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Direct NMR Observation and pK_a Determination of the Asp^{102} Side Chain in a Serine Protease

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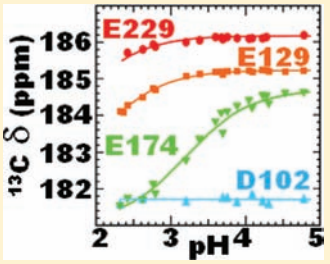
Supporting Information

ABSTRACT: The pK_a value of aspartic acid in the catalytic triad of serine proteases has been a pivotal element in essentially every mechanism proposed for these enzymes over the past 40 years, but has, until now, eluded direct determination. Here, we have used the multinuclear 3D-NMR pulse programs HCACO and HCCH-TOCSY to directly identify and study the side-chain resonances of the aspartate and glutamate residues in uniformly ^{13}C-labeled α-lytic protease. Resonances from four of the six residues were detected and assigned, including that of Asp^{102}, which is notably the weakest of the four. pH titrations have shown all of the carboxylate ^{13}C signals to have unusually low pK_a values: 2.0, 3.2, and 1.7 for Glu^{129}, Glu^{174}, and Glu^{229}, respectively, and an upper limit of 1.5 for Asp^{102}. The multiple H-bonds to Asp^{102}, long known from X-ray crystal studies, probably account for its unusually low pK_a value through preferential stabilization of its anionic form. These H-bonds probably also contribute to the weakness of the NMR resonances of Asp^{102} by restricting its mobility. The Asp^{102} $^{13}C^{\gamma}$ atom responds to the ionization of His^{57} in the resting enzyme and to the inhibitor-derived oxyanion in a chloromethyl ketone complex, observations that strongly support the assignment. The low pK_a value of Asp^{102} would appear to be incompatible with mechanisms involving strong Asp^{102}–His^{57} H-bonds or high pK_a values, but is compatible with mechanisms involving normal Asp^{102}–His^{57} H-bonds and moving His^{57} imidazole rings, such as the reaction-driven ring flip.



INTRODUCTION

The pK_a value of the aspartic acid found in the active site of serine proteases (Asp^{102}) has been pivotal to essentially every attempt to understand the mechanism of these enzymes since Blow first discovered the residue in 1963, a finding that led him to propose the "charge-relay" mechanism.[1] Blow's idea was that the burying of Asp^{102} within the hydrophobic interior of the protein might make it a sufficiently strong base to deprotonate the catalytic serine (Ser^{195}), in the resting enzyme, via a "relay" through the catalytic histidine (His^{57}). The generation of a seryl alkoxide ion in solutions of neutral pH, he argued, could be the key to understanding the catalytic power of these enzymes. This idea did not gain much acceptance as, among other reasons, it was widely interpreted as requiring a pK_a value $\geq$16 for Asp^{102}, a value generally regarded as unreasonably high, even for a "buried" carboxylate.

Hunkapillar et al. subsequently proposed a modification of the "charge-relay" hypothesis in which the idea of full proton transfer to Asp^{102} was retained, but was moved from the ground state to the transition state.[2] This modification eliminated the problematic free Ser^{195} alkoxide ion and reduced the pK_a value requirement of Asp^{102} from ~16 to ~7.0, as now Asp^{102} need only be basic enough to deprotonate His^{57}. Moreover, this time there was experimental support for the idea from an NMR study of α-lytic protease (αLP), whose authors assigned a pK_a value of 6.7 to Asp^{102} and assumed a value of <4.0 for His^{57}. However, subsequent ^{15}N and ^{13}C NMR studies eventually proved that the experimental support for the "modified charge-relay" hypothesis was misinterpreted, that His^{57} titrated normally, with a pK_a value of ~6.7, and that no conclusions could be drawn concerning the pK_a value of Asp^{102}.[3,4]

The next mechanism to capture significant attention proposed that the Asp^{102}–His^{57} H-bond becomes a special type of very short, very strong H-bond, termed a low-barrier hydrogen bond (LBHB) when His^{57} becomes protonated or when the enzyme-catalyzed reaction enters the transition state.[5] This hypothesis requires Asp^{102} and His^{57} to have matching pK_a values, and therefore that Asp^{102} must have a pK_a value of ~6.7.[6]

Our group has proposed a reaction-driven ring flip (RDRF) mechanism, in which the imidazole ring of His^{57} rotates ~180° about its C^{β}–C^{γ} axis between two H-bonded states in response to protonation or deprotonation of the imidazole ring.[7] This permits the enzyme to guide the reaction through multiple transition states, rather than to strongly bind and stabilize only one. Asp^{102} pK_a values $\leq$4.0 would be consistent with this mechanism, as it requires only that Asp^{102} be available as an H-bond acceptor for each of the two imidazole ring orientations, and that the H-bond formed with His^{57} not be so strong as to hinder the imidazole ring flip.

