projection, along the ^{13}C-axis (*F2*) restricted for the chemical shift range of 40.1-45.5 ppm for the $^{13}C^{\varepsilon}$ signals, is shown for the $^{1}H^{\alpha}$-$^{1}H^{\varepsilon 2}$ correlation signals (Fig. 1c). By taking advantage of the well-dispersed $^{1}H^{\alpha}$-$^{1}H^{\varepsilon 2}$ signals, the backbone $^{1}H^{\alpha}$-$^{13}C^{\alpha}$ signals (Fig. 1e) were readily correlated to the $^{1}H^{\varepsilon 2}$-$^{13}C^{\varepsilon}$ HSQC signals (Fig. 1d). Actually, all of the SAIL-Lys side chain ^{13}C signals were facilely and unambiguously assigned through the 3D HCCH TOCSY spectrum, yielding a complete set of the Lys side chain NMR chemical shifts, as summarized in Table 1. It should be noted that, since each one of the side chain methylene protons was *stereo-specifically* deuterated in SAIL-Lys; i.e., [U-^{13}C,^{15}N; β_2,γ_2,δ_2,ε_3-D_4]-Lys (Fig. 1f), all of the 21 Lys side chain methylene proton signals are *stereo-specifically* assigned to each of the β_3, γ_3, δ_3, and ε_2-^{1}H signals, thus providing precious clues to examine the local conformations of the Lys side chains in solution.

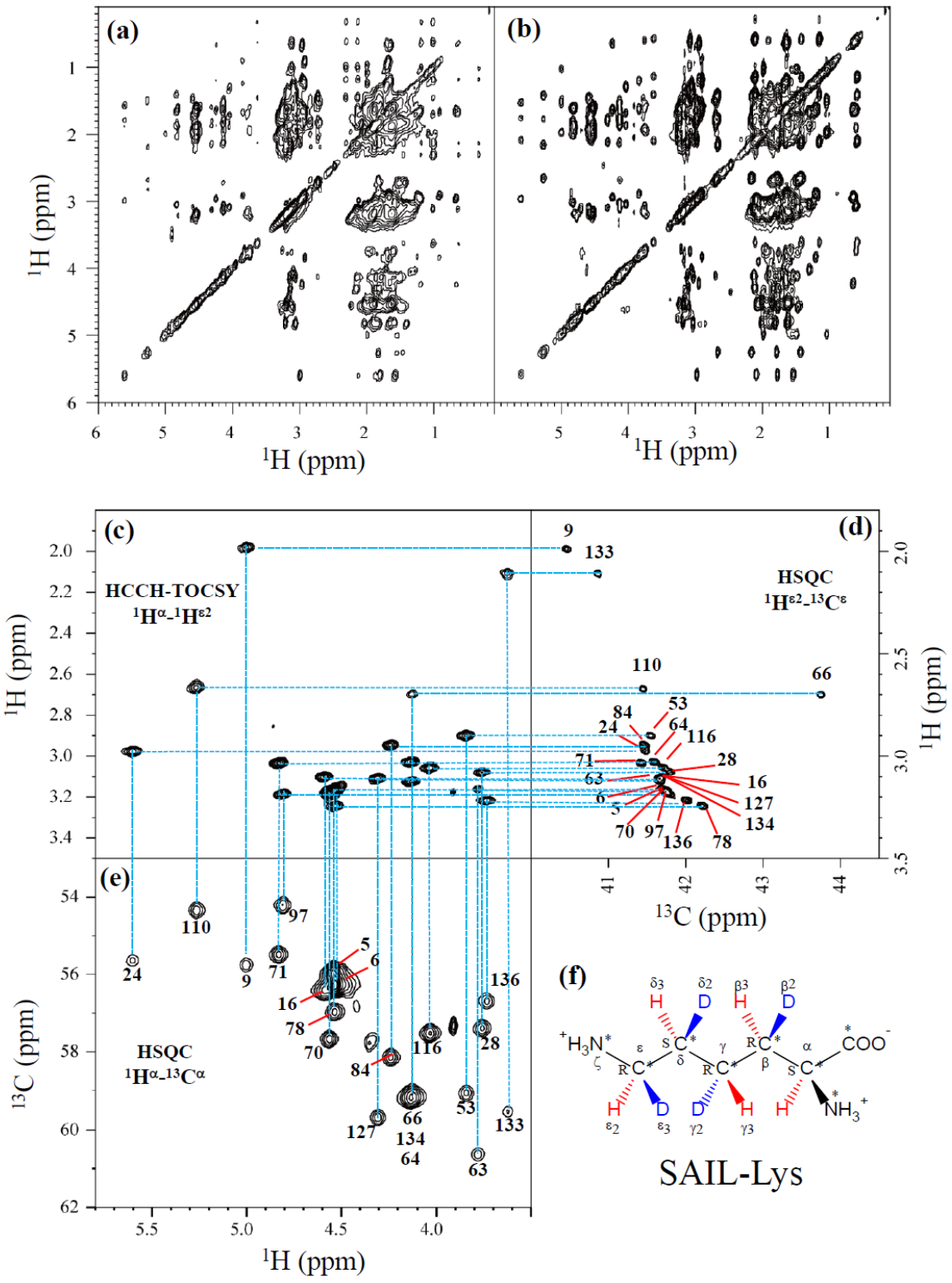


Figure 1: Sequential assignment of the Lys side chain signals for the SNase variant selectively

labeled with SAIL-Lys by the 3D HCCH TOCSY experiment. Panels (a) and (b) show a comparison of the *F1-F3* projections of the 3D HCCH TOCSY spectra obtained for the SNase variant selectively labeled with either [U-^{13}C, ^{15}N]-Lys (a) or SAIL-Lys (b). A complete side chain signal assignment was established for the SNase variant selectively labeled with SAIL-Lys by the correlation networks on the 3D HCCH TOCSY spectrum, starting from the backbone $^{1}H^{\alpha}$, $^{13}C^{\alpha}$ signals with assignments deposited in the BMRB (Entry #16123; Chimenti et al., 2011). For example, the $^{1}H^{\varepsilon 2}$-$^{13}C^{\varepsilon}$ HSQC signals in panel (d) were unambiguously correlated to the backbone $^{1}H^{\alpha}$-$^{13}C^{\alpha}$ HSQC signals in panel (e), through the $^{1}H^{\alpha}$-$^{1}H^{\varepsilon 2}$ correlation signals in panel (c), which represents the *F1-F3* projection of the 3D HCCH TOCSY spectrum along the ^{13}C-axis (*F2*) restricted for the $^{13}C^{\varepsilon}$ shift range of 40.1-45.5 ppm. The structure of SAIL-Lys, [U-^{13}C,^{15}N; β_2,γ_2,δ_2,ε_3-D$_4$]-Lys, was shown in panel (f). The spectrum was measured at 30 ºC on a Bruker Avance 600 spectrometer equipped with a TXI cryogenic probe.

~~^{15}N NMR signals of the 21 Lys residues. To illustrate the improved spectral quality obtained with the SAIL-Lys in lieu of [U-^{13}C,^{15}N]-Lys, a panel obtained for the *F1-F3* projection, along the ^{13}C-axis (*F2*) restricted for the chemical shift range of 40.1-45.5 ppm for the $^{13}C^{\varepsilon}$ signals, is shown for the $^{1}H^{\alpha}$-$^{1}H^{\varepsilon 2}$ correlation signals (Fig. 1e). By taking advantage of the well-dispersed $^{1}H^{\alpha}$-$^{1}H^{\varepsilon 2}$ signals, the backbone $^{1}H^{\alpha}$-$^{13}C^{\alpha}$ signals (Fig. 1c) were readily correlated to the $^{1}H^{\varepsilon 2}$-$^{13}C^{\varepsilon}$ HSQC signals (Fig. 1d). Actually, all of the SAIL-Lys side chain ^{13}C signals were facilely and unambiguously assigned through the 3D HCCH TOCSY spectrum, yielding a complete set of the Lys side chain NMR chemical shifts, as summarized in Table 1. It should be noted that, since each one of the side chain methylene protons was *stereo-specifically* deuterated in SAIL-Lys; i.e., [U-^{13}C,^{15}N; β_2,γ_2,δ_2,ε_3-D$_4$]-Lys (Fig. 1f), all of the 21 Lys side chain methylene proton signals are *stereo-specifically* assigned to each of the β_3, γ_3, δ_3, and ε_2-^{1}H signals, thus providing precious clues to examine the local conformations of the Lys side chains in solution.~~

3.2 Structural information inferable from the Lys side chain chemical shifts

Note that the chemical shifts in Table 1 for the 21 Lys residues in the SAIL-Lys labeled SNase variant are *not* corrected for the various isotope-induced shifts caused by the complicated isotope-labeling pattern of the SAIL-Lys structure (*see*, Fig. 1f). Based on comprehensive NMR data, we should be able to elucidate the *dual* role of the Lys side chains in terms of the conformational dynamics and functional properties of a protein in further detail, using various solution NMR

methods. In this section, we briefly interpret the chemical shift data to characterize the local conformational features by the ^{1}H, ^{13}C, and ^{15}N-signals compiled in Table 1, which should be followed by more extensive studies in the future. Although we have not yet attempted to collect the comprehensive NOEs, such as by using a *fully* SAIL-labeled SNase variant (Kainosho et al., 2006), it was obvious that the chemical shift data with exclusive and unambiguous assignments for the Lys residues contain an abundance of information on the side chain conformations and ionization states of the ζ-amino groups. As described above, the unusual chemical shifts of the Lys-66 side chain confirmed the deprotonated state of its ζ-amino group. We also obtained some interesting structural information for the other Lys residues with protonated ζ-amino groups. For example, the Lys-9 side chain exists in two conformational states in the crystalline state (PDB Entry #3HZX), which only differ in the χ^4-angle; i.e., *Form A* (*trans*, ~-175˚) and *Form B* (*gauche*$^+$,

