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

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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 pKₐ values of internal ionizable groups are usually different from the normal pKₐ values in water¹²⁻¹⁹ and are often tuned for specific biological purposes.⁴ Understanding the determinants of these pKₐ values is important for quantitative description of the structural basis of function in a large variety of biological processes.

The shift in the pKₐ of an internal group relative to the normal pKₐ in water is governed by differences in the polarity and polarizability experienced by the charge in the two environments (ΔGself) 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 pKₐ. One of the goals of this study was to examine the relative magnitude of these three determinants of the pKₐ values of internal groups.

The polarity and polarizability in the protein interior are usually lower than those in bulk water;
therefore, \( \Delta G_{\text{self}} \) 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).

Surprisingly, the apparent polarity and polarizability in the protein interior reported by internal ionizable groups are not as low as previously thought. 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fully for the loss of hydration experienced by a charged atom inside a protein (M.J.H., J.L.S., G.R.S., and B.G.-M.E, unpublished results).

Coulomb interactions between surface charges are usually weak because charges are screened effectively by water. In contrast, the Coulomb interaction of ion pairs sequestered from bulk solvent at protein–protein interfaces can be quite strong (3–5 kcal/mol). 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. Even if the effects are small, the sum of many small interactions could lead potentially to a large effect. 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.

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. 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. 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. 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 \( \Delta^+ \)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. Refinement statistics are shown in Supplementary Table 1. PHS nuclease was used for crystallographic studies instead of the \( \Delta^+ \)PHS form of nuclease that was used for equilibrium thermodynamic and NMR spectroscopy experiments because PHS/L38E crystallized and \( \Delta^+ \)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 \( \Delta^+ \)PHS nuclease. The \( pK_a \) values of these internal ionizable groups are superimposable.

The overall structure of the PHS/L38E variant is comparable to the structures of PHS nuclease (\( C^\alpha \) RMSD = 0.7 Å) and of the PHS/L38K variant (\( C^\alpha \) 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 \( C^\delta \) of Glu122 is shifted by 1.6 Å to establish a Lys38/Glu122 ion pair in the structure of PHS/L38K.

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 \( C^\alpha \) atoms of Asp and Glu residues are shown as red spheres. (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 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.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.

**pKₐ values of Glu38 and Asp38**

The pKₐ 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 (ΔG° unfolding) of a protein as a function of pH reports on the pKₐ values of all ionizable residues in the protein. The pKₐ value of a single group introduced by mutagenesis can be measured by subtracting ΔG° unfolding of the background protein (i.e., Δ+PHS/L38E) from ΔG° unfolding of the variant protein (i.e., Δ+PHS/L38E). Shifts in the pKₐ are reflected in the characteristic shape of the pH dependence of ΔG° unfolding.15,23,25 It was shown previously that the pKₐ of Lys38 was ≥10.4, comparable to the normal pKₐ of a Lys in water.35 In contrast, the pKₐ values of Glu38 and Asp38 were 7.0±0.3 and 6.8±0.3 pH units, respectively (Fig. 2). Relative to the normal pKₐ values of 4.4 and 4.0 for Glu and Asp in water, respectively, this corresponds to shifts in pKₐ of 2.6 and 2.8 pH units.

The measurement of pKₐ values by analysis of the pH dependence of stability is too imprecise for detailed investigation of the contribution of Coulomb interactions to the observed pKₐ value. An attempt was made to measure the pKₐ of Glu38 with NMR spectroscopy using the pH dependence of the Glu38 Cα resonance.40 Although the Cα 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 Cγ/Cδ 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 pKₐ of 7.0 for Glu38.

The pKₐ of Glu38 was also obtained by performing a global fit to the pH titrations of multiple resonances.45,46 Specifically, the pKₐ was obtained by analysis of the pH dependence of the 1H 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 pKₐ values are both 6.5 in Δ+PHS nuclease.40 In contrast, the pH dependence of the 1H 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 pKₐ value of 7.0±0.1, in excellent agreement with the pKₐ 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 pKₐ 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 pKₐ values extracted by global fit of NMR spectroscopy data and by linkage analysis are summarized in Table 1.