Recently, it has been proposed that the Asp^{102}–His^{57} H-bond is a short, ionic H-bond (SIHB) and that a network of such SIHBs exists along the substrate–enzyme interface, which

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together facilitate catalysis.[8] The SIHB mechanism differs from the RDRF mechanism in that it employs subtler movements of the catalytic residues, but is similar in the demands it makes of the Asp^{102} pK_a value and H-bonding characteristics.

All previous attempts to determine the pK_a value of Asp^{102} have been made by inference and/or indirect measurement. Most have come from examination of secondary inflection points or perturbations on the titration curves of NMR signals from His^{57}, such as its $H^{\varepsilon 2}$ resonance or its low-field (13−18 ppm) $H^{\delta 1}$ resonance.[9−12] Other methods have included deuterium exchange,[13] IR spectroscopy,[14] and pulse radiolysis.[15] These studies have reported values for the pK_a of Asp^{102} ranging from 2.8 to 7.0. A recent, ultrahigh-resolution X-ray crystal structure found evidence of Asp^{102} protonation (in combination with a neutral His^{57}) at pH 4.6 or higher in a complex of bovine trypsin with a semisynthetic, Kunitz-type peptide inhibitor.[16] Nevertheless, despite all of these efforts, the pK_a value of Asp^{102} in a resting or inhibited serine protease has not been convincingly demonstrated.

Developments in multinuclear 3D NMR have made it routine to assign protein backbones, and, with the aid of pulse programs such as HCACO[17] and HCCH-TOCSY,[18] their side chains as well. Here, we have employed an HCACO pulse sequence optimized for detection of aspartyl $^{13}C^{\gamma}$ and glutamyl $^{13}C^{\delta}$ resonances relayed through their vicinal protons.[19] In addition, the HCCH-TOCSY sequence was employed to distinguish aspartate from glutamate resonances, and to enable correlation to previously reported partial ^{13}C and ^{1}H assignments in αLP.[20]

The variation in chemical shift of the Asp^{102} side chain $^{13}C^{\gamma}$ atom is monitored as a function of pH using HCACO in ^{13}C-labeled αLP to determine its pK_a value directly. Repeating the pH titration in the presence of inhibitors phenylmethylsulfonyl fluoride (PMSF) or Ac-AlaAlaProVal-chloromethyl ketone (CMK) provides further support for the Asp^{102} assignment. The results indicate that Asp^{102} is unusually acidic, having a pK_a value less than 1.5. The implications for the mechanism of action of serine proteases are discussed.

EXPERIMENTAL SECTION

Unless otherwise noted, all reagents were purchased from Sigma-Aldrich Inc.

Preparation of α-Lytic Protease. In this Article, we employ exclusively wild-type αLP produced from its natural source, *Lysobacter enzymogenes* (ATCC 29487), as previously described.[21] A complete purification scheme is available in the Supporting Information.

Enzymatic Activity Measurements. Enzyme concentrations were determined from measurement of enzyme activity using Ac-Ala-Pro-Ala-pNA (Bachem) substrate as previously described.[22]

NMR Sample Preparation. For all samples, the concentration of αLP was adjusted to 2−3 mM by diluting with deionized H_2O or concentrating in Amicon Ultra-15 centrifugation cells. D_2O was added to a final concentration of 10% for field/frequency lock. pH of the enzyme solutions was adjusted by addition of small increments of sterile stocks of 0.1−1.0 M HCl or NaOH with constant stirring. CMK inhibited samples of αLP were prepared by titrating αLP with Ac-Ala-Ala-Pro-Val- CMK (synthesized in house[23]) in deionized H_2O at pH 8.75 until the enzyme activity was reduced to <1% of its initial value. Samples of αLP inhibited with PMSF were prepared by titrating with PMSF in dioxane at pH 8.75 until the enzyme activity was reduced to <10% of the starting values.

NMR Experiments. All spectra were run at 25 °C on the Tufts Bruker Avance 600 or Brandeis Bruker Avance 800 NMR spectrometers. Both spectrometers were equipped with $^{1}H/^{13}C/^{15}N$ triple resonance probes, with that of the 800 MHz spectrometer being a cryoprobe. The 1D ^{1}H NMR experiments employed the Bruker pulse sequence *zgcppr* with a spectral width of 14 ppm. ^{1}H and ^{13}C signals referenced to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).