	$^{13}C^{\alpha}$	$^{1}H^{\alpha}$	$^{13}C^{\beta}$	$^{1}H^{\beta3}$	$^{13}C^{\gamma}$	$^{1}H^{\gamma3}$	$^{13}C^{\delta}$	$^{1}H^{\delta3}$	$^{13}C^{\varepsilon}$	$^{1}H^{\varepsilon2}$	$^{15}N^{\zeta}$
K5	56.4	4.54	32.8	1.98	24.1	1.60	29.2	1.85	41.8	3.17	n.d.
K6	56.0	4.54	33.3	1.97	24.5	1.63	28.4	1.86	41.8	3.17	n.d
K9	55.9	5.00	34.5	1.56	25.1	1.49	28.8	1.04	40.5	1.98	30.8
K16	56.6	4.60	35.6	1.92	23.9	1.47	28.3	1.78	41.7	3.10	31.7
K24	55.8	5.61	34.3	2.10	25.2	1.54	29.5	1.77	41.5	2.98	32.0
K28	57.5	3.80	29.5	2.00	24.6	0.61	29.1	1.63	41.9	3.16	31.9
K53	59.2	3.84	31.5	1.64	24.7	1.21	28.8	1.61	41.6	2.90	31.8
K63	60.8	3.78	32.8	1.91	24.2	1.46	29.7	1.75	41.8	3.17	n.d.
K64	59.4	4.13	31.8	1.89	24.5	1.55	28.9	1.74	41.6	3.03	31.7
K66	*59.5*	*4.12*	*32.8*	*1.85*	*25.7*	*1.76*	*34.0*	*1.47*	*43.8*	*2.70*	*20.9*
K70	57.8	4.55	32.7	1.92	24.3	1.60	28.6	1.83	41.8	3.19	n.d.
K71	55.6	4.84	35.2	2.01	24.4	1.60	28.4	1.82	41.4	3.04	31.8
K78	57.1	4.53	33.0	2.06	24.1	1.67	28.5	1.90	42.2	3.24	31.5
K84	58.3	4.24	31.3	1.65	23.1	0.64	28.8	1.61	41.5	2.95	32.0
K97	54.2	4.81	32.9	1.89	24.6	1.62	28.7	1.79	41.8	3.19	n.d.
K110	54.4	5.27	35.2	2.17	25.1	1.42	29.2	1.79	41.5	2.68	31.7
K116	57.7	4.04	31.7	1.88	24.1	1.30	28.5	1.69	41.7	3.06	31.7
K127	59.3	4.31	31.9	2.12	24.9	1.77	29.1	1.83	41.7	3.12	31.4

K133	59.6	3.62	32.2	1.42	24.1	0.59	28.9	1.15	40.9	2.10	31.7
K134	59.4	4.13	32.0	2.15	24.4	1.65	29.5	1.76	41.8	3.13	31.6
K136	56.8	3.76	28.8	1.66	24.6	1.55	28.7	1.95	42.0	3.20	31.5
Avg. δ ppm	-	-	**33.1**	**1.95**	**24.5**	**1.54**	**28.9**	**1.68**	**41.7**	**3.09**	**31.7**

Table 1. The ^{1}H, ^{13}C, ^{15}N chemical shifts for the sidechains of the 21 Lys residues in Δ+PHS/V66K SNase selectively labeled with SAIL-Lys in D_2O. The ^{1}H and ^{13}C signals were assigned by the 3D HCCH-TOCSY experiment recorded on a Bruker 600 MHz equipment 30 ˚C, pH 8.0 using a 600 MHz NMR machine. The ^{15}N-signals were assigned by the HECENZ correlations and those denoted as "n.d." were not clearly observed. Since one of the prochiral methylene protons was stereo-specifically deuterated in the SAIL-Lys, i.e. [U-^{13}C,^{15}N; β_2,γ_2,δ_2,ε_3-D_4]-Lys, thus the observed ^{1}H-signals were unambiguously assigned. The chemical shifts for the engineered Lys-66, which has a deprotonated ζ-amino group, are shown italic. The averaged chemical shifts are obtained by excluding some of the outlying shifts, and the measurement errors were estimated as less than 0.2 and 0.02 ppm, for $^{13}C/^{15}N$- and ^{1}H-chemical shifts, respectively. All chemical shifts are not corrected for the isotope shifts.

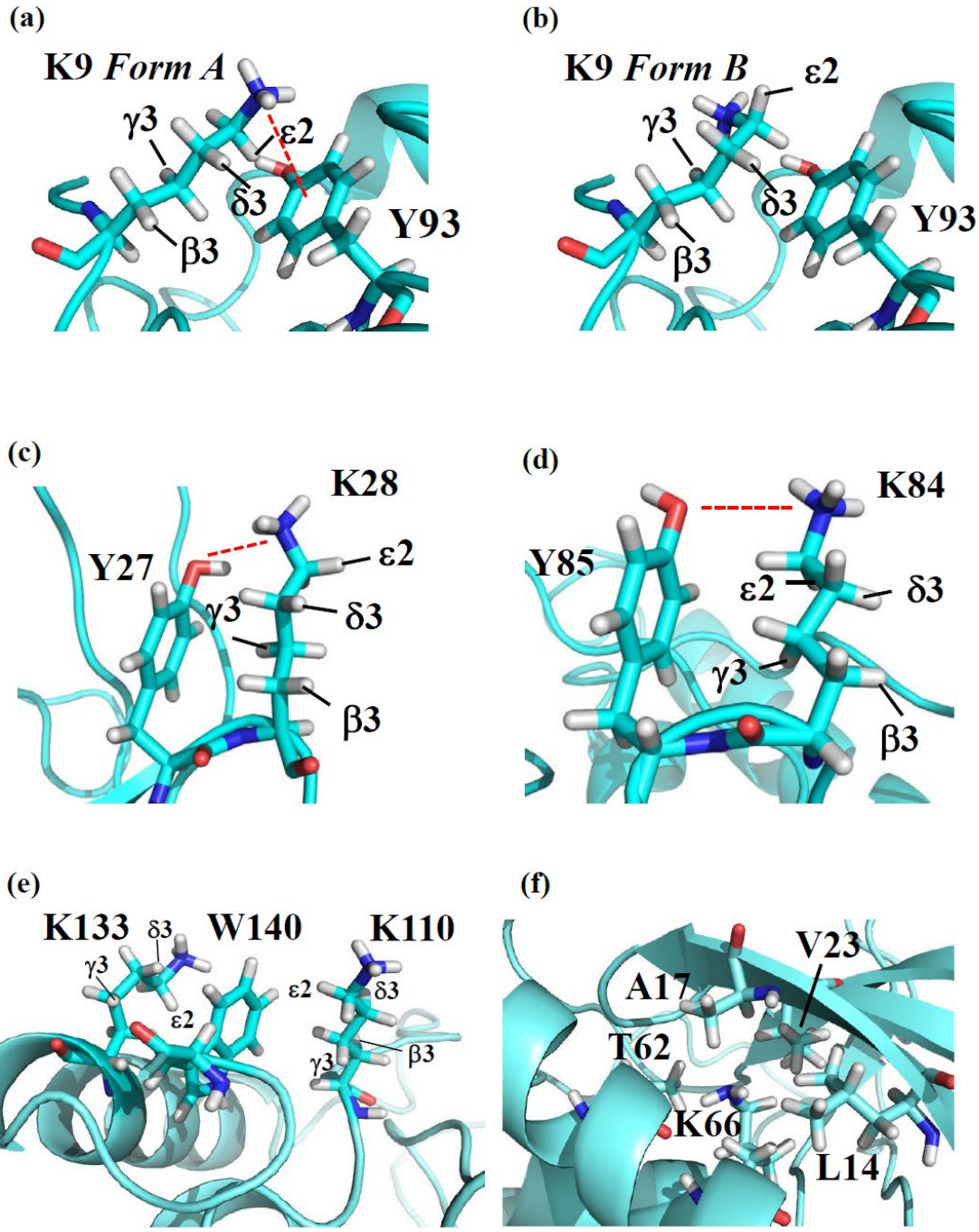


Figure 2: The local structures around the Lys residues, which exhibit unusual side chain chemical shifts, in the crystal structure of the SNase variant (PDB: 3HZX). The crystal structure of the SNase variant was solved as a complex with calcium ions and thymidine 3',5'-diphosphate. Therefore,

it may be slightly different from that in the free state. The figures were created with the PyMOL 2.4 software in order to highlight the relative orientations between the Lys side chains and nearby aromatic rings (a)-(e), and Lys-66 and the surrounding hydrophobic amino acids (f). The nomenclatures of the prochiral hydrogen atoms, shown as the suffixes in the figures, are according to the recommended atom identifiers (Markley et al., 1998).

