doi:10.1016/j.jmb.2009.03.039 J. Mol. Biol. (2009) 389, 34 47

JMB

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

The pK_a Values of Acidic and Basic Residues Buried at the Same Internal Location in a Protein Are Governed by Different Factors

Michael J. Harms[1], Carlos A. Castañeda[1], Jamie L. Schlessman[1,2], Gloria R. Sue[1], Daniel G. Isom[1], Brian R. Cannon[1] and Bertrand García-Moreno E.[1]*

[1]*Department of Biophysics, Johns Hopkins University, 3400 North Charles Street, Baltimore, MD 21218, USA*

[2]*Department of Chemistry, United States Naval Academy, 572 Holloway Road, Annapolis, MD 21402, USA*

Received 4 December 2008; received in revised form 6 March 2009; accepted 11 March 2009
Available online 24 March 2009

The pK_a values of internal ionizable groups are usually very different from the normal pK_a values of ionizable groups in water. To examine the molecular determinants of pK_a values of internal groups, we compared the properties of Lys, Asp, and Glu at internal position 38 in staphylococcal nuclease. Lys38 titrates with a normal or elevated pK_a, whereas Asp38 and Glu38 titrate with elevated pK_a values of 7.0 and 7.2, respectively. In the structure of the L38K variant, the buried amino group of the Lys38 side chain makes an ion pair with Glu122, whereas in the structure of the L38E variant, the buried carboxyl group of Glu38 interacts with two backbone amides and has several nearby carboxyl oxygen atoms. Previously, we showed that the pK_a of Lys38 is normal owing to structural reorganization and water penetration concomitant with ionization of the Lys side chain. In contrast, the pK_a values of Asp38 and Glu38 are perturbed significantly owing to an imbalance between favorable polar interactions and unfavorable contributions from dehydration and from Coulomb interactions with surface carboxylic groups. Their ionization is also coupled to subtle structural reorganization. These results illustrate the complex interplay between local polarity, Coulomb interactions, and structural reorganization as determinants of pK_a values of internal groups in proteins. This study suggests that improvements to computational methods for pK_a calculations will require explicit treatment of the conformational reorganization that can occur when internal groups ionize.

Keywords: pK_a values; internal ionizable groups; structure/function; energy calculations; electrostatics

Edited by C. R. Matthews

Introduction

A small fraction of ionizable residues in proteins are sequestered from water and buried in the protein interior.[1–3] These internal ionizable groups are essential for catalysis,[4–6] H^+/e transport,[7–10] and molecular recognition.[11] The pK_a values of internal ionizable groups are usually different from the normal pK_a values in water[12–19] and are often tuned for specific biological purposes.[4] Understanding the determinants of these pK_a values is important for quantitative description of the structural basis of function in a large variety of biological processes.

The shift in the pK_a of an internal group relative to the normal pK_a in water is governed by differences in the polarity and polarizability experienced by the charge in the two environments (ΔG_{self}) and by Coulomb interactions with the charges of other ionizable groups. Structural reorganization of the protein coupled to the ionization of internal groups can also influence their pK_a. One of the goals of this study was to examine the relative magnitude of these three determinants of the pK_a values of internal groups.

The polarity and polarizability in the protein interior are usually lower than those in bulk water;

*Corresponding author. E-mail address: bertrand@jhu.edu.

Abbreviations used: SNase, staphylococcal nuclease; THP, thymidine-3′,5′-diphosphate; HSQC, heteronuclear single quantum coherence; MCCE, multi-conformer continuum electrostatics; PDB, Protein Data Bank.

therefore, ΔG_{self} is generally unfavorable for buried ionizable groups. For this reason, the pK_a values of internal ionizable groups are usually shifted in the direction that favors the neutral state (i.e., increase in pK_a for acidic groups and depression for basic ones).[12–18] Surprisingly, the apparent polarity and polarizability in the protein interior reported by internal ionizable groups are not as low as previously thought.[15–17,20–25] In some cases, hydrogen bonds (i.e., high polarity) can actually compensate fully for the loss of hydration experienced by a charged atom inside a protein (M.J.H., J.L.S., G.R.S., and B.G.-M.E, unpublished results).[6,19]