The HCACO spectra were run at 600 MHz using the Bruker pulse sequence *hcacogp3d* optimized for side chain $-CH_2-C(=O)-$ moieties. Spectral offsets and sweep widths in ppm were O1P = 4.703, O2P = 39 (aliphatic carbon), O3P = 176 (carboxyl carbon), SW1 = 14, SW2 = 32, and SW3 = 22. Key delays were as follows: d1 = 1.2 s, d4 = 1.8 ms, and d21 = 1.2 ms. The numbers of data points acquired were TD1 = 2K, TD2 = 60, and TD3 = 122, requiring 3 days for number of scans NS = 16. For pH titrations, a 2D version of the HCACO spectrum was used (setting TD2 = 1) in which the $^{1}H-^{13}C$ carboxyl projections could be acquired in 45 min with NS = 16 (with some weaker spectra being run for 90 min and NS = 32).

The HCCH-TOCSY experiments were run at 800 MHz using the Bruker pulse sequence *hcchdigp3d*. Spectral offsets and sweep widths in ppm were O1P = 4.704, O2P = 39 (aliphatic carbon), O3P = 4.704 (^{1}H indirect), SW1 = 12, SW2 = 75, and SW3 = 12. Key delays were as follows: d1 = 1 s, d31 (mixing time) = 27.2 ms, d4 = 475 μs, d21 = 1.1 min, and d23 = 475 μs. The numbers of data points acquired were TD1 = 2000, TD2 = 64, and TD3 = 128, requiring 2 days for number of scans NS = 16.

The 600 MHz experiments were processed with Bruker's XWIN-NMR software, while the 800 MHz experiments were processed with Topspin 2.1. Additional spectral processing, display, plotting, and analysis were carried out with SPARKY freeware.

RESULTS

Assignment of Acidic Side Chains of αLP. There are six acidic residues in αLP: four glutamates and two aspartates. Glu^{32} and Glu^{129} are situated on the enzyme surface, Glu^{174} is located near His^{57} in the S2 substrate binding pocket, and Glu^{229} lies partly solvent-sequestered not far from Asp^{102}. Both

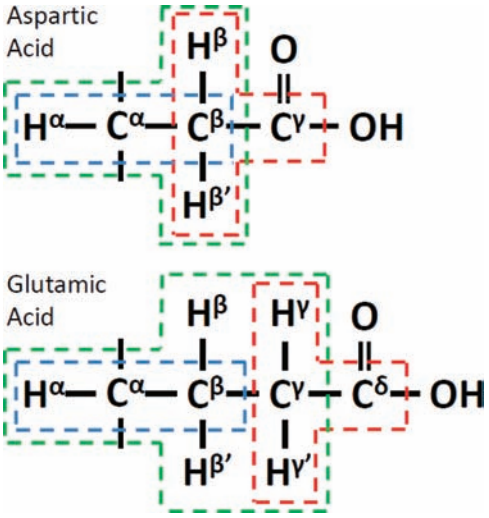

Figure 1. Correlation of acidic side-chain NMR ^{1}H and ^{13}C resonances. HCACO correlations are outlined in red. HCCH-TOCSY correlations are outlined in green. Previous assignments for αLP are outlined in blue.[20] Spatial assignments of H^{β} and $H^{\beta\prime}$ pairs and H^{γ} and $H^{\gamma\prime}$ pairs are unknown. We arbitrarily assign $H^{\beta\prime}$ and $H^{\gamma\prime}$ to the most upfield of the geminal pairs.

aspartate residues, Asp^{102} in the active site and Asp^{194} near the oxyanion hole, appear to be completely solvent shielded. The HCACO pulse sequence (outlined in red, Figure 1) transfers magnetization from aliphatic ^{1}H atoms (labeled H and H′) first to their directly bonded ^{13}C atoms, then to directly bonded ^{13}C

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carboxyl atoms, and then both steps in reverse for proton detection.

The ^{13}C signals from carboxylate carbons of glutamate and aspartate residues typically move ~4 ppm upfield as the group undergoes protonation, with the $^{13}C^{\delta}$ signal of glutamate moving from ~185 to ~181 ppm, and the $^{13}C^{\gamma}$ signal of aspartate moving from ~182 to ~178 ppm.[24,25] The aliphatic carbon atoms bound to the carboxylate groups of both glutamate and aspartate usually resonate at ~34−39 ppm,[26] while the protons on this carbon resonate at ~2.2−3.2 ppm.[27] Thus, the glutamate and aspartate resonances are found in a relatively small and uncrowded spectral region, with possible overlap only from the pH-independent glutamine side chains.