~~methods. In this section, we briefly interpret the chemical shift data to characterize the local conformational features by the ^{1}H, ^{13}C, and ^{15}N-signals compiled in Table 1, which should be followed by more extensive studies in the future. Although we have not yet attempted to collect the comprehensive NOEs, such as by using a *fully* SAIL-labeled SNase variant (Kainosho et al., 2006), it was obvious that the chemical shift data with exclusive and unambiguous assignments for the Lys residues contain an abundance of information on the side chain conformations and ionization states of the ζ-amino groups. As described above, the unusual chemical shifts of the Lys-66 side chain confirmed the deprotonated state of its ζ-amino group. We also obtained some interesting structural information for the other Lys residues with protonated ζ-amino groups. For example, the Lys-9 side chain exists in two conformational states in the crystalline state (PDB Entry #3HZX), which only differ in the χ^4-angle; i.e., *Form A* (*trans*, ~-175˚) and *Form B* (*gauche*$^+$,~~ ~+44˚), as shown in Fig. 2a and b, respectively (*see also*, Table A1). The significantly up-field shifted signals observed for Lys-9 relative to the averaged chemical shifts (Δδ, ppm) are obviously due to the aromatic ring current of Tyr-93; i.e., $^{15}N^{\zeta}$ (30.8 ppm, Δδ=-0.9 ppm), $^{13}C^{\varepsilon}/^{1}H^{\varepsilon 2}$ (40.5/1.98 ppm, Δδ=-1.2/-1.11ppm) and $^{1}H^{\delta 3}$ (1.04 ppm, Δδ=-0.64 ppm). These chemical shifts suggest the ζ-NH^{3+}-π interaction, as shown by the dashed red line (Fig. 2a). Therefore, the chemical shifts for Lys-9 strongly imply that the van der Waals interactions between the aliphatic side chain, as well as the *electrostatic interaction* between the positively charged ζ-HN^{3+} and the nearby aromatic ring of Tyr-93, simultaneously contribute to preferentially stabilize the *Form A* conformation in solution (Fig. 2a).

The high-field shifts of the side chain methylenes, induced by the neighboring aromatic rings, were also detected for Lys-28, Lys-84 and Lys-133. Considering the local structures of Lys-28 and Lys-84 in the crystal (Fig. 2c, d), the relative orientations between Lys-28 and Tyr-27, and Lys-84 and Tyr-85 seem to be similar to those in the crystal, and are responsible for the large high-field shifts for only their $^{1}H^{\gamma 3}$ signals; i.e., Lys-28: 0.61 ppm, Δδ=-0.93 ppm; Lys-84: 0.64 ppm, Δδ=-

0.90 ppm, while the other $^{13}C/^{1}H$ shifts remain within the average ranges (Table 1). The small but obvious low-field shifts for the $^{15}N^{\zeta}$ (Lys-28, Lys-84: 32.0 ppm, $\Delta\delta$= +0.3 ppm) might be caused by the electrostatic interactions between the O^{η} of Tyr-27/Tyr-85 and the N^{ζ} of Lys-28/Lys-84, respectively, as shown by the dashed red lines (Fig. 2 c, d). The bulky indole ring of Trp-140 seems to simultaneously stabilize the aliphatic chains of both Lys-133 and Lys-110, inducing the high-field shifts for some of the side chain signals; i.e., Lys-133 $^{13}C^{\varepsilon}/^{1}H^{\varepsilon 2}$ (40.9/2.10 ppm, $\Delta\delta$=-0.8/-0.99 ppm), $^{1}H^{\delta 3}$ (1.15 ppm, $\Delta\delta$=-0.53 ppm), $^{1}H^{\gamma 3}$ (0.59 ppm, $\Delta\delta$=-0.95 ppm) and $^{1}H^{\beta 3}$ (1.42 ppm, $\Delta\delta$=-0.53 ppm); Lys-110 $^{1}H^{\varepsilon 2}$ (2.68 ppm, $\Delta\delta$= -0.41ppm). These up-field shifted signals indicate that the van der Waals interactions between the methylene moieties of Lys-133 and Lys-110, with the hydrophobic indole ring of Trp-140 sandwiched in the middle, are also preserved in solution (Fig. 2e). Interestingly, the chemical shift differences between the two prochiral methylene protons attached to the ε-carbons of the Lys residues, observed for the SNase variant residue-selectively labeled with [U-^{13}C,^{15}N]-Lys, are only considerably large for the Lys-110 and -133 residues, while those for the other 19 Lys residues were much smaller than ~ 0.05 ppm, if present (Fig. A3). Since the $^{1}H^{\varepsilon 2}$ chemical shifts were observed at 0.15 and 0.17 ppm higher field than the $^{1}H\varepsilon^{3}$ chemical shifts for Lys-110 and -133, respectively, the conformations of these two Lys residues are likely to be similar to those in the crystal (Fig. 2e).

On the other hand, the striking chemical shifts observed for the Lys-66 residue, which is deliberately trapped within the hydrophobic environment engineered in the SNase variant (Fig. 2f), clearly reveal the strong influence of the ionization state of the ζ-amino group on the Lys side chain chemical shifts. As shown in Table 1, the $^{15}N^{\zeta}$ chemical shift of the ζ-ND_2 of Lys-66 in the SNase variant appears at an unusually high-field position, as compared to the averaged chemical shift range for the ζ-ND^{3+} in the other Lys residues; i.e., $^{15}N^{\zeta}$ (Lys-66: 20.9 ppm, $\Delta\delta$= -10.8 ppm), which is close to the value of the ζ-NH_2 chemical shift, 23.3 ppm, previously reported for Lys-66 in the [U-^{13}C,^{15}N]-SNase variant (André et al., 2007; Takayama et al., 2008). Apparently, the $^{15}N^{\zeta}$ chemical shifts provide a quite useful clue to distinguish between the deprotonated and protonated ζ-amino groups of Lys residues. However, the complete side chain assignment including the terminal ζ-^{15}N signals by conventional methods using a [U-^{13}C,^{15}N]-protein is usually laborious, and occasionally impossible.

The deprotonation of the ζ-amino group caused sizable ^{1}H and ^{13}C chemical shift changes down to the γ-position in the side chain, as observed for Lys-66; i.e., $^{13}C^{\varepsilon}/^{1}H^{\varepsilon 2}$ (43.8/2.70 ppm, Δδ= +2.1/-0.39 ppm), $^{13}C^{\delta}/^{1}H^{\delta 3}$ (34.0/1.47 ppm, Δδ= +5.1/-0.21 ppm), and $^{13}C^{\gamma}/^{1}H^{\gamma 3}$ (25.7/1.76 ppm, Δδ= +1.2/+0.22 ppm). These *deprotonation* shifts, especially on the $^{13}C^{\varepsilon}$ and/or $^{13}C^{\delta}$ chemical shifts, could therefore be used as a useful alternative index to characterize the ionization states of the ζ-amino groups of Lys residues in a protein, since they can be accurately and readily observed and assigned by using a protein selectively labeled with SAIL-Lys. It should be noted, however, that the side chain chemical shifts in general might significantly vary according to the local environments, such as the relative position to aromatic rings, and thus the results obtained exclusively from the side chain chemical shifts might not be absolutely reliable. To avoid any possible uncertainties in characterizing the ionization states of ζ-amino groups, an alternative approach using the deuterium-induced isotope shifts of the SAIL-Lys side chain ^{13}C signals must be developed.

3.3 Characterization of the ionization state of the ζ-amino group of Lys residues using the effects of deuterium-induced isotope shifts on the side chain ^{13}C and ^{15}N signals

In our previous studies investigating the effects of the deuterium-induced isotope shifts on the ^{13}C signals adjacent to polar functional groups with an exchangeable hydrogen, such as hydroxyl (OH) or sulfhydryl (SH) groups, we demonstrated that those isotope shifts are versatile indices for identifying residues, such as Tyr, Thr, Ser or Cys, with *exceptionally* slow hydrogen exchange rates (Takeda et al., 2014). For example, in a protein selectively labeled with [ζ-^{13}C]-Tyr, the Tyr residues have much slower hydrogen exchange rates for the η-hydroxyl groups than the isotope shift differences in the $^{13}C^{\zeta}$ signals, and exhibit well-resolved pairwise signals with nearly equal intensities in the 1D ^{13}C-NMR spectrum in H_2O-D_2O (1:1) (Takeda et al., 2009). The up- and downfield counterparts of the pairwise $^{13}C^{\zeta}$ signals correspond to those in D_2O and H_2O, respectively, and their relative intensities reflect the fractionation factors; i.e., [OD]/[OH]. Similar approaches have been developed for Ser, Thr and Cys residues, using the $^{13}C^{\beta}$ signals observed for proteins selectively labeled with [β-^{13}C; β, β-D_2]-Ser, [β-^{13}C; β-D]-Thr, and [β-^{13}C; β, β-D_2]-Cys, respectively (Takeda et al., 2010, 2011). Since the isolated $^{13}C^{\beta}(D_2)$ or $^{13}C^{\beta}(D)$ moieties in the

labeled amino acids give extremely narrow signals under the deuterium decoupling, the ^{13}C-NMR signals can be obtained with remarkably high sensitivities, especially with a ^{13}C-direct observing cryogenic probe. Interestingly, while the fractionation factors for the Ser and Thr hydroxyl groups; i.e., [OD]/[OH], are usually close to unity, as also for the Tyr residues, those for the Cys sulfhydryl groups; i.e., [SD]/[SH], are around 0.4-0.5 (Takeda et al., 2010, 2011). The methods are especially important, since the functional groups of the residues readily identified as having exceptionally slow hydrogen exchange rates are most likely to be involved in hydrogen bonding networks and/or located in distinctive local environments.