The agreement between the pKₐ 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
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).

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

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<tr>
<th>Variant</th>
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<th>His121</th>
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* Uncertainty in $pK_a$ value is ±0.1.

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

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<tr>
<th>Position</th>
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<th>Δ+PHS/L38E/E122Q</th>
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</table>

* Castaño et al. 40
b $pK_a$ value is ±0.1.
c Uncertainty in $pK_a$ value is ±0.1.
d Acid baseline fixed to Δ+PHS value.
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). 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. These spectra were compared to the spectra of Δ+PHS collected previously, 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; 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 pKₐ value of His121 (see next section). Overall, the changes in chemical shift are smaller in the L38K 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. Changes in the micro-environments of His residues can be probed by measuring their pKₐ 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 pKₐ values of 6.6 and 5.4, respectively. The pKₐ 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 pKₐ of His121 is depressed owing primarily to dehydration in a partially buried configuration. Histidine 121 is 6 Å from the O² of Glu38 and 9 Å from the N³ atom of Lys38 (Fig. 1b). In both the L38D and L38E variants, the pKₐ 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
hydrogen bonding network centered on His121 almost always cause the pKₐ value of His121 to increase relative to its pKₐ in Δ+PHS. Thus, it is likely that Glu38 and Asp38 perturb the hydrogen-bond network, possibly allowing more water to reach His121 than in Δ+PHS nuclease.

Lys38 is fully charged in the range of pH where His121 titrates; therefore, a Coulomb interaction between these residues is possible. An unfavorable Coulomb interaction would further depress the pKₐ of His121. This was not observed. Instead, as in the L38D and L38E variants, the pKₐ of His121 was elevated to 5.6 in the presence of Lys38. This small change is consistent with slight structural relaxation induced by the substitution of Leu38 with Lys or by the ionization of Lys38. This is fully consistent with the conclusion of our previous study of Lys38 and with the evidence from NMR spectroscopy of slight structural reorganization in the L38K variant.38

Coulomb interactions between surface and internal ionizable groups

Δ+PHS nuclease has 20 Asp and Glu residues. The pKₐ values of these groups are known.40 With the exception of Asp21, which has an elevated pKₐ, all carboxylic groups titrate with depressed or normal pKₐ values. The pKₐ values of surface Asp and Glu residues in the Δ+PHS/L38E and Δ+PHS/L38K variants were measured with NMR spectroscopy (Table 3).

Substitution of Leu38 with Glu did not alter the pKₐ values of any of the Asp or Glu residues (Table 3). This does not imply that Glu38 does not interact with these groups. Glu38 titrates with a pKₐ of 7.0, while most other acidic groups titrate with pKₐ values near 4.0; therefore, Glu38 is neutral during the titration of the other Asp and Glu residues and cannot affect their pKₐ values by Coulomb interaction. These data also show that the L38E substitution has no detectable impact on the structure of the protein and, thus, on the electrostatic environments of the surface Asp and Glu residues. If Asp21 had a significant Coulomb interaction with Glu38, its pKₐ of 6.5 would be affected. The lack of any detectable shift indicates that any Coulomb interaction between these two carboxylic groups is <0.1 kcal/mol. Overall, the absence of any measurable impact of substitutions of Leu38 on the pKₐ values of surface residues corroborates the results from spectroscopic experiments showing that the structure of

![HSQC spectra of the Δ+PHS (black), Δ+PHS/L38D (red), Δ+PHS/L38E (green), and Δ+PHS/L38K variants (blue) at pH 4.6–4.7. The four spectra are overall quite similar. Arrows highlight 4 of the 12 residues that are seen in every spectrum except that of Δ+PHS/L38K. Boxes identify residues used in global fits to extract pKₐ values of groups at position 38.](image-url)
### Table 4. pKₐ Values of Asp38 and Glu38 measured using global fit to 1H² chemical shift data

<table>
<thead>
<tr>
<th>Variant</th>
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<th>n</th>
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<td>−0.6</td>
</tr>
</tbody>
</table>

* Uncertainty in pKₐ value is ±0.1.

the variants is very similar if not identical with the structures of the background protein.