Coulomb interactions between surface charges are usually weak because charges are screened effectively by water.[26–30] In contrast, the Coulomb interaction of ion pairs sequestered from bulk solvent at protein–protein interfaces can be quite strong (3–5 kcal/mol).[31] Coulomb interactions between surface and internal groups in protein active sites have never been studied directly. Surface ionizable groups have been shown to have small but observable effects on enzyme activity.[32–34] Even if the effects are small, the sum of many small interactions could lead potentially to a large effect.[35,36] A complete understanding of interactions between internal and surface charges is necessary to understand contributions of surface ionizable residues to the properties of internal groups at active sites and interfaces.[4]

Staphylococcal nuclease (SNase) is an excellent model system for studying properties of internal ionizable groups systematically and for dissecting molecular determinants of their pK_a values. It has been shown that hyperstable variants of SNase can tolerate substitutions of 25 internal positions with Lys, Asp, Glu, and Arg.[37] The majority of these internal ionizable groups titrate with pK_a values shifted in the direction that promotes the neutral state, some by as much as 5.7 pK_a units (M.J.H., J.L.S., G.R.S., and B.G.-M.E, unpublished results).[15,16,22,23,25,38] We have shown previously that, although Lys38 in SNase is internal, it titrates with a normal or possibly elevated pK_a value. The pK_a is not depressed despite the amino group being secluded from bulk water in the crystal structure; water penetration facilitated by structural relaxation ensures hydration of the charged group.[38] In contrast, we show here that the pK_a of Glu38 and Asp38 is shifted significantly. The differences in the ionization behavior of Lys, Glu, and Asp at position 38 in SNase offer opportunities to examine contributions by the reaction field of bulk solvent, local polarity and polarizability, conformational reorganization, and Coulomb interactions to the pK_a values of these internal ionizable groups.

Results

Crystal structure of the L38E variant

Two hyperstable variants of SNase were used in this study: PHS and Δ+PHS. The structure of the PHS/L38E variant was solved to 2.0 Å and compared to the structures of PHS nuclease and the PHS/L38K variant.[38,39] Refinement statistics are shown in Supplementary Table 1. PHS nuclease was used for crystallographic studies instead of the Δ+PHS form of nuclease that was used for equilibrium thermodynamic and NMR spectroscopy experiments because PHS/L38E crystallized and Δ+PHS/L38E did not. PHS nuclease contains six residues (44–49) in a dynamic loop and two point mutations (F50G and N51V) that are not present in Δ+PHS nuclease. The structures of Δ+PHS and PHS variants are superimposable.[39,40]

The overall structure of the PHS/L38E variant is comparable to the structures of PHS nuclease (C^α RMSD = 0.7 Å) and of the PHS/L38K variant (C^α RMSD = 0.4 Å), even in the region surrounding Glu38 (Fig. 1a). The primary difference between the PHS/L38E and PHS/L38K structures is the position of Glu122. This residue is in the same position in the structure of PHS and PHS/L38E, whereas the C^δ of Glu122 is shifted by 1.6 Å to establish a Lys38/Glu122 ion pair in the structure of PHS/L38K.[38] Residues 113–115 in the structure of PHS/L38E are