Although the idea of estimating the hydrogen exchange rates by the deuterium-induced isotope shifts on the ^{13}C nuclei adjacent to functional groups with exchangeable hydrogens was originally exploited years ago, for the backbone amide groups in the residue-selectively labeled proteins with [C'-^{13}C]-amino acid(s) (Kainosho and Tsuji 1982; Markley and Kainosho, 1993), it has not yet been applied for the Lys ζ-amino groups. Having established the complete assignment for the 21 Lys residues in the SNase variant selectively labeled with SAIL-Lys (Table 1), we next examined the deuterium-induced chemical shifts in detail for the Lys side chain signals. In the case of Lys residues, the NMR signals of the ζ-amino ^{15}N and ε- or δ-carbon ^{13}C signals would be plausible candidates for probing the deuterium substitution effects. There have only been a few reports on the isotope shifts of the δ- and ε-^{13}C for the Lys-residues induced by the deuteration of ζ-amino groups (Hansen, 1983; Dziembowska et al., 2004). However, apparently no comprehensive studies have applied the deuterium-induced isotope shifts to characterize the ionization states of Lys residues.

We first examined the 1D ^{13}C- and ^{15}N-NMR spectra of [$^{15}N_2$]-Lys in D_2O and H_2O, at pH 8 and 30 ºC, to choose the suitable NMR probes to distinguish between the deprotonated and protonated ζ-amino groups (Fig. 3). The ζ-^{15}N signal appears at ~1 ppm up-field in D_2O relative to that in H_2O (Fig. 3a), and the aliphatic ^{13}C signals of [$^{15}N_2$]-Lys at the natural abundance also showed isotope shifts, $\Delta\delta$[$^{13}C^i$ (in D_2O)- $\delta^{13}C^i$ (in H_2O)]; i.e., $^{13}C^{\alpha}$, -0.25 ppm; $^{13}C^{\beta}$, -0.20 ppm; $^{13}C^{\gamma}$, -0.03 ppm; $^{13}C^{\delta}$, -0.17 ppm; and $^{13}C^{\varepsilon}$, -0.31 ppm (Fig. 3b). Although the isotope shifts for

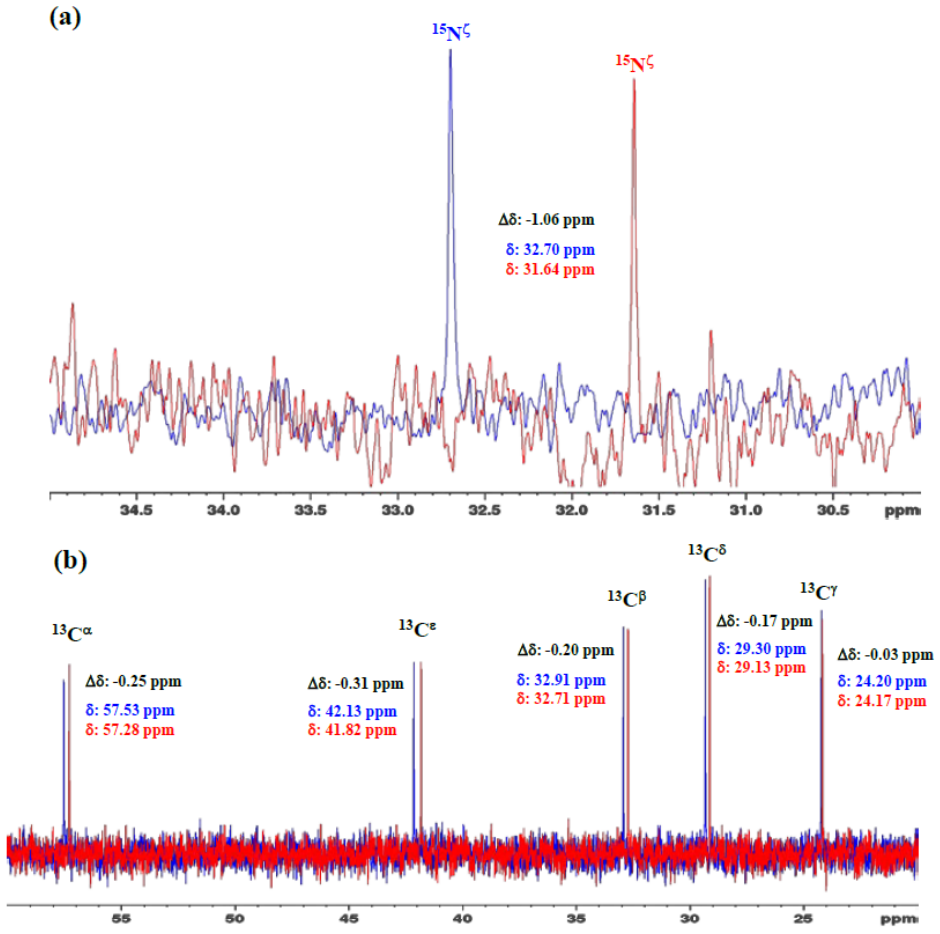
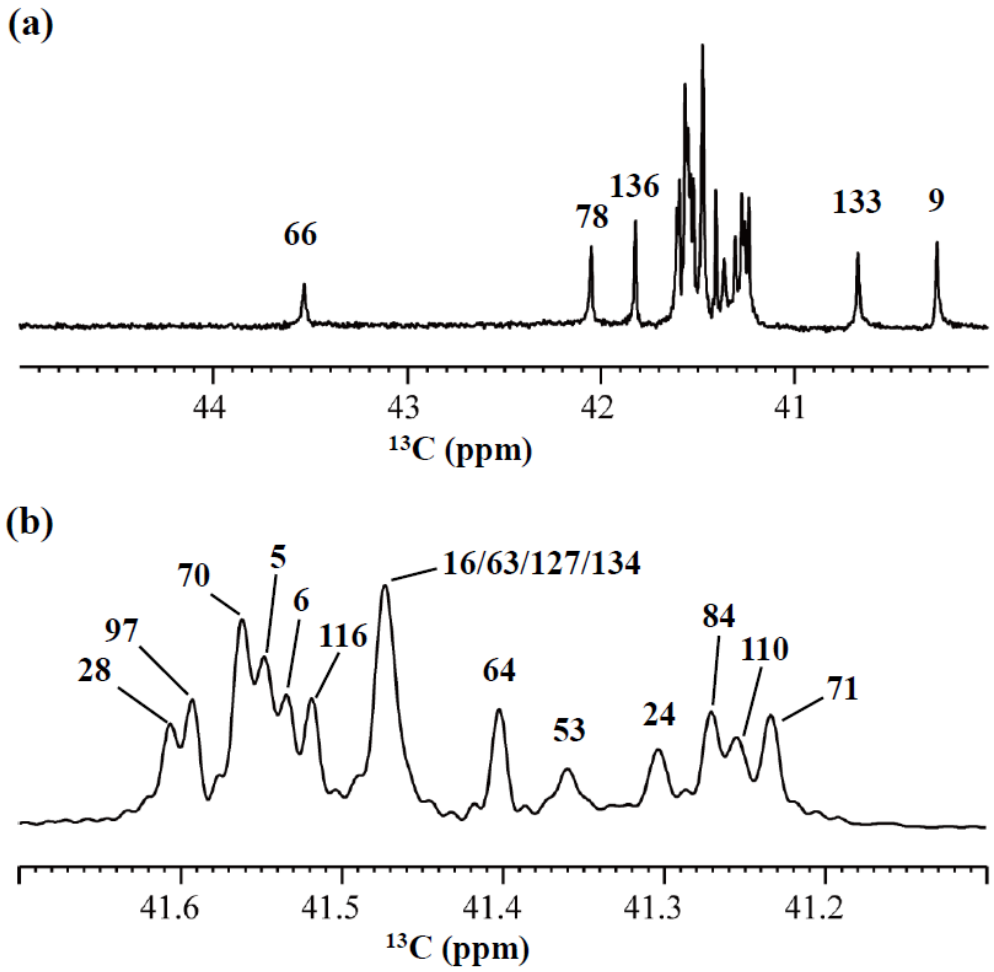


Figure 3: 1D ^{15}N- and ^{13}C-NMR spectra of [$^{15}N_2$]-lysine free in H_2O and D_2O. The 96.3 MHz 1D ^{15}N-NMR spectra (Figure 3a) and 239.0 MHz 1D ^{13}C-NMR spectra (Figure 3b) of [$^{15}N_2$]-lysine were measured at 30 ºC on a Bruker Avance III 950 spectrometer with a TCI cryogenic probe, using ~70 mM solutions of either 20 mM Tris buffer prepared with H_2O (or D_2O) at pH (or pD) 8.0. The NMR spectra and the chemical shifts, δ ppm, shown in blue and red, are those for the H_2O and D_2O buffer solutions, respectively. The deuterium-induced shifts, Δδ ppm : δ (in D_2O) - δ (in H_2O) for the $^{15}N^{\zeta}$ and side chain ^{13}C signals are shown in black.