Five double mutants were made (L38E/D77N, L38E/E122Q, L38E/E122D, L38E/R126Q, and L38D/E122Q) to probe interactions between Asp38 and Glu38 with neighboring ionizable residues directly. The pKₐ value of the internal Glu38 or Asp38 was measured by global fit of the pH dependence of ¹H² chemical shifts measured with NMR spectroscopy. The measured pKₐ values are listed in Table 4. These pKₐ values can be readily converted to apparent ΔG_ij values by multiplying the difference in pKₐ between the background protein and the variants with neutral substitutions by RTln(10). To measure the interaction between Glu38 and Asp122, we measured ΔpKₐ using the L38E/E122Q variant as a background. The observed ΔG_ij values were strongly distance dependent and ranged from 0 to 1.5 kcal/mol (Table 5).

Unlike Asp38 or Glu38, which are neutral at low pH, Lys38 is ionized below pH 10. Any Coulomb interaction with Lys38 should therefore alter the pKₐ values of surface carboxylic groups. In particular, Lys38 is involved in a 2.7-Å ion pair with Glu122 in the crystal structure of the L38K variant. A conservative estimate of the possible pKₐ shift can be made with Coulomb's law using the dielectric constant of pure water. This indicates that an interaction of 1.5 kcal/mol between Lys38 and Glu122 is possible. This would shift the pKₐ of Glu122 by 1.1 units. The measured pKₐ values of the carboxylic groups in the Δ+PHS/L38K variant are shown in Table 3. Surprisingly, no shifts in pKₐ value were observed. This experiment demonstrates conclusively that the ion pair between Lys38 and Glu122 observed in the crystal structure is not present in solution and that Lys38 does not interact with any carboxylic acid residues in the protein. This behavior is fully consistent with a structural rearrangement leading to hydration of the charged side chain of Lys38.

### Structure-based pKₐ calculations

Reproducing the shifts in pKₐ values in multiple sites in a protein is still a difficult challenge for structure-based electrostatics calculations. To test the ability of computational methods to calculate Coulomb interactions accurately, we used five computational methods to calculate the pKₐ of Glu38 in the L38E, L38E/D77N, L38E/E122Q, L38E/E122D, and L38E/R126Q variants. The pKₐ values of Asp38 (in the L38D variant) and His8 and His121 were also calculated. Overall, the calculations attempted to reproduce 12 unique pKₐ values: Glu38 in the five variants listed above (Table 4), Asp38 in the L38D variant (Table 4), and His8 and His121 in the L38E, L38E/E122Q, and L38D variants (Table 2).

A variety of different computational methods were tested: PROPKA, the single site (S/FDPB) and full-site PARSE (F/FDPB) implementation of finite difference Poisson–Boltzmann electrostatics, the pH-adapted conformer FDPB method (PAC), and multi-conformer continuum electrostatics (MCCE). PROPKA uses a set of empirical rules to estimate pKₐ values from structure. The pKₐ of Glu38 calculated with PROPKA was 5.3. The group was classified as buried and experienced an unfavorable dehybridization energy (2.1 kcal/mol) offset by favorable polar interactions with the Tyr91 side chain and His121/Glu122 backbone (−1.3 kcal/mol). No Coulomb interactions were calculated because PROPKA only treats Coulomb interactions between two residues if they are both classified as buried and within 7 Å of one another. Only His121 meets both criteria, but no interaction energy was calculated because the calculated pKₐ of His121 (5.1) is below that of Glu38; the PROPKA algorithm excludes possible interaction because the groups are never charged simultaneously. The lack of explicit Coulomb interactions causes PROPKA to be entirely insensitive to substitution of residues at position 77, 122, or 126 with uncharged analogs. The overall RMS error for the calculated versus experimental pKₐ values was 1.0 (Table 6).