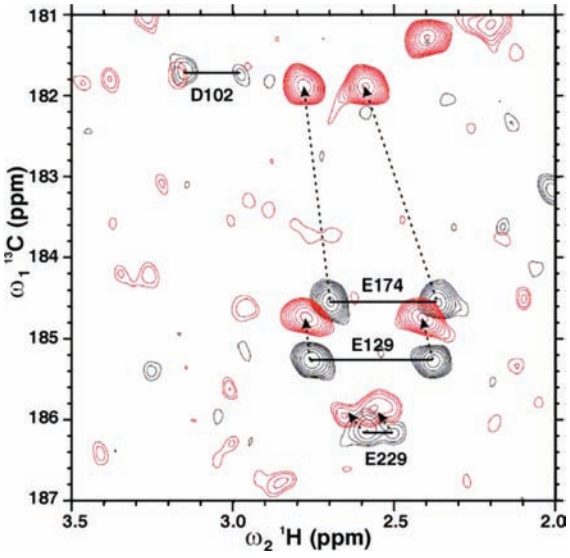

Figure 2. Superimposed 2D projections (^{1}H and carboxyl ^{13}C dimensions) of 600 MHz HCACO of αLP at 25 °C and two different pH values: pH 4.20 in black, pH 2.79 in red. The assignments, labeled here in single-letter code, result from HCCH-TOCSY experiments and further pH variations, including some on various inhibited forms of αLP. Asp[194] was not located in this or previous studies.[20] Glu[32] was also impossible to define due to strong overlap with other peaks.

Figure 2 shows typical 2D projections of HCACO spectra of αLP and illustrates the linear migration of the various contours as pH is varied from 4.20 (black) to 2.79 (red). Such 2D projections were typically recorded within 1−2 h. The resonances assigned to Asp[102] were much weaker than those of freely rotating surface glutamates Glu[129] and Glu[174], which we attribute to hindered mobility of Asp[102] side chains increasing linewidths and decreasing signal-to-noise, as will be discussed below. Glu[229] is also weaker, probably due to hindered rotation in the salt bridge in which it participates.

αLP samples are at their most stable at ~pH 4, where autoproteolysis is minimal. In titrations such as those shown here, we pushed the pH in small increments from 4.8 to 2.3, where acid denaturation decreases the sample lifetime. In other 2D HCACO titrations, the pH was raised incrementally from 4 toward 9, where autoproteolysis degrades the sample with increasing rapidity. The extent of sample denaturation was monitored using 1D ^{1}H NMR, with special attention paid to the high field signals at −0.76 and −0.95 ppm, indicative of deeply buried methyl groups (Figure S1, Supporting Information). These signals first widen slightly as pH is lowered, then broaden and disappear as acid denaturation takes hold, whereupon the titration and NMR measurements were stopped. αLP is far more stable in complex with Ac-AlaAlaProVal-CMK, a potent, irreversible inhibitor, which forms a covalent diadduct between the O^{γ} atom of Ser[195] and the $N^{\varepsilon 2}$ atom of His[57].[23] Complexation with the CMK caused little perturbation in ^{1}H and ^{13}C chemical shifts, the largest change being an increase of ~0.2 ppm for the $^{13}C^{\gamma}$ of Asp[102].

With the aid of 3D HCCH-TOCSY of CMK-inhibited αLP (Figure S2, Supporting Information) and its correlation to previous $^{1}H^{\alpha}$, $^{13}C^{\alpha}$, and $^{13}C^{\beta}$ assignments,[20] we were able to assign four of the six acidic residues in αLP, as shown in Table 1.

Titration of αLP Acidic Residues. Titrations of several preparations of αLP were conducted employing 2D HCACO projections at many pH values in the range 2.32−8.27. As shown in Figure 2, the $^{13}C^{\gamma}/^{13}C^{\delta}$ resonances are far more sensitive to pH variation than are their vicinal ^{1}H resonances. Although denaturation becomes more rapid as pH is lowered to 2.3, the absence of any discontinuity in the titration curves combined with the fact that at least 70% of the active enzyme could always be recovered argues for the validity of the low pH points in Figure 3.

At pH values above 5, $^{13}C^{\gamma}/^{13}C^{\delta}$ chemical shift changes are small, so Figure 3 displays only the pH region from 2 to 5. Standard errors for readability of the well-contoured Glu[129] and

Table 1. Chemical Shift Assignments (ppm) for Acidic Residues of αLP and Inhibited Complexes at pH 4.0

	C^{α}	H^{α}	C^{β}	H^{β}	$H^{\beta'}$	C^{γ}	H^{γ}	$H^{\gamma'}$	C^{δ}
Asp[102]	55.1[a]	5.68[a]	40.2[a]	3.18[a]	2.87[a]		N/A	N/A	N/A
			40.1[b]	3.19[b]	2.89[b]	181.9[b]			
	54.8[c]	5.74[c]	40.1[c]						
Glu[129]	58.2[a]	4.28[a]	30.6[a]	2.06[a]	1.85[a]	37.6[a]	2.65[a]	2.30[a]	
						37.4[b]	2.65[b]	2.29[b]	185.3[b]
	58.2[c]	4.41[c]	30.6[c]						
Glu[174]	59.4[a]	3.94[a]	30.3[a]	1.98[a]	1.82[a]	37.6[a]	2.58[a]	2.37[a]	
						37.9[b]	2.58[b]	2.36[b]	183.8[b]
	58.9[c]	4.10[c]	29.7[c]						
Glu[229]	55.1[a]	5.00[a]	32.6[a]	2.16[a]	1.99[a]		2.53[a]	2.36[a]	
						36.1[b]	2.53[b]	2.36[b]	186.1[b]
	54.9[c]	5.14[c]	32.6[c]						