$^{13}C^{\alpha}$ and $^{13}C^{\beta}$ are due to the deuteration of the α-amino group, those for $^{13}C^{\delta}$ and $^{13}C^{\varepsilon}$ are obviously due to the deuteration of the ζ-amino group. Considering the finding that the $^{13}C^{\varepsilon}$ of Lys gives an isolated signal far from the others and exhibits a ~1.8-fold larger isotope shift as compared to $^{13}C^{\delta}$,

356 the $^{13}C^{\varepsilon}$ and $^{15}N^{\zeta}$ signals seem to be good candidates for probing the ionization states of Lys
357 residues in the SNase variant.

358 Although the $^{15}N^{\zeta}$ and $^{13}C^{\varepsilon}$ chemical shifts for the Lys residues can be measured by the HECENZ
359 and ^{1}H-^{13}C ct-HSQC experiments, respectively, using the SNase variant selectively



360

361 **Figure 4: 125.7 MHz {^{1}H, ^{2}D}-decoupled 1D-^{13}C-NMR spectrum for the SNase variant**
362 **selectively labeled with [ε-^{13}C; ε,ε-D_2]-Lys.** The spectra were measured at 25 ºC, pH 8.0, in D_2O
363 solution on an Avance 500 spectrometer equipped with a DCH cryogenic probe. Although only a
364 few discrete $^{13}C^{\varepsilon}$ signals are apparent in Figure 4a, the congested spectral region around 41-42 ppm
365 shows well-separated signals due to their narrow line-widths of 1-2 Hz (Figure 4b). All of the 1D
366 NMR signals for $^{13}C^{\varepsilon}$ were readily assigned by using the chemical shift data obtained from the 3D
367 HCCH TOCSY experiment for the SNase variant selectively labeled with SAIL-Lys (*see* Sect. 3.1).

labeled with [U-^{13}C,^{15}N]-Lys or SAIL-Lys, it was rather difficult to determine the accurate isotope shifts of the $^{15}N^{\zeta}$ and $^{13}C^{\varepsilon}$ signals for all 21 Lys residues by these methods. Especially, the accurate chemical shift measurement for an individual $^{13}C^{\varepsilon}$ signal was hampered by the insufficient quality

	$\delta^{15}N^{\zeta}$ in H_2O	$\delta^{15}N^{\zeta}$ in D_2O	$\Delta\delta^{15}N^{\zeta}$ ppm	$\delta^{13}C^{\varepsilon}$ in H_2O	$\delta^{13}C^{\varepsilon}$ in D_2O	$\Delta\delta^{13}C^{\varepsilon}$ ppm
K5	n.d.	n.d.	n.d.	41.89	41.54	-0.35
K6	n.d.	n.d.	n.d.	41.87	41.53	-0.34
K9	31.9	30.8	-1.1	40.55	40.26	-0.29
K16	32.9	31.8	-1.1	41.80	41.47	-0.33
K24	33.1	31.8	-1.3	41.66	41.31	-0.35
K28	33.0	32.0	-1.0	41.92	41.62	-0.30
K53	32.9	31.8	-1.1	n.d.	41.36	n.d.
K63	n.d.	n.d.	n.d.	41.80	41.47	-0.33
K64	32.7	31.7	-1.0	41.72	41.41	-0.31
K66	*22.7*	*20.9*	***-1.8***	*43.75*	*43.54*	***-0.21***
K70	n.d.	n.d.	n.d.	41.89	41.55	-0.34
K71	32.8	31.8	-1.0	41.55	41.24	-0.31
K78	32.7	31.5	-1.2	42.37	42.09	-0.28
K84	33.1	32.0	-1.1	41.64	41.36	-0.28
K97	n.d.	n.d.	n.d.	41.91	41.59	-0.32
K110	32.8	31.6	-1.2	41.65	41.26	-0.39
K116	32.8	31.7	-1.1	41.86	41.52	-0.34
K127	32.6	31.4	-1.2	41.80	41.50	-0.30
K133	32.8	31.7	-1.1	40.96	40.67	-0.29
K134	32.6	31.6	-1.0	41.80	41.50	-0.30
K136	32.5	31.5	-1.0	42.12	41.82	-0.30
Avg. δ, Δδ ppm	32.7	31.6	**-1.1 +/- 0.1**	41.72	41.40	**-0.32 +/- 0.02**

Table 2. Deuterium-induced isotope shifts for the sidechain $^{15}N^{\zeta}$ and $^{13}C^{\varepsilon}$ signals of the 21 Lys residues in Δ+PHS/V66K SNase. The $^{15}N^{\zeta}$ and $^{13}C^{\varepsilon}$ chemical shift data in H_2O and D_2O were obtained for the SNase labeled either with SAIL-Lys or [ε-^{13}C; ε,ε-D_2]-Lys, respectively. Note that the 1D $^{13}C^{\varepsilon}$ data measured at 125.7 MHz the 1D ^{13}C-spectra are much higher precision as compared to those by the 2D HSQC using the SNase labeled with SAIL-Lys. The averaged chemical shifts in the last row are obtained for the Lys residues with protonated

ζ-amino groups, except for Lys-66 (italic) which has a deprotonated ζ-amino group. The averaged Δδ values show the difference between the averaged $^{15}N^{\zeta}$ and $^{13}C^{\varepsilon}$, except for Lys-66 which are the difference between its $^{15}N^{\zeta}$ and $^{13}C^{\varepsilon}$ shifts in H_2O and D_2O. Negative Δδ values indicate the chemical shifts in D_2O are up-field shifted due to deuteration of the ζ-amino groups.

of the ct-HSQC spectrum, even for the protein labeled with SAIL-Lys (Fig. A3). Therefore, we used [ε-^{13}C; ε, ε-D_2]-Lys to reduce the line-widths of the $^{13}C^{\varepsilon}$ signals for the Lys-residues in the SNase variant. As expected, the 1D ^{13}C-NMR spectra of the SNase variant selectively labeled with [ε-^{13}C; ε, ε-D_2]-Lys showed remarkably well-resolved signals with line-widths less than 2 Hz, under the $^{1}H/^{2}D$ double decoupling conditions (Fig. 4). Note that the weak background signals due to the naturally abundant ^{13}C nuclei were filtered out in this spectrum (Fig. A2). By referring to the chemical shifts in Table 1, which were determined by the 3D HCCH TOCSY experiment for the SNase labeled with SAIL-Lys, all of the 1-D $^{13}C^{\varepsilon}$ signals were unambiguously assigned (Fig. 4 a, b). The chemical shifts of $^{13}C^{\varepsilon}$ are slightly different among the data sets, because the isotope shifts induced by the nearby isotopes on the $^{13}C^{\varepsilon}$ signals are different for SAIL-Lys and [ε-^{13}C; ε, ε-D_2]-Lys (Tables 1, 2). The $^{13}C^{\varepsilon}$ chemical shifts in H_2O and D_2O, which were accurately determined by the 1D ^{13}C-NMR spectra, are presented in Fig. 5. At a glance, the $^{13}C^{\varepsilon}$ spectra in Fig. 5a and 5c look almost the same, since the signals moved up-field with a constant increment of -0.32 +/- 0.02 ppm, except for the $^{13}C^{\varepsilon}$ signal of Lys-66 (Table 2). Since the $\delta^{13}C^{\varepsilon}$ values in H_2O and D_2O are very close to those for the *free* [$^{15}N_2$]-Lys (Fig. 3b), the ζ-amino groups are protonated in H_2O and deuterated in D_2O, and thus the averaged deuterium-induced isotope shift was designated as $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_3^{+}$-$N^{\zeta}H_3^{+}$]. Similarly, the averaged $\Delta\delta^{15}N^{\zeta}$ [$N^{\zeta}D_3^{+}$-$N^{\zeta}H_3^{+}$], excluding the value for Lys-66, was determined to be -1.1+/- 0.1 ppm, which was also close to the *free* [$^{15}N_2$]-Lys (Fig. 3a). The $\Delta\delta^{13}C^{\varepsilon}$ and $\Delta\delta^{15}N^{\zeta}$ for Lys-66, which are -0.21 and -1.8 ppm (Table 2), respectively, confirmed that the ζ-amino group of this residue is deprotonated at pH 8, and should be designated as $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$] and $\Delta\delta^{15}N^{\zeta}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$]. Interestingly, the fact that the averaged $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_3^{+}$-$N^{\zeta}H_3^{+}$], -0.32 ppm, was ~1.5 times larger than the $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$] for Lys-66, -0.21 ppm, might suggest that the deuterium-induced isotope shift on $^{13}C^{\varepsilon}$ is proportional to the number of hydrogen atoms on the ζ-amino groups. In contrast, the averaged $\Delta\delta^{15}N^{\zeta}$ [$N^{\zeta}D_3^{+}$-$N^{\zeta}H_3^{+}$], -1.1 ppm, was much smaller that of the $\Delta\delta^{15}N^{\zeta}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$] for Lys-66, -1.8 ppm.