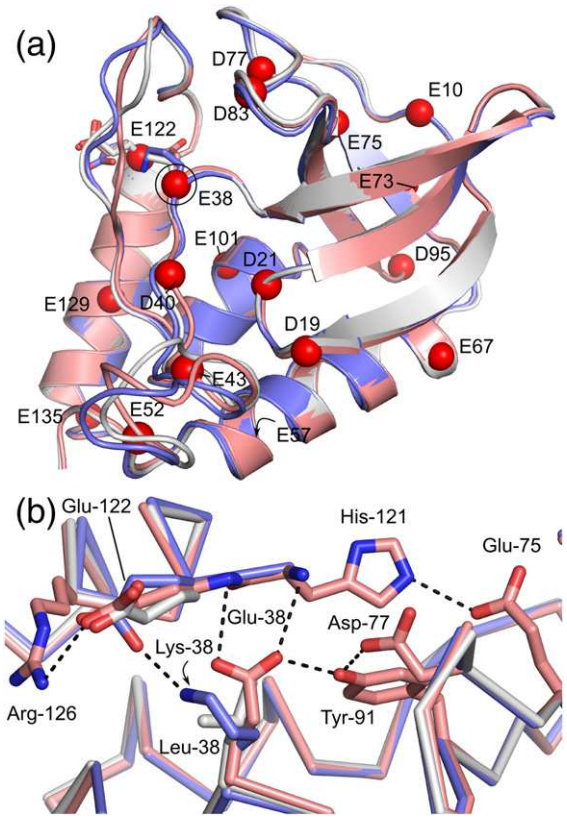

Fig. 1. Crystal structure of PHS/L38E (pink, PDB accession code 3D6C) overlaid on the structures of PHS/L38K (blue, PDB accession code 2RKS) and PHS nuclease (white, PDB accession code 1EY8). (a) The global fold of the protein is not perturbed. The C^α atoms of Asp and Glu residues are shown as red spheres. Glu38, Lys38, and Glu122 are shown as sticks. (b) Microenvironment of Glu38 and Lys38. Ionizable residues within 8.4 Å of Glu38 are shown in stick, and hydrogen bonds are shown as broken lines.

in a slightly different conformation than in the other structures owing to the presence of an inhibitor [thymidine-3′,5′-diphosphate (THP)] that is present in the structure of PHS/L38E nuclease and absent in the other structures.

The oxygen atoms of the Glu38 side chain are completely solvent inaccessible in the structure of PHS/L38E. The nearest crystallographic water molecule is 5.4 Å from the Glu38 $O^{\varepsilon 1}$ atom. Thus far, this is the only crystal structure of an SNase variant with an internal oxygen atom in which the atom is not hydrated by an internal water molecule.[16,41] Interactions with internal water molecules might be precluded by hydrogen bonds between the carboxylic group of Glu38 and the backbone amides of Thr120 and His121 and the hydroxyl group of Tyr91 (Fig. 1b). The hydrogen bond to Tyr91 directly links Glu38 into an extensive hydrogen bond network.[29,30,42,43]

Although SNase is a basic protein, the ionizable residues nearest to Glu38 in the crystal structure of the PHS/L38E variant are acidic: Asp77 (4.0 Å) and Glu122 (5.1 Å). The next nearest ionizable residues are basic: His121 (6.5 Å) and Arg126 (6.6 Å) (Fig. 1b). The proximity of these residues makes them ideal for direct measurement of Coulomb interactions between surface charges and the carboxylic groups of Asp38 and Glu38.

pK_a values of Glu38 and Asp38

The pK_a values of Glu38 and Asp38 were measured by analysis of the pH dependence of protein stability. This method takes advantage of the thermodynamic linkage between proton binding and stability.[44] Measurement of the unfolding free energy ($\Delta G°_{H2O}$) of a protein as a function of pH reports on the pK_a values of all ionizable residues in the protein. The pK_a value of a single group introduced by mutagenesis can be measured by subtracting $\Delta G°_{H2O}$ of the background protein (i.e., Δ+PHS nuclease) from $\Delta G°_{H2O}$ of the variant protein (i.e., Δ+PHS/L38E). Shifts in the pK_a are reflected in the characteristic shape of the pH dependence of $\Delta\Delta G°_{H2O}$.[15,23,25] It was shown previously that the pK_a of Lys38 was ≥10.4, comparable to the normal pK_a of a Lys in water.[38] In contrast, the pK_a values of Glu38 and Asp38 were 7.0±0.3 and 6.8±0.3 pH units, respectively (Fig. 2). Relative to the normal pK_a values of 4.4 and 4.0 for Glu and Asp in water, respectively, this corresponds to shifts in pK_a of 2.6 and 2.8 pH units.

