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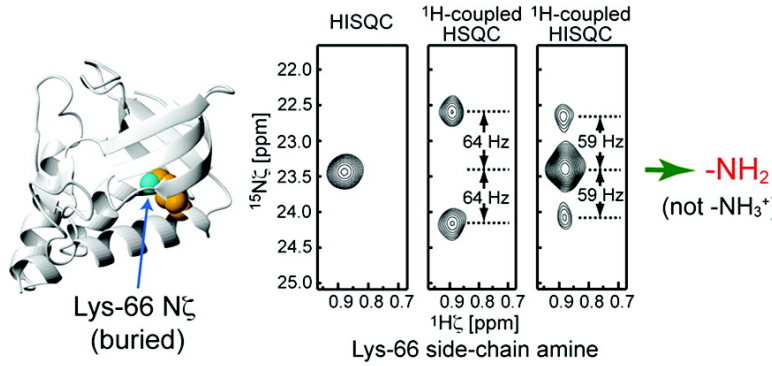

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Direct Evidence for Deprotonation of a Lysine Side Chain Buried in the Hydrophobic Core of a Protein

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The amine (Nζ) groups of the lysine (Lys) side chains in proteins typically titrate with pK_a values near 10.5; therefore, they are usually protonated (NH_3^+) at neutral pH. The positively charged NH_3^+ groups are usually found at the protein surface, where they interact extensively with water. In efforts to understand the molecular determinants of the pK_a values of internal ionizable groups, which are essential for catalysis and bioenergetics, a Lys residue was introduced into the hydrophobic core of staphylococcal nuclease (SNase) by engineering the V66K substitution with site-directed mutagenesis.[1,2] Two completely independent equilibrium thermodynamic methods were used to show that the pK_a of Lys-66 is 5.6.[1−3] Crystal structures obtained at neutral pH show that the Lys side chain is indeed internal and buried deeply in the hydrophobic core of the protein (Figure 1A).[2] The structures suggest that the pK_a of Lys-66 is depressed owing to the dehydration experienced in the buried state, which is not compensated by interactions with other polar or ionizable groups. Judging from its pK_a, Lys-66 should be deprotonated (NH_2) at neutral pH. Here, we report the first ever $^1H-^{15}N$ heteronuclear NMR spectra recorded on a Lys in the NH_2 state and discuss the highly unusual spectroscopic signature of a neutral Lys in a hydrophobic environment.

Direct observation of NH_3^+ groups with $^1H-^{15}N$ correlation experiments at high pH is difficult owing to rapid hydrogen exchange with water, which results in severe line-broadening in the ^1H dimension. The rapid hydrogen exchange can also broaden ^{15}N line shapes through the scalar relaxation of the second kind.[4,5] This indirect effect is suppressed in the $^1H-^{15}N$ HISQC experiment optimized for the Nζ groups of Lys, which leads to significant sensitivity gain.[5] Even with this technique, no cross peaks were observed at pH 8.0 from the 20 NH_3^+ groups from Lys in the wild-type SNase owing to very rapid hydrogen exchange. On the other hand, under the same conditions, the V66K variant exhibited a single cross peak at a very unique and unusual position: ^1H 0.81 ppm and ^{15}N 23.3 ppm at 6 °C (Figure 1B). By using $^1H/^{13}C/^{15}N$ triple resonance experiments, this HISQC cross peak was assigned to the Lys-66 Nζ group (Supporting Information), which is buried in the hydrophobic core in the crystal structure.

The observed ^1H and ^{15}N chemical shifts for this Nζ group are too abnormal to correspond to NH_3^+. Typical chemical shifts for the NH_3^+ groups of Lys residues in proteins are 7−8 ppm for ^1H and 31−34 ppm for ^{15}N.[5−7] Neither aromatic rings nor paramagnetic ions that could cause the abnormal chemical shifts are present near the Nζ group of Lys-66 in the structures of the V66K variants. The abnormal ^1H and ^{15}N chemical shifts were therefore interpreted as belonging to the Nζ group of Lys-66 in the NH_2 state. This notion is supported by the observation that ^1H chemical shifts for sp^3-type alkyl-NH_2 groups of small compounds are typically ∼1−2 ppm,[8] although chemical shifts for nitrogen-attached ^1H nuclei in protein NMR spectroscopy are mostly >6 ppm. Note that, except for the amine groups of N-termini and of the Lys side chains, all nitrogen atoms in proteins are of the planar sp^2-type. Furthermore, the $^{15}N\zeta$ chemical shift of free Lys in water at pH 12.7, where Lys should be in the neutral state, has been reported to be 25.5 ppm.[7] We also studied *n*-butylamine ($CH_3CH_2CH_2CH_2NH_2$) in a hydrophobic solvent as a model system that mimics the Lys side chain in the hydrophobic core (Supporting Information). In this case, the amine group of *n*-butylamine is deprotonated owing to the absence of a proton donor. ^1H and ^{15}N chemical shifts for the amine group of the *n*-butylamine were found to be ∼1.4 and ∼22 ppm, respectively, depending on solvent. Overall, the data strongly suggest that the ^1H and ^{15}N chemical shifts observed for the Nζ group of Lys-66 (Figure 1B) correspond to the NH_2 state rather than to the NH_3^+ state. The single ^1H resonance for the two prochiral NH_2 protons can be explained by the rapid chiral inversion of sp^3 nitrogen occurring with a relatively low energy barrier (∼7 kcal/mol),[9] for which the Eyring equation predicts submicrosecond time scale.