### Table 5. Apparent Coulomb interactions between Glu38 and other ionizable residues

<table>
<thead>
<tr>
<th>Interaction</th>
<th>ΔG_a (kcal/mol)</th>
<th>r_g (Å)</th>
<th>Interaction</th>
<th>ΔG_a (kcal/mol)</th>
<th>r_g (Å)</th>
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* Uncertainty is 0.1 kcal/mol.
* ΔpKₐ estimated using L38E/E122Q variant.
* Distance estimated using structural model.
The overall RMS for all 13 measured pK\textsubscript{a} values of unfavorable Coulomb interactions (2.5 kcal/mol).

<table>
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<th>Residue</th>
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<th>PROPKA</th>
<th>FDPB</th>
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<th>F</th>
<th>PAC</th>
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<td>2.1</td>
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</table>

The results of FDPB calculations were highly dependent on the choice of protein dielectric constant. Using S/FDPB, the calculated pK\textsubscript{a} of Glu38 ranged from 19.2 when \( \varepsilon = 4 \) to 5.4 when \( \varepsilon = 20 \). Likewise, the pK\textsubscript{a} of Glu38 calculated using F/FDPB ranges from 19.2 when \( \varepsilon = 4 \) to 5.2 when \( \varepsilon = 20 \). The pK\textsubscript{a} of 7.0 of Glu38 was reproduced by dielectric constants of 12 and 11 with the S/FDPB and F/FDPB methods, respectively. The dielectric constant that reproduced the experimental pK\textsubscript{a} of Glu38 was 4.9 and the overall RMS error of 1.2 and 2.1 for S/FDPB and F/FDPB, respectively (Table 6). The relatively large RMS value is due to a large error in the calculated pK\textsubscript{a} of His121 (8.0 and 10.2). If this is excluded from the RMS calculation, both methods have RMS errors of 0.4. S/FDPB and F/FDPB overestimate the apparent Coulomb interactions by 75% and 25%, respectively.

The PAC method generates ensembles of possible side-chain positions at pH extremes, calculates electrostatic potentials of each configuration using FDPB, and Boltzmann weights these ensembles as a function of pH. To test the best-case scenario for PAC calculations, we used a variety of parameter combinations to maximize agreement with experiment. For PAC, it was found that minimizing side-chain positions was the most important user-adjustable parameter, lowering the RMS error by 20%. Even so, the calculations performed poorly. The calculated pK\textsubscript{a} of Glu38 was 4.9 and the overall RMS error of the pK\textsubscript{a} values was 3.4 pH units (Table 6). On average, the apparent Coulomb interactions calculated using this method were 3.7 times higher than those of the experiment.

MCCE couples conventional FDPB calculations to side-chain rotamer sampling with a Monte Carlo method. MCCE does not allow for arbitrary adjustment of the protein dielectric constant without extensive reparameterization; therefore, these calculations used the default \( \varepsilon = 4 \) values of 4 or 8. The calculations with \( \varepsilon = 4 \) failed to converge, giving pK\textsubscript{a} values for Glu38 that were >14. Only calculations using \( \varepsilon = 8 \) are reported here. The calculated pK\textsubscript{a} value of Glu38 was 8.5, and the overall RMS error was 1.4 pH units (Table 6). The apparent Coulomb interactions reported by MCCE were, on average, twice as strong as observed experimentally. A correlation plot of the experimental and calculated apparent Coulomb interactions for each calculation type is shown in Supplementary Fig. 1.