The measurement of pK_a values by analysis of the pH dependence of stability is too imprecise for detailed investigation of the contribution of Coulomb interactions to the observed pK_a value. An attempt was made to measure the pK_a of Glu38 with NMR spectroscopy using the pH dependence of the Glu38 C^{δ} resonance.[40] Although the C^{δ} resonance could be assigned at low pH, the peak entered intermediate exchange above pH 5.6 and could not be followed at higher pH values. Resonances corresponding to the C^{γ}/C^{δ} atoms of Glu73, Glu75, Asp77, Asp83, and Glu122 all showed a secondary apparent titration centered at pH 7.0. At positions 75 and 77, the magnitude of the secondary transition was greater than 0.5 ppm. All of the data from NMR spectroscopy are consistent with a pK_a of 7.0 for Glu38.

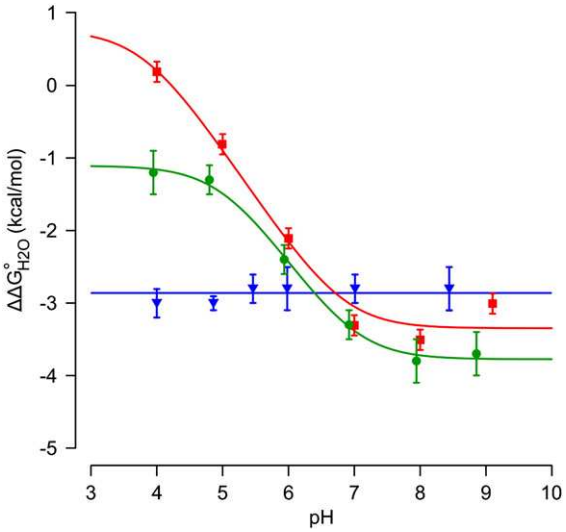

Fig. 2. pH dependence of $\Delta\Delta G°_{H2O}$ for the Δ+PHS/L38D (■), Δ+PHS/L38E (●), and Δ+PHS/L38K (▼) variants. Continuous lines are fits to the data (see Materials and Methods). Error bars are propagated from GdnHCl denaturation fit errors.

The pK_a of Glu38 was also obtained by performing a global fit to the pH titrations of multiple resonances.[45,46] Specifically, the pK_a was obtained by analysis of the pH dependence of the ^{1}H chemical shift of six backbone amides (Thr33, Phe34, Arg35, Glu75, Gly88, and Leu89). The titration events monitored by these amide backbone atoms in Δ+PHS nuclease are shown in Fig. 3a. No changes larger than 0.06 ppm were observed over the pH range studied. A small transition centered at pH 6.3±0.3 is visible for positions 34, 35, 75, and 89, most likely reflecting the titration of His8 or Asp21, whose pK_a values are both 6.5 in Δ+PHS nuclease.[40] In contrast, the pH dependence of the ^{1}H chemical shift of the same six amides in the Δ+PHS/L38E variant (Fig. 3b) reflects a large transition. A global fit of the modified Hill equation to this transition yielded a pK_a value of 7.0±0.1, in excellent agreement with the pK_a of Glu38 determined using linkage thermodynamics and the value inferred from the titration of carboxylic acids. A similar analysis of the L38D variant showed that Asp38 has a pK_a of 7.2±0.1, which is also in agreement with the value of 6.8±0.3 obtained by analysis of the pH dependence of stability of the Δ+PHS/L38D variant. The pK_a values extracted by global fit of NMR spectroscopy data and by linkage analysis are summarized in Table 1.

The agreement between the pK_a values measured from equilibrium thermodynamic data and from the global fit of titrations of backbone amide resonances suggests that the values obtained by NMR are