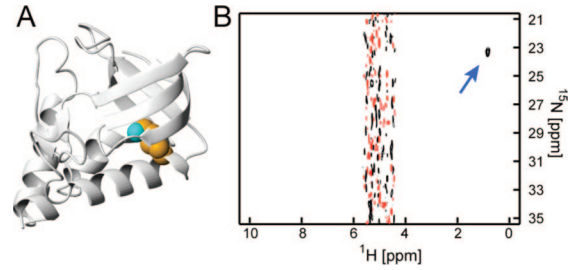

Figure 1. (A) Crystal structure of the V66K variant of SNase (PDB entry 2SNM[2]). The side chain of Lys-66, buried in the hydrophobic core, is shown in space-fill representation (Nζ, cyan). (B) Lysine amine selective $^1H-^{15}N$ HISQC spectrum[5] recorded on 1 mM ^{15}N-labeled Δ-PHS/V66K variant of SNase[1] at pH 8.0 and 6 °C (positive contours, black; negative, red). The blue arrow indicates the resonance from the Nζ group of Lys-66 in the NH_2 state. Owing to the rapid water exchange at high pH, the NH_3^+ groups of the 20 Lys residues (typical chemical shifts: ^1H, 7−8 ppm; ^{15}N, 31−34 ppm) do not show up. The spectrum was measured with a Varian NMR system operated at ^1H frequency of 800 MHz; 1600 scans were accumulated per FID.

^{15}N multiplets for the Nζ group provide more straightforward and clear evidence for the deprotonation of Lys-66 at pH 8.0. In terms of $^1H-^{15}N$ heteronuclear NMR, the NH_2 and NH_3^+ states are AX_2 and AX_3 spin systems, respectively. In the F1-^1H-coupled HSQC experiment, an AX_3 spin system exhibits a 3:1:1:3 quartet due to a modulation of $(\cos^3 \pi J t_1 - 2 \sin^2 \pi J t_1 \cos \pi J t_1)\cos \Omega_A t_1$, whereas AX_2 exhibits a 1:0:1 "triplet" due to $(\cos^2 \pi J t_1 - \sin^2 \pi J t_1)\cos \Omega_A t_1$ (note that coherence transfers such as $2N_yH_z^a \rightarrow 2N_yH_z^b$ occurring during the t_1 period also generate observable magnetizations). The F1-coupled

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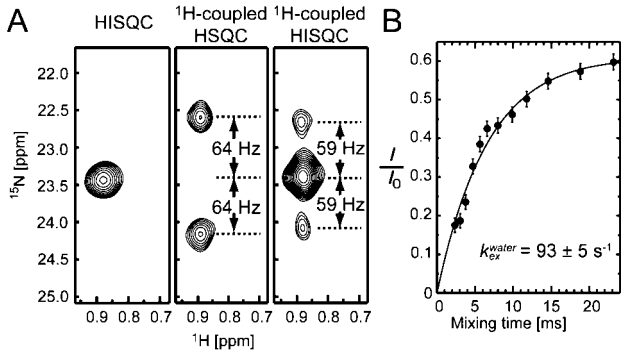

Figure 2. (A) Evidence for the deprotonated state (NH_2) of the amine group of Lys-66 in the Δ+PHS/V66K variant of SNase. The F1-coupled HSQC spectrum should exhibit a 3:1:1:3 quartet for NH_3^+ and a 1:0:1 triplet for NH_2, provided that the relaxation rates for individual components are identical. The pulse sequence of the F1-^{1}H-coupled HISQC was designed to observe 1:3:3:1 quartet for NH_3^+ and 1:2:1 triplet for NH_2 (Supporting Information). The splitting widths for the 1:0:1 and 1:2:1 triplets appear to be different primarily due to different effects of self-decoupling on these two rather than to the partial overlaps of individual components (see main text and Supporting Information). The spectra were measured at pH 8.0 and −1 °C. (B) Buildup curve for the CLEANEX-HSQC[10] signal arising from exchange between water and the Lys-66 NH_2 group at pH 8.0 and −1 °C. The vertical axis represents the ratio of signal intensity I to the reference intensity I_0. The value of k_{ex}^{water} was determined as described.[10]