**Discussion**

**Difference in ionization properties of Lys, Glu, and Asp at position 38**

Previous experimental and computational studies with the L38K variant suggested that the pK\textsubscript{a} of Lys38 was governed by structural reorganization and water penetration, with little or no contribution from the buried ion pair between Lys38 and Glu122 observed in the crystal structure.\textsuperscript{38} We have now confirmed this with NMR spectroscopy. The HSQC spectrum of this variant shows no peaks for residues 113–123 at any pH value studied. Although chemical shifts are sensitive to factors other than structural relaxation, the data are consistent with reorganization of this segment of the protein and exchange with solvent. The shift in the pK\textsubscript{a} of His121 from a depressed value towards a more normal value is opposite from the shift expected from Coulomb interactions between His121 and Lys38. However, it is fully consistent with enhanced hydration of His121 owing to structural relaxation in this region of the protein. Finally, Lys38 does not shift the pK\textsubscript{a} value of any acidic residue in the protein. This is particularly striking for Glu122, which is part of a 2.7-Å ion pair in the crystal structure of the L38K variant but apparently insensitive to the charge in Lys38. This can only be explained if Lys38 is fully hydrated when charged and if the structure of the L38K variant in solution is different from the crystal structure.

In contrast to the behavior of Lys38, the pK\textsubscript{a} values of Glu38 and Asp38 are higher than normal pK\textsubscript{a} values by 2.6 and 3.2 pH units, respectively. These two carboxylic groups experience substantial Coulomb interactions with multiple neighboring groups. There are several possible explanations for this difference in behavior between Lys38 and the carboxylic groups at this same position. The first is proximity to solvent. Although Lys38 and Glu38 are internal in the crystal structures of the PHS/L38K and PHS/L38E variants, the side chains point towards the protein–water interface and they are much closer to bulk water than the internal ionizable groups of acidic and basic residues at internal positions 66 and 92 studied previously.\textsuperscript{15–17,21–23,58} The shorter side chain of Glu38 is further from bulk water than the longer Lys38 side chain and, therefore, will experience the reaction field of bulk water more weakly.
This would contribute to the shift in its $pK_a$. Consistent with expectation, the shifts in $pK_a$ correlate inversely with side-chain length. We also suspect that the short length of the side chain of Asp38 is responsible for its not being fully dehydrated. A model of the L38D variant made from the crystal structure of the LPH/L38E variant (see Materials and Methods) showed that Asp38 forms a single hydrogen bond with its own backbone but is too short to reach any other hydrogen-bonding partners without substantial structural rearrangement. Water molecules have been found in association with the carboxylic oxygen atoms of internal Asp and Glu in all previous structures of SNase variants with internal Glu or Asp.$^{16,41}$ To examine the possibility that internal water molecules can satisfy the hydrogen-bonding potential of the side chain of Asp38, the DOWSER algorithm,$^{59}$ which places internal water molecules in polar cavities in crystal structures, was applied to the model of the L38D variant. DOWSER identified two potential water molecules in the region between Asp38 and the 113–119 loop, which make hydrogen bonds with Asp38, thereby linking this residue to the backbones of Asn118 and Glu122.

The difference in the $pK_a$ of Lys38 versus Glu38 or Asp38 is also consistent with the ability of carboxylic residues to form better hydrogen bonds than internal lys residues. In the crystal structure, the amino group of Lys38 makes an ion pair with the carboxylic side chain of Glu122 and one bond to the backbone of Ala112. In contrast, Glu38 forms hydrogen bonds to the side chain of Tyr91 and to the backbone amides of Thr120 and His121. If the predictions with the DOWSER method are correct, Asp38 forms a hydrogen bond to its own backbone and two hydrogen bonds to internal water molecules. The polar network that Asp38 and Glu38 establish might rigidify this part of the protein and thereby prevent these groups from being solvated through water penetration. This would contribute to the shift in $pK_a$ values in the direction that was observed.