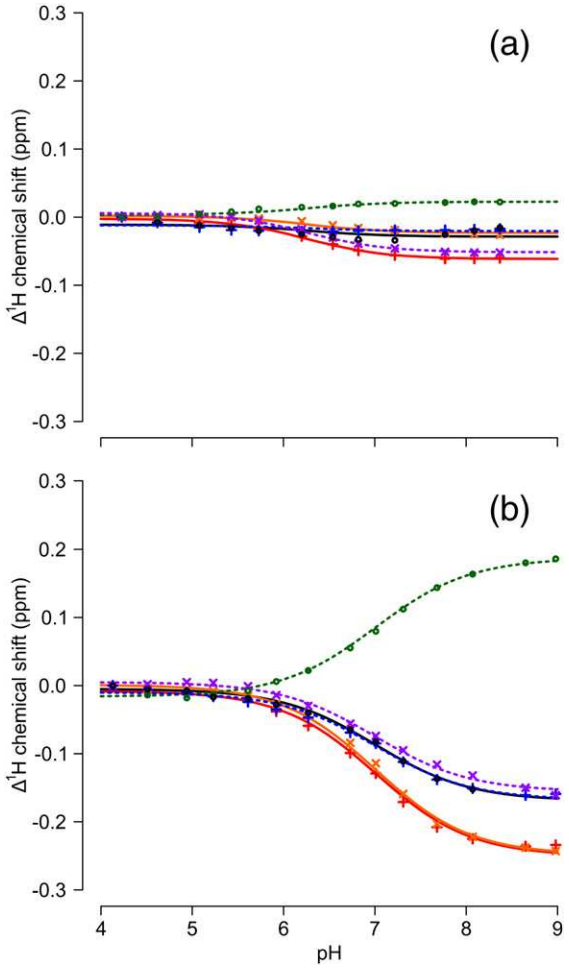

Fig. 3. Apparent titration of ^{1}H backbone resonances in Δ+PHS nuclease (a) and the Δ+PHS/L38E variant (b). Series are Thr33 (●), Phe34 (+), Arg35 (×), Glu75 (●), Gly88 (+), and Leu89 (×). Lines indicate a global fit to the apparent titration of all residues.

accurate. However, the NMR experiment does not follow the amino acid of interest directly. Other groups could be responsible for the observed transition. The pK_a values of all residues that titrate between pH 4.6 and 8.5 in Δ+PHS nuclease were measured in the Δ+PHS/L38E variant to examine this possibility. The pK_a values of His8, His121, and Asp21 were found to be 6.5, 5.7, and 6.5, respectively, in the Δ+PHS/L38E variant (Tables 2 and 3).

Table 1. Comparison of pK_a values determined by linkage analysis and NMR spectroscopy

		Linkage analysis		NMR	
Variant	Residue	pK_a	±	pK_a	±
Δ+PHS/L38E	Glu38	7.0	0.3	7.0	0.1
Δ+PHS/L38E/E122Q	Glu38	—	—	6.2	0.1
Δ+PHS/L38D	Asp38	6.8	0.3	7.2	0.1
Δ+PHS/L38D/E122Q	Asp38	6.9	0.3	6.6	0.1

—, pK_a could not be determined using linkage analysis because variant unfolds in an apparent three-state manner.

Table 2. pK_a values and Hill coefficients of His residues measured using NMR spectroscopy

	His8		His121	
Variant	pK_a[a]	n	pK_a[a]	n
Δ+PHS	6.6	1.0	5.4	0.8
Δ+PHS/E122Q	6.5	1.0	5.3	0.9
Δ+PHS/L38K	6.5	1.0	5.6	1.0
Δ+PHS/L38E	6.5	1.0	5.7	0.9
Δ+PHS/L38E/E122Q	6.4	1.1	5.7	0.9
Δ+PHS/L38D	6.5	1.0	5.7	0.9
Δ+PHS/L38D/E122Q	6.5	1.1	5.8	0.9

[a] Uncertainty in pK_a value is ±0.1.

This demonstrates that none of these groups are responsible for the apparent titration near pH 7 monitored with NMR spectroscopy. The ^{1}H chemical shifts of multiple groups appear to be reporting on the proton titration of Asp38 or Glu38.