[a]HCCH-TOCSY data at 800 MHz of αLP inhibited with CMK, pH 4 and 25 °C. [b]HCACO data at 800 MHz of αLP inhibited with CMK, pH 4 and 25 °C. [c]Published data at 500/600 MHz of αLP at pH 4 and 35 °C.[20]

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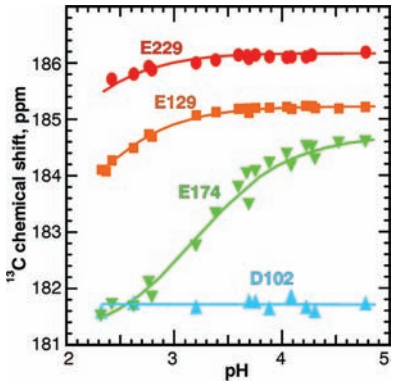

Figure 3. Carboxyl ^{13}C chemical shifts at 600 MHz and 25 °C versus pH for WT αLP and calculated pH titration curves.

Glu^{174} resonances are estimated at ±0.04 ppm, and ±0.06 ppm for the weaker Glu^{229} and Asp^{102} resonances. In addition, the Glu^{174} data exhibit larger scatter than the others, perhaps due to slight variations in the ionic strength between different sample preparations affecting this residue more than others.

The calculated ^{13}C chemical shift versus pH titration curves for Glu^{129} and Glu^{174} shown in Figure 3 were obtained by nonlinear, least-squares fit of the data using GnuPlot 3.5 to the function $f(x) = (K{\cdot}b + a{\cdot}10^{-x})/(K + 10^{-x})$, where K is acid disassociation constant, a is chemical shift of acidic form, b is chemical shift of base, and x is pH. The results are shown in Table 2. The limited number of low pH data points for Glu^{229} necessitated the assumption that it undergoes a chemical shift change of 3.80 ppm upon protonation, the same as for Glu^{174}, for curve fitting.

Confirmation of Asp^{102} Assignment. To confirm the all-important assignment of Asp^{102}, we examined the behavior of Asp $^{13}C^{\gamma}$ and Glu $^{13}C^{\delta}$ resonances over the pH range 5−9 in resting enzyme, CMK-inhibited enzyme, and PMSF-inhibited enzyme. For each case, a unique ionization is known to occur in the active site near Asp^{102} that could perturb its $^{13}C^{\gamma}$ resonance. In resting enzyme, His^{57} undergoes ionization with a pK_a value ~6.9.[28] In the CMK complex, His^{57} itself does not titrate, but it does respond to the ionization of the CMK oxyanion, which has a pK_a value of ~7.5.[23,29] In the PMSF complex, His^{57} is ejected from the active site upon protonation.[30] Figure 4 shows that the resonance at 181.73 ppm, tentatively assigned to the $^{13}C^{\gamma}$ of Asp^{102} based on NMR connectivities (Table 1), responds to each of these events.

In resting enzyme, the 181.73 ppm ^{13}C signal undergoes a 0.4 ppm chemical shift change with a pK_a value of 6.8 ± 0.2, which corresponds closely to the known pK_a value of His^{57}.[28] The 0.4 ppm chemical shift change is one-tenth that expected for titration of the Asp^{102} $^{13}C^{\gamma}$ itself and, moreover, moves in the opposite direction, arguing against interpretations that this change may reflect titration of Asp^{102}. In the CMK-inhibited

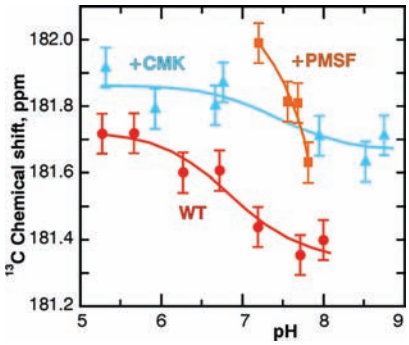

Figure 4. Asp^{102} $^{13}C^{\gamma}$ chemical shifts at 600 MHz and 25 °C versus pH. Perturbations correlate with changes in adjacent His^{57} imidazole ring for αLP and its complexes with Ac-AAPV-chloromethyl ketone (CMK) and phenylmethanesulfonyl fluoride (PMSF) (also see Table 3). Wild-type (WT) αLP and +CMK data are fit to calculated pH curves, yielding pK_a values of 6.8 ± 0.2 and 7.5 ± 0.5, respectively.