The differences in the properties of Lys38 and Glu38 or Asp38 probably also reflect differences in the $pH$ dependence of stability of the different variant proteins. The stability of the L38K variant near pH 10, where Lys normally titrates, is lower than the stability of the L38D and L38E variants near pH 4, where Asp and Glu normally titrate. At pH 10, the local stability of the loop composed of residues 113–119 does not appear to be sufficiently high to maintain Lys38 buried in the neutral state. Instead, the loop appears to adopt alternative, more open conformations that allow Lys38 to be charged, hydrated, and probably only partially buried. We showed previously that a subtle structural reorganization is sufficient to promote water penetration and solvation of the charged side chain of Lys38.$^{38}$ Near pH 4, the local stability of the 113–119 loop is sufficiently high to maintain Glu38 and Asp38 buried in the neutral state; therefore, the $pK_a$ values of these internal groups are shifted upwards. Because the stability of the L38E and L38D variants decrease with increasing $pH$, this loop might shift towards a more open, alternative conformation when Asp38 and Glu38 titrate near pH 7. This is analogous to what was observed in the L38K variant. Consistent with this hypothesis, several resonances in the HSQC spectra of the L38E variant corresponding to this loop disappear when Glu38 ionizes. The disappearance of these peaks is somewhat difficult to interpret; it could reflect structural reorganization but it could also be due to the electrostatic effects of the charge of Glu38 on the amides.$^{39}$

**Coulomb interactions between internal and surface ionizable residues**

How do the observed Coulomb interaction energies between internal and surface groups compare to previously measured interactions between surface ionizable moieties? The distance dependence of $\Delta G_{ij}$ between internal and surface charges is plotted in Fig. 6 and compared to previously measured long-range interactions between surface charges.$^{29,30}$ A single $\Delta G_{ij}$ versus $r_{ij}$ plot is sufficient to describe all data. This indicates that the interactions between internal and surface groups are as weak as those between surface groups, at least for these internal groups, which are close to the protein–water interface. The effective dielectric constant obtained by fitting Coulomb’s law with a Debye–Hückel parameter to the $\Delta G_{ij}$ obtained between Glu38 and nearby surface residues is $35 \pm 3$, which is as high as the value of $46 \pm 8$ obtained by including $\Delta G_{ij}$ from interactions between surface groups, measured previously.$^{29,30}$ The high effective dielectric constant is fully consistent with the high local polarity in the microenvironment of Glu38 and subtle structural reorganization coupled to its ionization.$^{60}$
Another interesting feature of the measured Coulomb interactions is the asymmetry of the Glu38/Asp122 and Asp38/Glu122 interactions. The apparent Coulomb interaction of Glu38/Asp122 is 1.5 kcal/mol, whereas the apparent Coulomb interaction of Asp38/Glu122 is 0.8 kcal/mol. This is surprising: how could simply reversing the placement of the charges significantly alter the observed energy of interaction? Simple structural models of the L38D and L38E/E122D variants suggest an explanation. In these models, the nearest side-chain oxygen atoms of the Glu38/Asp122 pair were 5.3 Å apart and the Asp38/Glu122 atoms were 3.6 Å apart. Due to their burial, neither Asp38 nor Glu38 can change position without a steric clash. Asp122 and Glu122, however, are on the surface and therefore not tethered in a fixed position. Glu, with its extra methylene carbon, is simply longer and better able to respond to the repulsive interaction with Asp38.

Implications for structure-based pKₐ calculations

The pKₐ values of internal ionizable residues are especially useful to test models for structure-based calculation of electrostatic energies and pKₐ values. In general, it is not sufficient to test the validity of these methods simply by reproducing pKₐ values, especially when the methods include ad hoc modifications to improve their performance. In these cases, the experimental pKₐ values can be reproduced accurately but for entirely incorrect physical reasons. The utility of the data in this study is that it contributes additional constraints such as the magnitude of pairwise Coulomb interactions that must be satisfied to ensure that pKₐ values are reproduced for the right physical reasons. To reproduce these data, a computational method must correctly capture both the self-energy and Coulomb interaction energies experienced by ionizable residues at a single position, and it must also reproduce the differences in the ionization properties of Lys38 and Asp38 or Glu38.