Spectroscopic probes of structural rearrangement

Structural reorganization associated with the substitution of Leu38 with Asp or Glu or with the ionization of Asp, Glu, and Lys at position 38 was probed by circular dichroism (CD), Trp fluorescence, and NMR spectroscopy. The intrinsic fluorescence of Trp140, which caps the C-terminal end of helix 3, has been shown to be a robust reporter of the global integrity of SNase.[47] Trp fluorescence at neutral pH was insensitive to the substitution of Leu38 with ionizable groups (data not shown). CD spectra of all variants in the far-UV range were also indistinguishable from one another at pH 4, 7, and 10 (Fig. 4),

Table 3. pK_a values of Asp and Glu residues measured using NMR spectroscopy

	Δ+PHS[a]	Δ+PHS/L38E		Δ+PHS/L38K	
Position	pK_a[b]	pK_a[b]	ΔpK_a[c]	pK_a[b]	ΔpK_a[c]
D19	2.2	<2.5	—	<2.5	—
D21	6.5	6.6	0.0	6.5	0.0
D40	3.9	3.9	0.0	3.8	−0.1
D77	<2.5	<2.5	—	—	—
D83	<2.5	<2.5	—	<2.5	—
D95	2.2	2.3	0.1	2.1[d]	0.0
D143	3.8	3.8	0.0	4.0	0.2
D146	3.9	3.9	0.0	4.0	0.1
E10	2.8	3.1	0.2	3.1[d]	0.2
E43	4.3	4.4	0.0	4.5	0.2
E52	3.9	4.0	0.1	4.1	0.2
E57	3.5	3.6	0.1	3.6	0.1
E67	3.8	3.8	0.1	3.9	0.1
E73	3.3	3.4	0.1	3.3[d]	0.0
E75	3.3	3.3	0.1	—	—
E101	3.8	3.9	0.1	3.9	0.0
E122	3.9	3.8	−0.1	3.9	0.0
E129	3.8	3.9	0.1	3.8	0.0
E135	3.8	3.8	0.1	3.9	0.1
E142	4.5	4.5	0.0	4.6	0.1

[a] Castañeda *et al.*[40]
[b] Uncertainty in pK_a value is ±0.1.
[c] Uncertainty in ΔpK_a value is ±0.1.
[d] Acid baseline fixed to Δ+PHS value.

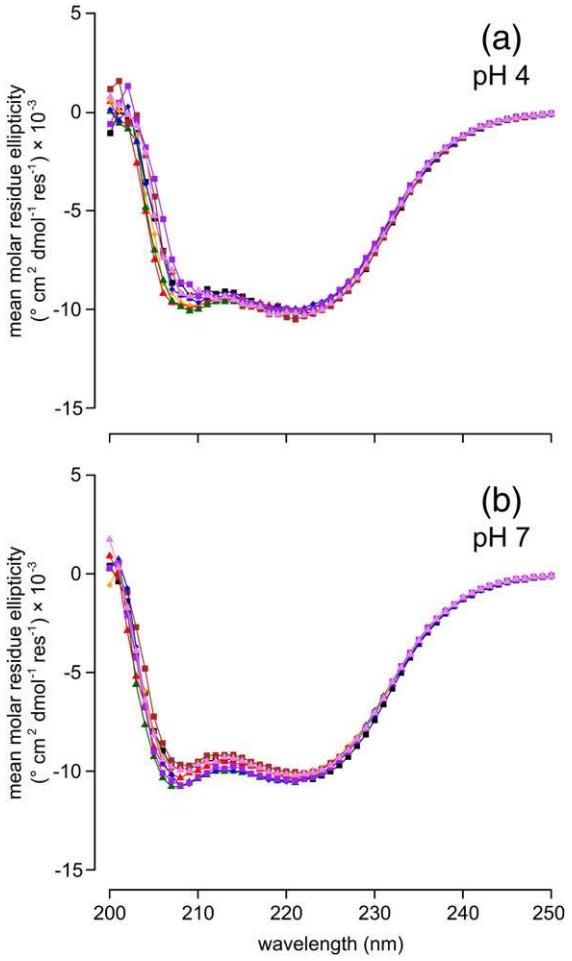

Fig. 4. Far-UV CD spectra of at pH 4 (a) and pH 7 (b). Series are Δ+PHS (▼), Δ+PHS/L38E (●), Δ+PHS/L38E/D77N (●), Δ+PHS/L38E/E122Q (●), Δ+PHS/L38E/E122D (●), Δ+PHS/L38E/R126Q (●), Δ+PHS/L38D (■), and Δ+PHS/L38D/E122Q (■).

suggesting that none of the substitutions altered the secondary structure of the protein significantly, even when the internal Lys, Asp, or Glu groups were charged. Similarly, 70–90% of the 131 peaks in the ^{15}N–^{1}H heteronuclear single quantum coherence (HSQC) spectrum of Δ+PHS nuclease at pH 4.5 were identifiable by visual inspection on the spectrum of each variant (Fig. 5).[40] Overall, the spectroscopic probes suggest that the substitutions did not affect the structure of the protein over a wide range of pH.