complex, the $^{13}C^{\gamma}$ resonance delineates a titration curve with a pK_a value of 7.5 ± 0.5, in agreement with the known pK_a value of 7.5 for the enzyme-bound oxyanion in this complex.[23,29] The direction of the chemical shift change is the same as observed for this resonance in the resting enzyme but smaller in magnitude (0.2 ppm), as would be expected given the increased distance of Asp^{102} from the site of protonation. In the PMSF complex, the Asp^{102} $^{13}C^{\gamma}$ resonance disappears as the pH is lowered from 8 to 7, again consistent with the known dramatic change that occurs in this complex as the pH is lowered, that is, expulsion of the imidazole ring from the active site.[30] In contrast, none of the glutamyl $^{13}C^{\delta}$ resonances showed an appreciable response to pH changes in the range between 5 and 9 with one exception: the Glu^{229} $^{13}C^{\delta}$ moves +0.2 ppm with pK_a of ~6.8 in resting enzyme, indicating it weakly senses the ionization of His^{57}. These observations together with the NMR connectivities from Table 1 strongly support the assignment of the ^{13}C carboxyl resonance at 181.73 ppm to that of the Asp^{102} C^{γ} in fully active enzyme.

pK_a Values of αLP Acidic Residues. We have directly determined pK_a values of 2.0 ± 0.1 and 3.2 ± 0.1 for Glu^{129} and Glu^{174} and have provided evidence that the pK_a value of Glu^{229} is about 1.7. As shown in Figure 2, the Asp^{102} resonance is virtually unchanged until pH 2.3, where acid denaturation takes over. The titration curve of Glu^{129} has changed measurably by pH 3.0, a full unit above its pK_a value. Asp^{102} shows no movement as low as pH 2.3; therefore, we conservatively estimate from Figure 3 that the pK_a value of Asp^{102} is less than 1.5.

DISCUSSION

Of the four carboxyl groups whose pK_a values are determined in Table 2, only that belonging to Glu^{174}, located in the solvent-

Table 2. Calculated ^{13}C Carboxyl Chemical Shifts and pK_a Values of αLP Titratable Side Chains Based on pH Titrations in Figure 3

	Asp^{102}	Glu^{129}	Glu^{174}	Glu^{229}
best fit pK_a	<1.5	2.0 ± 0.1	3.2 ± 0.1	1.7 ± 0.1[a]
δ_{base} (ppm)	181.74 ± 0.08	185.26 ± 0.01	184.7 ± 0.1	186.19 ± 0.02
δ_{acid} (ppm)	~178[b]	181.7 ± 0.5	180.9 ± 0.2	182.4[a]
$\delta_{base} - \delta_{acid}$ (ppm)	~−3.7	−3.53 ± 0.5	−3.80 ± 0.2	−3.80[a]

[a]Because of paucity of data, $\delta_{base} - \delta_{acid}$ was assumed to be −3.80 ppm. [b]δ_{acid} assumed from typical literature values.[24,25]