We also measured the 1D ^{13}C-NMR spectrum of the SNase variant selectively labeled with [ε-^{13}C; ε, ε-D_2]-Lys in H_2O-D_2O (1:1), to search for the Lys residues with slowly exchanging ζ-amino groups. Obviously, there are no such residues in the SNase variant at pH 8 and 30 ºC, as

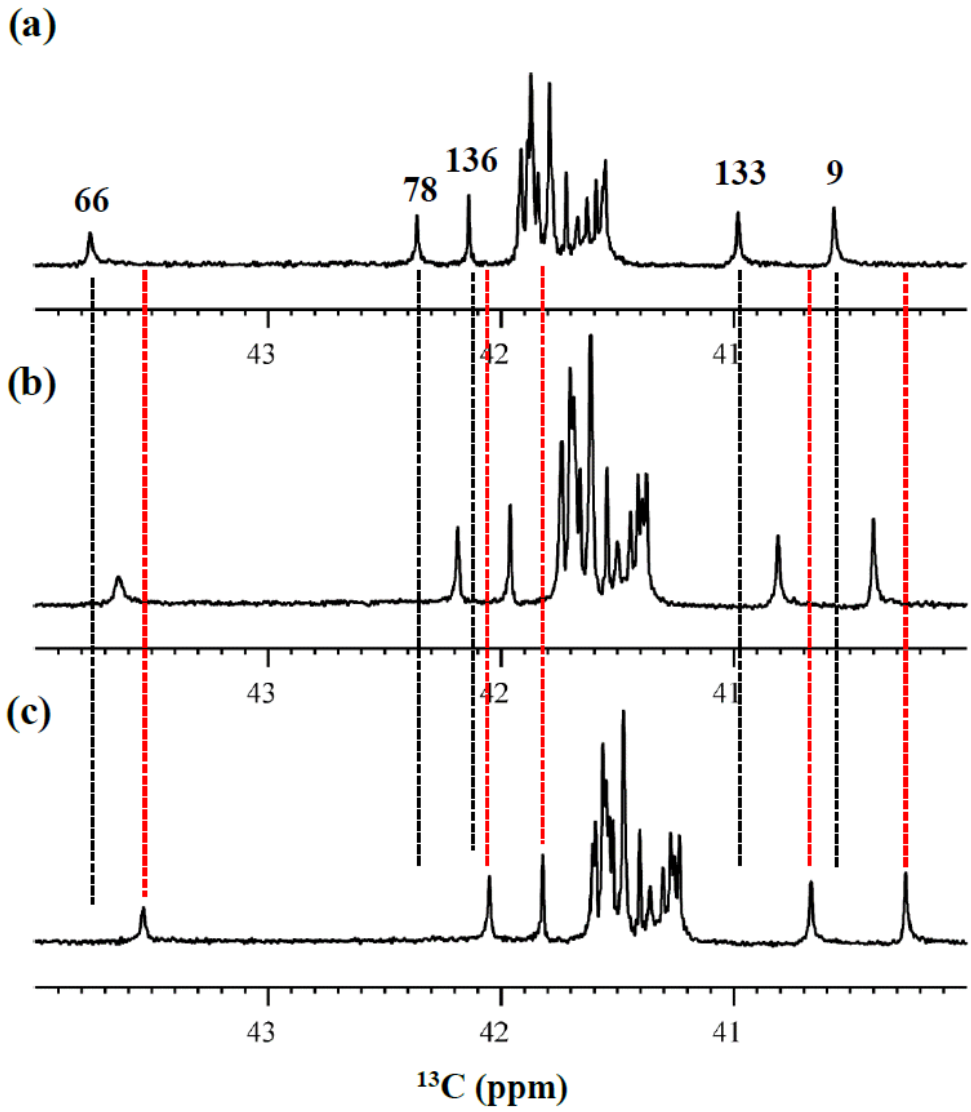


Figure 5: Isotope shifts on the $^{13}C^{\varepsilon}$ signals of the Lys residues in the SNase variant selectively labeled with [ε-^{13}C;ε,ε- D_2]-Lys, caused by the deuterium substitutions for the ζ-amino groups. The 125.7 MHz {^{1}H, ^{2}D}-decoupled 1D ^{13}C NMR spectra were measured at 25 ºC, pH 8.0, in either H_2O (Figure 5a), H_2O:D_2O (1:1) (Figure 5b), or D_2O (Figure 5c) solutions on an Avance 500 spectrometer equipped with a DCH cryogenic probe in H_2O (a), H_2O: D_2O (1:1) (b),

and D_2O (c) solutions. The vertical black and red dotted lines show the chemical shifts observed in 100% H_2O and D_2O, respectively. The complete data for the deuterium-induced isotope shifts for the sidechain $^{15}N^{\zeta}$ and $^{13}C^{\varepsilon}$ signals are summarized in Table 2.

shown in Fig. 5b. Due to the rapid hydrogen exchange rates for all 21 Lys residues in this protein, the observed isotope shifts on $^{13}C^{\varepsilon}$ were exactly half of the $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_2$-$N^{\zeta}H_2$] for Lys-66 or $\Delta\delta^{13}C^{\varepsilon}$ [$N^{\zeta}D_3^+$-$N^{\zeta}H_3^+$] for the rest of the Lys residues. The hydrogen exchange rate cinstant (k_{ex}) for the ζ-amino group of Lys-66 in the SNase variant, which is deeply embedded in the hydrophobic cavity originally occupied by Val-66 in the wild-type SNase, was 93+/-5 s^{-1} at pH 8 and -1 ºC (Takayama et al., 2008). Therefore, the hydrogens on the ζ-amino groups in all 21 Lys residues in the SNase variant are rapidly exchanging, and thus the observed chemical shifts for the $^{13}C^{\varepsilon}$ of Lys-66 and the rest of the Lys residues in H_2O-D_2O (1:1) are the time-averages for three isotopomers, NH_2, NHD, and ND_2, with nearly a 1:2:1 ratio for Lys-66, and for four isotopomers, NH_3^+, NH_2D^+, NHD_2^+, and ND_3^+, with a ratio of 1:3:3:1. Since the time-averaged signals for Lys-66 and other Lys residues in H_2O-D_2O (1:1) appeared exactly in the middle of the spectra observed in H_2O and D_2O (Fig. 5a, c), the fractional factors for the isotopomers are nearly identical, as statistically random distributions.

4 Conclusions

In this article, we have shown that comprehensive NMR information can be obtained by the cutting-edge isotope-aided NMR technologies for the Lys side chain moieties, comprising a long *hydrophobic* methylene chain and a *hydrophilic* ζ-amino group, to facilitate hitherto unexplored investigations toward elucidating the *dual* nature of the Lys residues in a protein. The unambiguously assigned ^{13}C signals, together with the stereo-specifically assigned prochiral protons for each of the long consecutive methylene chains, which first became available by the stereo-array isotope labeling (SAIL) method, provide unprecedented opportunities to examine the conformational features around the Lys residues in detail. The ionization states of the ζ-amino groups of Lys residues, which play crucial roles in the biological functions of proteins, could be readily characterized by the deuterium-induced isotope shifts on the ε-^{13}C signals observed by the 1D ^{13}C-NMR spectroscopy of a protein selectively labeled with [ε-^{13}C; ε,ε-D_2]-Lys. Both methods should work equally well for larger proteins, for which previous NMR approaches were rarely

444 applicable. Therefore, these methods will contribute toward clarifying the structural and functional
445 roles of the Lys residues in biologically important proteins.
446

447 Acknowledgements:

448 This work was supported by Grants-in-Aid in Innovative Areas (21121002, 26119005) to M.K.,
449 and also in part by the Kurata Memorial Hitachi Science and Technology Foundation and by a
450 Grant-in-Aid for Scientific Research (C) (25440018) to M.T.
451

452 References:

453 André, I., Linse, S., and Mulder, F. A.: Residue-specific pKa determination of lysine and
454 arginine side chains by indirect ^{15}N and ^{13}C NMR spectroscopy: application to apo
455 calmodulin, J. Am. Chem. Soc., 129, 15805-15813. https://doi.org/10.1021/ja0721824,
456 2007.
457 Barbas, C. F. 3rd, Heine, A., Zhong, G., Hoffmann, T., Gramatikova, S., Björnestedt, R., List,
458 B., Anderson, J., Stura, E. A., Wilson, I. A., and Lerner, R. A.: Immune versus natural
459 selection: antibody aldolases with enzymic rates but broader scope, Science, 278, 2085-
460 2092. https://doi: 10.1126/science.278.5346.2085, 1997.
461 Cavanagh, J., Fairbrother, W. J., Palmer, A. G., Rance, M., and Skelton, J. J.: Protein NMR
462 Spectroscopy: Principles and Practice, Academic Press, New York, 2007.
463 Chimenti MS, Castañeda CA, Majumdar A, García-Moreno E B. Structural origins of high
464 apparent dielectric constants experienced by ionizable groups in the hydrophobic core of a
465 protein. J. Mol. Biol., 405, 361-377, https://doi:10.1016/j.jmb.2010.10.001, 2011.
466 Clore, G. M., Bax, A., Driscoll, P. C., Wingfield, P. T., and Gronenborn, A. M.: Assignment of
467 the side-chain ^{1}H and ^{13}C resonances of interleukin-1 beta using double- and triple-
468 resonance heteronuclear three-dimensional NMR spectroscopy, Biochemistry, 29, 8172-
469 8184, https://doi: 10.1021/bi00487a027, 1990.
470 Damblon, C., Raquet, X., Lian, L. Y., Lamotte-Brasseur, J., Fonze, E., Charlier, P., Roberts, G.
471 C., Frère, J. M.: The catalytic mechanism of beta-lactamases: NMR titration of an active-site
472 lysine residue of the TEM-1 enzyme, Proc. Natl. Acad. Sci. USA, 93, 1747-1752,
473 https://doi: 10.1073/pnas.93.5.1747, 1996.