Although the global structure of the proteins remained intact, the previous investigation of the Δ+PHS/L38K variant suggested that flexibility of the loop containing residues 113–119 allowed water to penetrate the protein to solvate the charged moiety of the side chain of Lys38. To probe the conformation of this loop in these variants, we used an HNN experiment to assign all backbone ^{15}N–^{1}H peaks in the HSQC spectra of the Δ+PHS/L38E and Δ+PHS/L38K variants at pH 4.6.[48] These spectra were compared to the spectra of Δ+PHS collected previously,[40] at pH values between 2.8 and 9.0 in steps of ~0.4 pH units.

At pH 4.5, 137 peaks were evident for Δ+PHS nuclease, 138 for the Δ+PHS/L38E variant, and only 125 for the Δ+PHS/L38K variant. In the spectrum of Δ+PHS nuclease, only three peaks in the region of interest entered intermediate exchange (i.e., millisecond timescale) with increasing pH: Tyr113 disappeared above pH 5.7; Lys116 and Gly117 disappeared above pH 7.2. Larger changes were observed in the spectrum of the Δ+PHS/L38E variant. The peaks for seven residues (78, 80, 114, and 117–120) all entered exchange above pH 6.3, concomitant with ionization of Glu38. Without further information, it cannot be determined if this is due to structural relaxation or due to the change in the electrostatic environment of the groups owing to the ionization of Glu38. Changes in proton chemical shift as large as 0.4 ppm were also observed in helix 3 (residues 123–130), around Tyr91 (residues 88–92), and in the residues adjacent in sequence to position 38 (residues 34–39).

Unlike Glu38 and Asp38, Lys38 was ionized over the entire pH range under investigation. Twelve peaks are missing in this spectrum at all pH values studied. Of these, nine peaks correspond to a contiguous stretch from Tyr113 to Glu122 (Fig. 4). The remaining three missing peaks are Lys38, Lys78, and Gln80. Changes in ^{15}N–^{1}H chemical shifts should not be overinterpreted;[49] however, the absence of these peaks is consistent with increased exchange of the amide protons with solvent. This interpretation is also consistent with the previous investigation of the L38K variant and with the measured pK_a value of His121 (see next section).[38] Overall, the changes in chemical shift are smaller in the Lys38 variant than in the Glu38 variant, having a maximum shift of 0.2 ppm. The largest changes are limited to the N-terminal end of helix 3.

Structural reorganization probed with His121

The properties of His residues of SNase have been characterized extensively.[29,30] Changes in the microenvironments of His residues can be probed by measuring their pK_a values by 1D ^{1}H NMR, a method that has high accuracy and precision >0.1 pH units. The Δ+PHS variant of nuclease only contains two of the four His residues normally present in wild-type SNase: His8 and His121. In Δ+PHS nuclease, His8 and His121 titrate with pK_a values of 6.6 and 5.4, respectively. The pK_a of His8 was entirely insensitive to the presence and ionization of Asp, Glu, or Lys at position 38 (Table 2). This was expected because His8 is 17 Å from position 38.

It has been shown previously that the pK_a of His121 is depressed owing primarily to dehydration in a partially buried configuration.[30] His121 is 6 Å from the $O^{\varepsilon 1}$ of Glu38 and 9 Å from the N^{ζ} atom of Lys38 (Fig. 1b). In both the L38D and L38E variants, the pK_a value of His121 was elevated from 5.4 to 5.7 (Table 2). This cannot be due to a Coulomb interaction because His121 and the internal carboxylic groups do not ionize in the same range of pH. It has been observed previously that perturbations to the

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.