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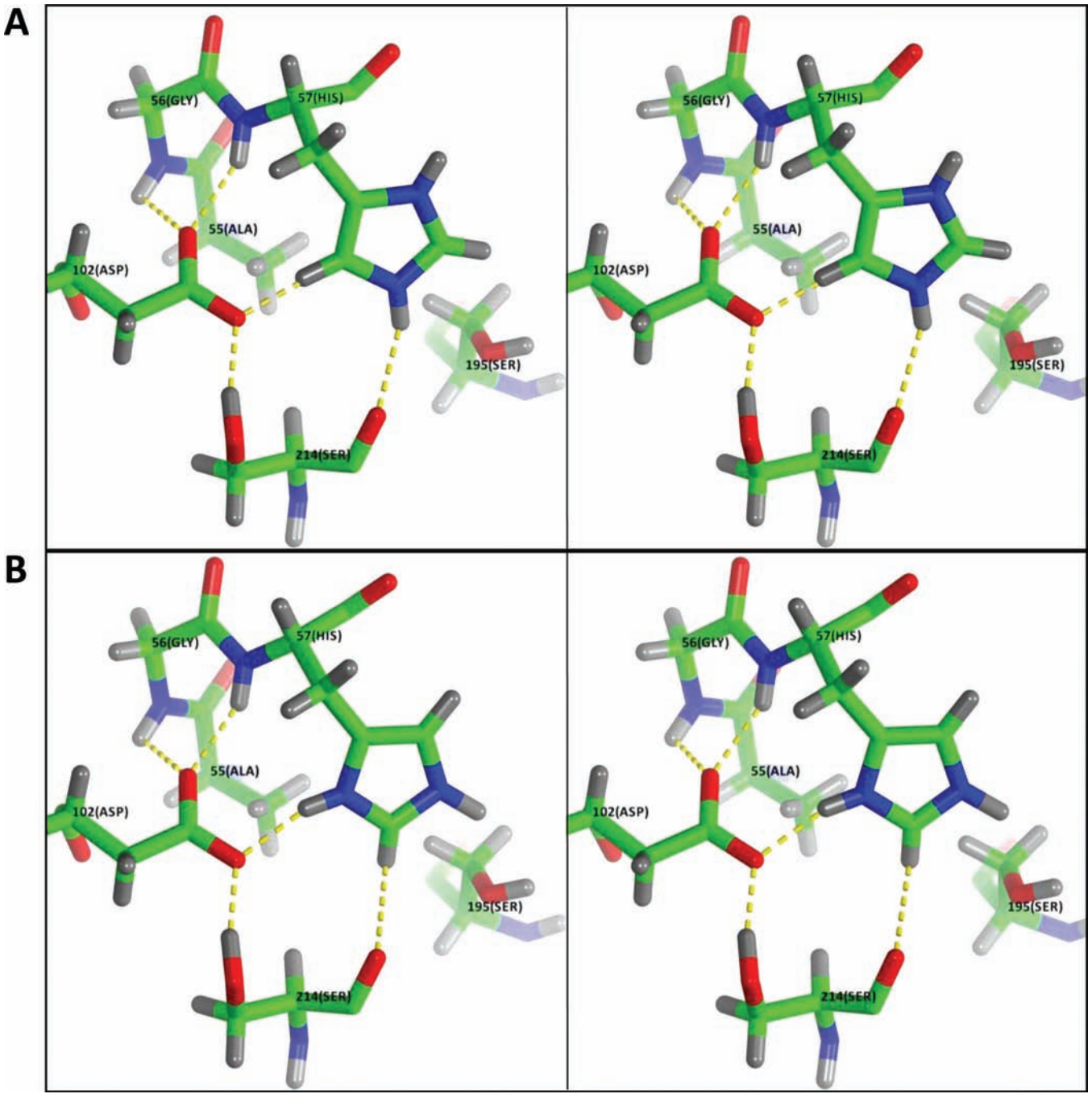

Figure 5. The catalytic tetrad of αLP in stereo using 2H5C,[8] showing H-bonding network around the Asp^{102} anion, decreasing its basicity and hindering its rotational motility. (A) Normal structure. (B) Intermediate with His^{57} imidazole ring rotated 180° as proposed in our RDRF mechanism.[7] This conformation of the active site His is observed in the X-ray crystal structure (1YJC) of subtilisin BPN in 50% dimethylformamide.[40]

accessible S2 binding pocket, does not interact with an opposing plus charge. Its pK_a value of 3.2 ± 0.1 is therefore the closest to the typical value for glutamates (4.2 ± 0.9).[31] Glu^{129} is in close proximity to Lys^{165} and Arg^{230}, which could stabilize its negative charge, lowering its pK_a value to 2.0 ± 0.1. The partially buried Glu^{229} is close to Arg^{103} and Arg^{230}, which in similar fashion could depress its pK_a value to 1.7.

The pK_a value of Asp^{102} of less than 1.5 is significantly lower than what is typical for an aspartyl residue (3.5 ± 1.2)[31] and lower than any previous value attributed to the catalytic aspartate in a serine protease.[10−12,31]

Within the active site of αLP, Asp^{102} is surrounded by hydrophobic amino acids Ala^{55}, Gly^{56}, Phe^{94}, Val^{177}, and Leu^{180}. However, it also participates in a number of H-bonds to the backbone amides of Ala^{55} and Gly^{56}, the H$^{\delta 1}$ of His^{57}, and the hydroxyl group of Ser^{214}, which forms the shortest, best aligned, and perhaps strongest of these H-bonds (Figure 5A).[8] The unusually low pK_a value of Asp^{102} could result from an electrostatic interaction with the positively charged imidazole group of His^{57}. However, such an interaction should produce equal and opposite effects on each partner. So, if the pK_a value of Asp^{102} is lowered by ≥ 2 pK_a units, as it appears to be here,

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then the pK_a value of His^{57} should be increased by ≥ 2 units. However, the largest ΔpK_a unit effect that could potentially be attributed to an Asp−His electrostatic interaction is about +0.8, as His^{57} has a pK_a of ∼6.9, while that of monomeric histidine has a pK_a of ∼6.1. The remainder of the ≥ 2 unit depression in the pK_a value of Asp^{102} must be due to the stabilizing interactions supplied by the other H-bond partners. The small Asp^{102}-mediated effect on the pK_a of His^{57} is consistent with results showing that Asp^{102} does not greatly affect the intrinsic nucleophilicity of His^{57}.[32] There is, nevertheless, no doubt that Asp^{102}, and therefore the Asp^{102}−His^{57} interaction, is crucial to catalysis, as indicated by the 10 000-fold decrease in catalytic activity of the D102N mutant.[32] Thus, these observations suggest that the primary role of Asp^{102} in catalysis may be to properly orient the imidazole ring throughout the reaction via H-bonding, rather than to stabilize the development of charge on His^{57}.