Dziembowska, T., Hansen, P. E., and Rozwadowski, Z.: Studies based on deuterium isotope effect on ^{13}C chemical shifts, Prog. Nucl. Magn. Reson. Spectrosc., 45, 1-29, https://doi: 10.1016/j.pnmrs.2004.04.001, 2004.

Farmer, B. T. II, and Venters, R. A.: Assignment of aliphatic side-chain $^{1}HN/^{15}N$ resonances in perdeuterated proteins, J. Biomol. NMR, 7, 59-71, https://doi: 10.1007/BF00190457. 1996.

Fitch, C. A., Karp, D. A., Lee, K. K., Stites, W. E., Lattman, E. E., and García-Moreno, E. B.: Experimental pK(a) values of buried residues: analysis with continuum methods and role of water penetration, Biophys. J., 82, 3289-3304, https://doi: 10.1016/s0006-3495(02)75670-1, 2002.

Gao, G., Prasad, R., Lodwig, S. N., Unkefer, C. J., Beard, W. A., Wilson, S. H., and London, R. E.: Determination of lysine pK values using [5-^{13}C]lysine: application to the lyase domain of DNA Pol beta, J. Am. Chem. Soc., 128, 8104-8105, https://doi: 10.1021/ja061473u, 2006.

García-Moreno, B., Dwyer, J. J., Gittis, A. G., Lattman, E. E., Spencer, D. S., and Stites, W. E.: Experimental measurement of the effective dielectric in the hydrophobic core of a protein, Biophys. Chem., 64, 211-224. https://doi.org/10.1016/S0301-4622(96)02238-7, 1997.

Hansen, P. E.: Isotope effects on nuclear shielding, Annu. Rep. NMR Spectrosc., 15, 105-234, 1983.

Harris, T. K., and Turner, G. J.: Structural basis of perturbed pKa values of catalytic groups in enzyme active sites, IUBMB Life, 53, 85-98, https://doi: 10.1080/15216540211468, 2002.

Highbarger, L. A., Gerlt, J. A., and Kenyon, G. L.: Mechanism of the reaction catalyzed by acetoacetate decarboxylase. Importance of lysine 116 in determining the pKa of active-site lysine 115, Biochemistry, 35, 41-46. https://doi: 10.1021/bi9518306, 1996.

Iwahara, J., Jung, Y. S., and Clore, G. M.: Heteronuclear NMR spectroscopy for lysine NH_3 groups in proteins: unique effect of water exchange on ^{15}N transverse relaxation, J. Am. Chem. Soc., 129, 2971-2980. https://doi: 10.1021/ja0683436, 2007.

Kainosho, M., and Tsuji, T. Assignment of the three methionyl carbonyl carbon resonances in Streptomyces subtilisin inhibitor by a carbon-13 and nitrogen-15 double-labeling technique. A new strategy for structural studies of proteins in solution, Biochemistry, 21, 6273-6279. https://doi: 10.1021/bi00267a036, 1982.

Kainosho, M., Torizawa, T., Iwashita, Y., Terauchi, T., Ono, A. M., and Güntert, P.: Optimal

isotope labelling for NMR protein structure determinations, Nature, 440, 52–57, https://doi: 10.1038/nature04525, 2006.

Kesvatera, T., Jönsson, B., Thulin, E., and Linse, S.: Measurement and modelling of sequence-specific pKa values of lysine residues in Calbindin D9k. J. Mol. Biol., 259, 828-839, https://doi.org/10.1006/jmbi.1996.0361, 1996.

Liepinsh, E., Otting, G., and Wüthrich, K.: NMR spectroscopy of hydroxyl protons in aqueous solutions of peptides and proteins, J. Biomol. NMR, 2, 447-465, https://doi: 10.1007/BF02192808, 1992.

Liepinsh, E., and Otting, G.: Proton exchange rates from amino acid side chains-implications for image contrast, Magn. Reson. Med., 35, 30-42, https://doi: 10.1002/mrm.1910350106, 1996.

Markley, J. L., Bax, A., Arata, Y., Hilbers, C. W., Kaptein, R., Sykes, B. D., Wright, P. E., Wüthrich, K.: Recommendations for the presentation of NMR structures of proteins and nucleic acids, Eur. J. Biochem., 256, 1-15, https://doi.10.1046/j.1432-1327.1998.2560001.x, 1998.

Markley, J. L., and Kainosho, M.: Stable isotope labeling and resonance assignments in larger proteins. in NMR of Macromolecules, Oxford University Press, 101-152, 1993.

Otting, G., and Wüthrich, K.: Studies of protein hydration in aqueous solution by direct NMR observation of individual protein-bound water molecules, J. Am. Chem. Soc., 111, 1871-1875, https://doi.org/10.1021/ja00187a050, 1989.

Otting, G., Liepinsh, E., and Wüthrich, K.: Proton exchange with internal water molecules in the protein BPTI in aqueous solution, J. Am. Chem. Soc., 113, 4363-4364, https:// doi.org/10.1021/ja00011a068, 1991.

Poon, D. K., Schubert, M., Au, J., Okon, M., Withers, S. G., and McIntosh, L. P.: Unambiguous determination of the ionization state of a glycoside hydrolase active site lysine by ^{1}H-^{15}N heteronuclear correlation spectroscopy, J. Am. Chem. Soc., 128, 15388-15389, https://doi: 10.1021/ja065766z, 2006.

Segawa T., Kateb F., Duma L., Bodenhausen G., Pelupessy P.: (2008) Exchange rate constants of invisible protons in proteins determined by NMR spectroscopy. ChemBioChem, 9, 537-542, https: //doi:10.1002/cbic.200700600, 2008.

Stites, W. E., Gittis, A. G., Lattman, E. E., and Shortle, D.: In a staphylococcal nuclease mutant

the side-chain of a lysine replacing valine 66 is fully buried in the hydrophobic core, J. Mol. Biol., 221, 7-14, https://doi: 10.1016/0022-2836(91)80195-z, 1991.

Takayama, Y., Castañeda, C. A., Chimenti, M., García-Moreno, B., and Iwahara, J.: Direct evidence for deprotonation of a lysine side chain buried in the hydrophobic core of a protein, J. Am. Chem. Soc., 130, 6714-6715, https://doi: 10.1021/ja801731g, 2008.

Takeda, M., Jee, J., Ono, A. M., Terauchi, T., and Kainosho, M.: Hydrogen exchange rate of tyrosine hydroxyl groups in proteins as studied by the deuterium isotope effect on Cζ chemical shifts, J. Am. Chem. Soc., 131, 18556-18562, https:// doi: 10.1021/ja907911y, 2009.

Takeda, M., Jee, J., Terauchi, T., and Kainosho, M.: Detection of the sulfhydryl groups in proteins with slow hydrogen exchange rates and determination of their proton/deuteron fractionation factors using the deuterium-induced effects on the ^{13}Cβ NMR signals, J. Am. Chem. Soc., 132, 6254-6260, https://doi: 10.1021/ja101205j, 2010.

Takeda, M., Jee, J., Ono, A. M., Terauchi, T., and Kainosho, M.: Hydrogen exchange study on the hydroxyl groups of Serine and Threonine residues in proteins and structure refinement using NOE restraints with polar side-chain groups, J. Am. Chem. Soc., 133, 17420-17427, https://doi: 10.1021/ja206799v, 2011.

Takeda, M., Miyanoiri, Y., Terauchi, T., Yang, C.-J., and Kainosho, M.: Use of H/D isotope effects to gather information about hydrogen bonding and hydrogen exchange rates, J. Magn. Reson., 241, 148-154, https://doi: 10.1016/j.jmr.2013.10.001, 2014.

Terauchi, T., Kamikawai, T., Vinogradov, M. G., Starodubtseva, E. V., Takeda, M., and Kainosho, M.: Synthesis of stereoarray isotope labeled (SAIL) lysine via the "head-to-tail" conversion of SAIL glutamic acid, Org. Lett., 13, 161-163, https:// doi: 10.1021/ol1026766, 2011.