Our experimental data expose several weaknesses in current computational methods. For example, the attempts to reproduce the experimental pKₐ values with PROPKA revealed the importance of medium-range electrostatics interactions, which can be an important determinant of pKₐ values. These interactions are neglected in PROPKA; thus, it fails to reproduce the experimental pKₐ values (Table 6). Inclusion of this term would likely bring the calculated pKₐ from 5.3 closer to the experimentally measured value. On the other hand, all of the continuum calculations overestimated the strength of Coulomb interactions, especially the PAC and MCCE methods. This reflects the fact that because the PAC and MCCE methods attempt to model side-chain relaxation explicitly, lower protein dielectric constants are used. The failure of these methods demonstrates that these models neglect important energetic terms in their Boltzmann-weighted ensembles or that relaxation involving the protein backbone occurs on the timescale of the experiments, which is not treated by PAC or MCCE.

Conventional FDPB calculations did better than the PAC and MCCE methods; however, it is important to emphasize that in these calculations, the protein dielectric constant was used as a tunable variable to maximize agreement between calculated and measured pKₐ values. FDPB calculations with a static structure cannot predict pKₐ values of internal ionizable groups. Both the S/FDPB and F/FDPB calculations slightly overestimated the strength of unfavorable Coulomb interactions. As the calculation reproduced the pKₐ of Glu38, the strength of the favorable polar interactions is overestimated or the energetic penalty of dehydration is underestimated, or both. If slight relaxation of the structure occurs in solution, the strength of Coulomb interactions, polar interactions, and the energetic penalty of dehydration would all decrease. Such a process would explain the discrepancy between the experimental and computational results.

Our study demonstrates that very different factors can govern the pKₐ values of different types of ionizable residues buried at the same location in a protein. Water penetration and structural reorganization are the primary determinants of the pKₐ of Lys38. In contrast, favorable polar interactions and unfavorable dehydration and Coulomb interactions determine the pKₐ values of Asp38 and Glu38. Some amount of structural reorganization also influences the pKₐ values of Asp38 and Glu38, as demonstrated by the fact that relatively high protein dielectric constants were needed to reproduce their pKₐ values in structure-based calculations with static structures.

If structure-based electrostatics calculations are going to be useful predictors of structure/function relationships, they must be able to reproduce the pKₐ values of internal ionizable groups. To this end, they must be able to identify a priori the cases in which the ionization of an internal group triggers structural reorganization. They must also be able to distinguish these cases from cases in which the ionization of an internal group does not promote structural reorganization. Because the probability that a protein undergoes local structural reorganization coupled to the ionization of an internal group is governed by the free energies of the folded protein and of the low-lying excited states, improved methods for structure-based pKₐ calculations will require methodology for improved conformational sampling and improved ability to estimate the stability of proteins.

Materials and Methods

Staphylococcal nuclease

Two hyperstable variants of SNase, known as PHS and Δ+PHS, were used for crystallography and equilibrium thermodynamic experiments, respectively. Both variants contain three substitutions: P117G, H124L, and S128A. Δ+PHS nuclease has two additional mutations (G50F and
were added in later rounds of refinement. The side-chain aliphatic carbon (C\text{\textalpha}, C\text{\textbeta}, and C\text{\textgamma}) assignments of Glu38 were collected using the C–C total correlated spectroscopy (CCO/NH) experiment. Through modification of the C\text{\textdelta}-detect HBBHGCBCGCO pulse sequence to include a total correlated spectroscopy element, the Glu38 C\text{\textdelta} resonance was determined by correlation with its side-chain aliphatic carbon assignments (C. Castañeda and A. Majumdar, unpublished results).