Ser^{214} is highly conserved in serine proteases and is often consider the fourth member of a "catalytic tetrad".[28,33] In αLP, removal of Ser^{214} reduces activity but also appears to increase the pK_a value of His^{57}, indicating that the His^{57}−Asp^{102} interaction is strengthened by removal of the Asp^{102}−Ser^{214} interaction.[34] Analogous mutations in trypsin and thrombin have effects on catalytic rate constants, but no information is available on the effect of these mutations on the pK_a value of His^{57}. Direct pK_a value measurements of Asp^{102} and His^{57} in these mutants could help to clarify the role of Ser^{214} on the microenvironment of Asp^{102} and its effect on the pK_a value of Asp^{102}.

We have proposed a "reaction-driven ring flip" (RDRF) mechanism in response to the Wang−Polgar−Jencks dilemma questioning how serine protease-catalyzed reactions can proceed in the forward direction at all.[35−37] Our mechanism involves rotation of ∼180° of the His^{57} imidazole ring about its C_β−C_γ axis,[7] which satisfies the dilemma in elegant fashion while offering a role for Derewenda's H-bond between the carbonyl oxygen of Ser^{214} and the $C^{\varepsilon 1}$−H of His^{57}, which is conserved in all serine proteases.[33] The two His^{57} conformations, shown in Figure 5A and B, are held in place by H-bonds between Asp^{102}, Ser^{214}, Ser^{195}, and a water/substrate molecule bound in the active site cleft. These two rotamers must be easily interchangeable, requiring H-bonds from His^{57} to surrounding groups including Asp^{102} and Ser^{214} strong enough to align His^{57}, but not so strong as to restrict flipping. An invariantly negatively charged Asp^{102} and relatively weak Asp−His interaction fits wells with this mechanism. Some X-ray crystal studies and theoretical calculations claim that active site motions far more subtle than a His^{57} ring flip are sufficient for catalysis, but it is untrue as claimed that there is any evidence against the RDRF theory to date.[38] Recently, Scheiner has discussed previous theoretical calculations of serine protease catalytic models and did a thorough analysis of the energetics of the RDRF model.[39] This full ab initio calculation with polarized basis set included the Ser^{214} carbonyl group, the oxyanion hole, the catalytic water, restriction of the four side chain motions from fixed C^α, and full consideration of the dielectric medium. Although not providing definitive evidence in favor of the RDRF theory, Scheiner's work supports its energetic feasibility. Given the likely affect of the Ser^{214} hydroxyl group and the rest of the Asp^{102} H-bond network on the pK_a value of Asp^{102}, it would seem appropriate to include these interactions in any future models. Physical evidence of His^{57} in the flipped configuration within the active site of a fully active serine proteases has been reported in an X-ray crystallographic study of subtilisin in 50% DMF.[40]

Table 3. Secondary Effects Caused by Neighboring Group Ionization (i.e., His^{57} Deprotonation or CMK Oxyanion Formation) on Carboxyl ^{13}C Chemical Shifts of αLP and CMK Complex Calculated from pH Titrations

	Asp^{102}	Asp^{102} (+CMK)	Glu^{229}
best fit pK_a	6.8 ± 0.2	7.5 ± 0.5	6.7 ± 0.6
δ_{base} (ppm)	181.34 ± 0.03	181.67 ± 0.05	186.40 ± 0.03
δ_{acid} (ppm)	181.73 ± 0.03	181.85 ± 0.03	186.17 ± 0.03
$\delta_{base} - \delta_{acid}$ (ppm)	0.39 ± 0.04	0.18 ± 0.06	−0.23 ± 0.04

CONCLUSION

Here, we report the first direct observation of the $^{13}C^\gamma$ NMR resonance from the aspartic acid in the catalytic triad of a serine protease and direct measurement of its pK_a value. The results demonstrate this residue has a pK_a of less than 1.5 in resting enzyme, a value that is lower than any previously suggested, and one that is compatible with RDRF or SIHB mechanisms requiring a relatively weak Asp−His interaction, but not with mechanisms requiring strong H-bonds or high Asp^{102} pK_a values, such as the "modified charge-relay" and LBHB mechanisms.

ASSOCIATED CONTENT

Supporting Information

Complete purification scheme for αLP. ^{1}H NMR spectra of high field methyl group region versus pH (Figure S1). HCCH-TOCSY assignment for Asp^{102} (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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