Appendices

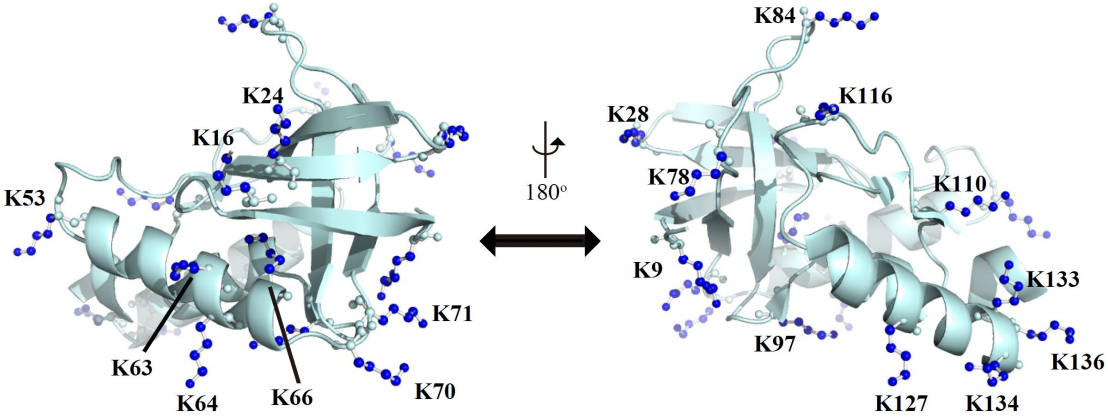


Figure A1: Distribution of the Lys residues in the crystalline structure of the Δ+PHS/V66K variant of SNase (PDB#: 3HZX). All of the side-chain moieties of the Lys residues, which are shown by the ball-and-stick model in blue, exist on the protein surface, except for the Lys-66 (K66). This engineered residue is locked in the protein interior that is originally occupied by the Val sidechain in the wild-type protein. Two Lys residues, K5 and K6, were not visible in the X-ray analysis of the SNase complexed with calcium and thymidine 3',5'-diphosphate and thus it may be slightly different from that in the free state. The figure was created using the Pymol 2.4 software.

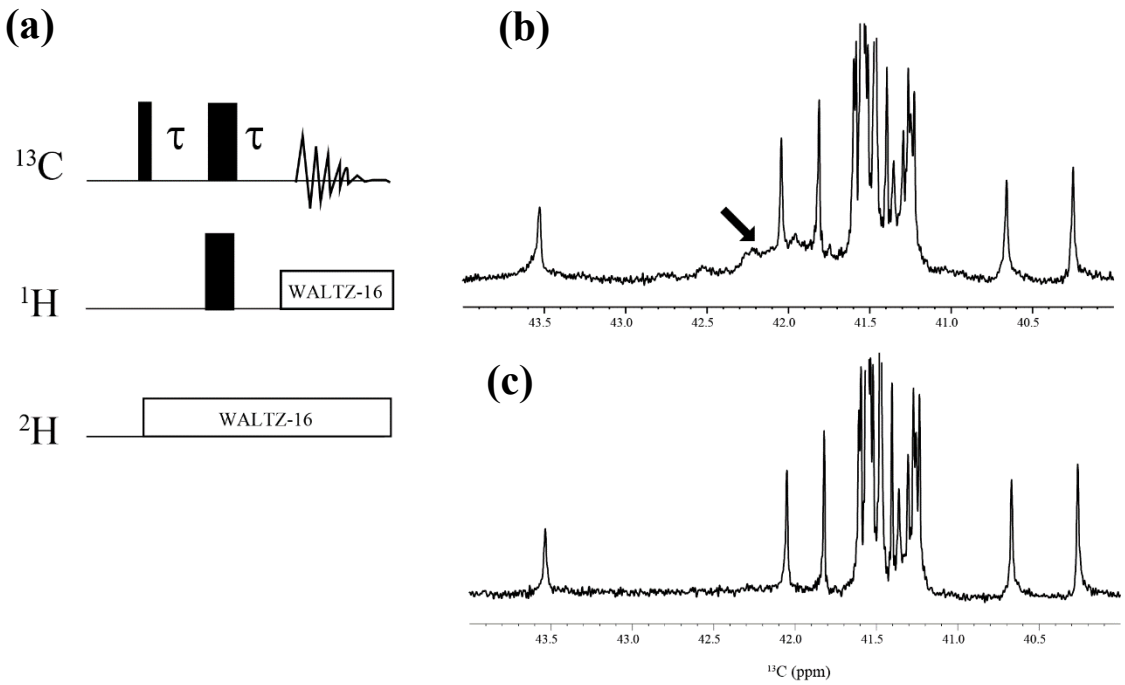


Figure A2: {^{1}H, ^{2}D}-1D ^{13}C NMR spectra for the SNase variant selectively labeled with [ε-^{13}C;ε,ε-D$_2$]-Lys. The 125.7 MHz ^{13}C NMR spectra were measured at 25 ˚C on a Bruker Avance 500 spectrometer equipped with a ^{13}C-observing DCH cryogenic probe. The broad background signals observed in the spectrum (b), indicated by a thick arrow, are due to the natural abundant ^{13}C atoms bound to proton(s), which are readily filtered out to give the spectrum (c), by using the pulse scheme shown in (a). The narrow and wide bars in the scheme represent 90 and 180 º rectangular pulses, respectively, and are applied along the x-axis at τ =1.7 ms, which corresponds to 1/4 $^{1}J_{CH}$. The SNase variant was dissolved in 100 % D_2O buffer, containing 20 mM sodium phosphate and 100 mM potassium chloride at pH 8.0.

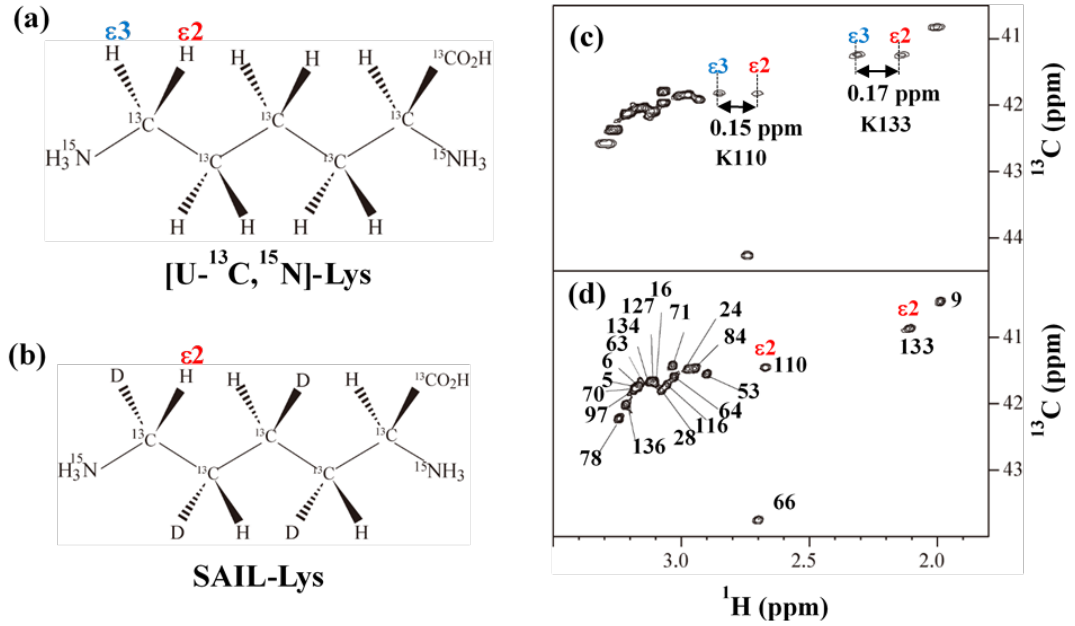


Figure A3: Comparison between the $^{13}C^{\varepsilon}$ regions of the 2D ^{1}H-^{13}C constant time (ct-) HSQC spectra obtained for the SNase variant selectively labeled with [U-^{13}C,^{15}N]-Lys (a) and [U-^{13}C, ^{15}N;β_2,γ_2,δ_2,ε_3-D_4]-Lys, SAIL-Lys (b). Since ε-carbons for the [U-^{13}C,^{15}N]-Lys residues are attached to the two prochiral protons, $^{1}H^{\varepsilon 2}$ and $^{1}H^{\varepsilon 3}$, a pairwise correlation signals, namely $^{1}H^{\varepsilon 2}$-$^{13}C^{\varepsilon}$ and $^{1}H^{\varepsilon 3}$-$^{13}C^{\varepsilon}$, can be observed for each of the ε-carbons (c). However, considerable large chemical shift difference between the prochiral ε-methylene protons were observed only for K110 (Δδ, 0.15 ppm) and for K133 (Δδ, 0.17 ppm), and the other 19 Lys residues showed the differences less than ~0.05 ppm. On the other hand, ε-carbons for the SAIL-Lys residues are attached only to the ε2-protons, all of the correlation signals are automatically assigned to $^{1}H^{\varepsilon 2}$ (d). C^{ε} peaks are labeled with their assignment. The spectra were measured at 30 ˚C on an Avance 600 spectrometer equipped with a TXI cryogenic probe.

595

	χ^1	χ^2	χ^3	χ^4
K5	n.d.	n.d.	n.d.	n.d.
K6	n.d.	n.d.	n.d.	n.d.
K9 ***Form A***	-73	169	-167	-175
Form B	-78	171	-153	44
K16	-171	177	32	-127
K24	-170	157	146	171
K28	-80	177	161	149
K53	-171	164	-164	164
K63	-180	-172	160	73
K64	164	179	174	166
K66	-102	108	81	-73
K70	82	135	171	78
K71	176	164	141	-158
K78	-69	-72	-174	-144
K84	65	-166	166	161
K97	175	176	-155	-144
K110	-83	170	-180	177
K116	-148	134	99	-165
K127	168	66	164	-67
K133	171	156	18	-179
K134	-134	155	-71	126
K136	-68	-112	-143	-50

596

597 **Table A1: List of the dihedral angles (χ^1, χ^2, χ^3, χ^4) in the crystalline state (PDB #3HZX).**