The pK\textalpha values of Asp and Glu residues were determined by monitoring the pH dependence of their C\text{\textdelta} resonance. For indirect pK\textalpha determination of carboxylic residues using 1H chemical shifts, a series of 15N–1H HSQC spectra were collected in a similar manner. These experiments were performed and processed as previously described. For pK\textalpha determination of histidine residues in the C–P38 selection, the titration (Figs. 2 and 4); (3) the residues were within close spatial proximity of position 38. The fit minimized the root mean square deviation of the C\text{\textdelta} resonance assigned in all variants and did not exhibit exchange over the course of the titration (Figs. 2 and 4); (3) the residues were within close spatial proximity of position 38. The fit minimized the root mean square deviation of the C\text{\textdelta} resonance assigned in all variants and did not exhibit exchange over the course of the titration (Figs. 2 and 4). The RMSD between the calculated and measured chemical shifts:

\[
\text{RMS} = \sum_{i=1}^{N} \sqrt{\frac{1}{pH} \sum_{pH} (\delta_{i,\text{calc}}(pH) - \delta_{i,\text{meas}}(pH))^2}
\]
where \( N \) is the number of resonances included in the fit and \( \delta_{i,\text{meas}} \) is the experimentally measured chemical shift. \( \delta_{i,\text{calc}} \) is described by the modified Hill equation:

\[
\delta_{i,\text{calc}} = \frac{\delta_{i,\text{acidic}} + \delta_{i,\text{basic}} \cdot 10^{(p(H^-) - p_K)} / (1 + 10^{(p(H^-) - p_K)})}
\]

(2)

where \( \delta_{i,\text{acidic}} \) and \( \delta_{i,\text{basic}} \) are the acidic and basic baselines of the apparent titration. A single \( p_K \) and the Hill coefficient (\( n \)) were used for all resonances included in the fit. Minimization was done using the \( \text{optin} \) function inside the R 2.7.1 statistical environment.\(^{78}\) To ensure that the \( p_K \) and Hill coefficient did not depend on the resonances chosen, we performed bootstrap sampling of resonances included in the fit. The list of resonances was also expanded to include resonances that only met two of the three inclusion criteria. In all cases, the bootstrap error was less than 0.05 pH units for the \( p_K \) value. The errors reported in the paper reflect the maximum possible systematic error in pH measurement rather than the fit error.

**Structural models**

Models of the PHS/L38D, PHS/L38E/D77N, PHS/L38E/E122Q, PHS/L38E/E122D, and PHS/L38E/R126Q variants were made using the structure of the PHS/L38E variant as a template. The positions of the atoms of the side chain that was mutated in silico were minimized using the CHARMM22 force field, as described previously.\(^{23}\) All other atoms were held fixed.

**Structure-based electrostatics calculations**

PROPKA calculations were performed using PDB2PQR 1.1.2.\(^{56,79}\) Conventional FDPB calculations were done using the University of Houston Brownian Dynamics package, version 5.1.\(^{80}\) FDPB calculations were done using two parameter sets: single site (S/FDPB)\(^{51,32}\) and full site (F/FDPB) using the PARSE parameter set.\(^{53}\) All calculations were performed at 100 mM ionic strength as described previously.\(^{33}\) MCCE calculations were done using MCCE 2.2.\(^{56,27}\) The parameters distributed with the program were used for the \( \varepsilon_{\text{p}} = 4 \) and \( \varepsilon_{\cdot} = 8 \) calculations. Calculations were done at 100 mM ionic strength. PAC calculations were done using the Karlsberg+ web interface.\(^{54,35}\) Side-chain positions were minimized and salt bridges were randomized at high and low pH. Results were found to be insensitive to choice of solvation model. Values reported herein were calculated using the vacuum solvation model.

**Accession code**

Coordinates and structure factors have been deposited in the PDB with accession number 3D6C.

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**Acknowledgements**

We gratefully acknowledge Dr. Ananya Majumdar for assistance with NMR experiments and for

\(^{†}\) Downloaded from http://pdb2pqr.sourceforge.net/
\(^{‡}\) Downloaded from http://www.ncbi.nlm.nih.gov/Structure/nciblast/
§ http://agknapp.chemie.fu-berlin.de/karlsberg/

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**Supplementary Data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.03.039

**References**