^{1}H−^{15}N HSQC spectrum recorded on the Nζ group of Lys-66 is shown in Figure 2A. The observed multiplet was indeed 1:0:1 with $|^1J_{NH}| = 64$ Hz. Interestingly, the J-coupling is significantly smaller than those observed for the NH_3^+ groups of Lys (∼74 Hz).[5,7] We designed the F1-^{1}H-coupled HISQC experiment to observe the 1:3:3:1 quartet arising from a modulation of $\cos^3 \pi J t_1 \cos \Omega_A t_1$ for AX_3 and the 1:2:1 triplet from $\cos^2 \pi J t_1 \cos \Omega_A t_1$ for AX_2 (Supporting Information). The ^{15}N multiplet observed for the Nζ group of Lys-66 with this experiment was a triplet, as shown in Figure 2A. These data unambiguously indicated that the Nζ group of Lys-66 is in the NH_2 state. It is intriguing that splitting widths for the 1:0:1 and 1:2:1 triplets appear to be slightly different. This is mainly due to self-decoupling effect, as described below.

Perhaps surprisingly, the NH_2 group of Lys-66 exhibits relatively rapid hydrogen exchange with water despite being buried in the hydrophobic core. We measured the water exchange rate k_{ex}^{water} using the CLEANEX-HSQC experiment.[10] The value of k_{ex}^{water} was found to be highly dependent on temperature and as rapid as 93 ± 5 s^{-1} even at −1 °C (Figure 2B). The rapid water exchange rate dominates the relaxation of the antiphase ^{15}N transverse magnetizations such as $2H_zN^+$ and $4H_zH_zN^+$ through the scalar relaxation of the second kind.[5] In fact, the apparent ^{15}N transverse relaxation rates obtained from Lorentzian line shape fitting against ^{1}H−^{15}N HSQC and HISQC signals from NH_2 group of Lys-66 at −1 °C were 125 ± 3 and 36 ± 4 s^{-1}, respectively, and the difference roughly corresponds to k_{ex}^{water}. The observed k_{ex}^{water} rate still reflects the protection due to burial because the rate for an exposed lysine amine group is estimated to be ∼700 s^{-1} at −1 °C and pH 8.0 from previous investigations of the NH_3^+ of free Lys and the empirical equation for the temperature dependence.[11,12]

The rapid water exchange causes the partial self-decoupling effect on the ^{15}N transverse magnetization. As described previously for the AX spin system, self-decoupling causes a singlet even in the presence of J-coupling when the difference between relaxation rates for antiphase and in-phase terms is much greater than $2\pi J$.[13] If the difference is comparable to $2\pi J$, the J-splitting appears to be smaller than the actual J-coupling.[14,15] We simulated the self-decoupling due to the water exchange for the ^{15}N transverse magnetizations of the NH_2 spin system (Supporting Information). Interestingly, the effects of the self-decoupling on the 1:2:1 triplet in the ^{1}H-coupled HISQC experiment and the 1:0:1 triplet in the ^{1}H-coupled HSQC are significantly different (Figure S4 in Supporting Information), which accounts well for the different J-splittings observed for 1:2:1 triplet (59 Hz) and 1:0:1 triplet (64 Hz).

The fact that the internal NH_2 group of Lys-66 exhibits rapid water exchange is consistent with the presence of a dynamic process such as local unfolding or water penetration,[16] which may be enhanced by the instability owing to the burial of the ionizable group in a hydrophobic region of the protein. Although the rapid water exchange reduces the sensitivity of the NMR experiments with direct ^{1}Hζ detections, Nζ groups can also be analyzed using triple resonance experiments with detection at nonlabile ^{1}Hε/^{1}Hδ with higher sensitivity.[6,17] The identification of NMR signals representing a clear signature of the Nζ group of Lys residues when they are in the NH_2 state will allow detailed studies of determinants of the pK_a values therefore interpreted as belonging to the Nζ group of Lys-66 in internal Lys residues in proteins and kinetic and thermodynamic aspects of ionization of internal groups in proteins.

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Supporting Information Available: Resonance assignment of the Lys-66 Nζ group; NMR data on *n*-butylamine; pulse sequence of the 2D ^{1}H−^{15}N F1-^{1}H-coupled HISQC experiment; simulation of the self-decoupling effect for NH_2 system. This material is available free of charge via the Internet at http://pubs.acs.org.

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