^{15}N and ^{1}H NMR Spectroscopy of the Catalytic Histidine in Chloromethyl Ketone-Inhibited Complexes of Serine Proteases†

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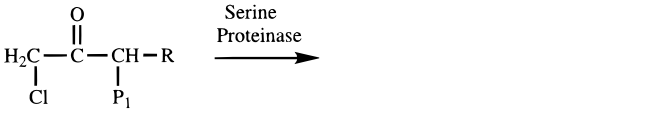
Received June 20, 1995; Revised Manuscript Received December 14, 1995⊗

ABSTRACT: The hemiketal hydroxyl groups in chloromethyl ketone (cmk) complexes of trypsin and chymotrypsin have been reported to ionize to the oxyanion with pK_a values 2−4 pK_a units below expectations for such a functional group on the basis of the behavior of the hemiketal carbon atom in ^{13}C NMR spectra [Finucane, M. D., & Malthouse, J. P. G. (1992) *Biochem. J. 286*, 889−900]. The low pK_a indicates the enzymes selectively stabilize the oxyanion form of the bound inhibitor, and therefore that cmk complexes may be good models of enzyme-mediated transition-state stabilization. However, the ^{13}C NMR studies could not rule out His57 as the titrating group. Here we report the behavior of the ring ^{15}N atoms of His57 in the Ala-Ala-Pro-Val−cmk complex of α-lytic protease. Both $N^{\delta 1}$ and $N^{\epsilon 2}$ of His57 respond to an ionization with a pK_a of ∼7.5, but His57 itself does not titrate as $N^{\epsilon 2}$ remains alkylated and $N^{\delta 1}$ remains bonded to a proton over the entire pH range. The species titrating with a pK_a of ∼7.5 must therefore be the hemiketal hydroxyl. The results also show that the ^{1}H NMR signal from the proton in the Asp-His hydrogen bond behaves in a characteristic manner in cmk complexes and can be used diagnostically to confirm that His57 does not titrate and to measure the pK_a of the hemiketal hydroxyl in cmk−protease complexes without resorting to ^{15}N-labeling. We have used the behavior of this signal to directly confirm that His57 does not titrate in the trypsin and chymotrypsin complexes that were the subjects of the original ^{13}C NMR studies.

Chloromethyl ketones (cmk's) (**1**) are irreversible inhibitors of serine proteases. Although the mechanism of inhibition remains the subject of debate (Prorok et al., 1994), the structure of the final complex formed has been well-characterized by X-ray diffraction analysis (Bode et al., 1992; James et al., 1980; Navia et al., 1989). It includes two covalent bonds between the inhibitor and the enzyme, one between the methylene group of the inhibitor and $N^{\epsilon 2}$ of His57,[1] the second between the carbonyl carbon of the inhibitor and O^{γ} of Ser195 as shown in **2** below.



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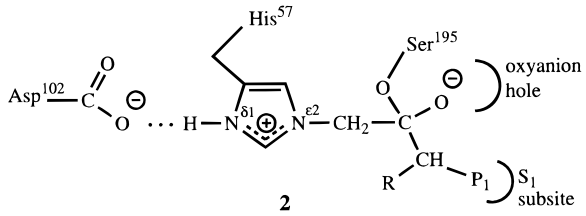

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In the complex the carbonyl carbon of the inhibitor is tetrahedral with the oxygen atom oriented toward the oxyanion hole as shown in structure **2**. Crystal structures of several cmk−serine protease complexes have indicated that the keto oxygen atom is close enough to the amide NH groups of Gly193 and Ser195 to form strong hydrogen bonds. These are the NH groups that constitute the oxyanion hole and which are believed to contribute to catalysis by stabilizing the tetrahedral substrate oxyanion formed during catalysis. Thus, the structure of these complexes geometrically and electronically resembles that of the putative tetrahedral intermediate formed during catalysis. Chloromethyl ketone inhibitor−enzyme complexes are therefore of interest as potential models of enzyme-mediated transition-state stabilization.

The crystal structures, however, do not reveal whether the bound hemiketal oxygen atom is protonated or ionized; the ionized oxyanion form is illustrated in structure **2** above. This question has been addressed by Malthouse and coworkers in ^{13}C NMR studies of cmk complexes specifically ^{13}C-labeled at the hemiketal carbon atom (Finucane et al., 1989; Finucane & Malthouse, 1992; Malthouse et al., 1983, 1985; O'Connell & Malthouse, 1995; Primrose et al., 1985; Scott et al., 1986). These investigators have interpreted the pH dependence of the hemiketal ^{13}C signal as indicating that the hemiketal hydroxyl group ionizes to the oxyanion with a pK_a value of ∼7.9 in a ZLCK−trypsin complex and of ∼8.7 in a TPCK−chymotrypsin complex. These results indicate under the conditions most crystallogaphic work on cmk complexes has been carried out that the hemiketal exists

† Supported by Research Grant GM 27927 from the National Institutes of Health. Presented in part at the ASBMNB/ACS-DBC, San Diego, CA, May 30−June 3, 1993, and at the American Chemical Society Meeting, Chicago, IL, August 20−25, 1995.

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⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1996.

[1] The chymotrypsinogen numbering system is used when specifying the residues of the catalytic triad in the enzymes of the chymotrypsin family.

[2] Abbreviations: NMR, nuclear magnetic resonance; X-cmk, the chloromethyl ketone derivative of peptide X; Z-, benzyloxy- ; TPCK, tosyl Phe-chloromethyl ketone; TLCK, tosyl Lys-chloromethyl ketone.

0006-2960/96/0435-2437$12.00/0

as the protonated hydroxyl rather than as the ionized oxyanion. These results nevertheless also indicate that trypsin and chymotrypsin substantially stabilize the oxyanion form of the bound hemiketal as the observed pK_a values are two to four units below expectations for such a functional group.

The above ^{13}C NMR studies provide the first direct evidence that serine proteases are indeed able to stabilize an enzyme-bound tetrahedral and negatively charged species as has been widely supposed. However, these findings have additional significance because they suggest it may be possible to dissect the oxyanion component of enzyme-mediated transition-state stabilization from other components and to directly quantify it through measurement of the hemiketal pK_a in cmk complexes. Such measurements could prove useful in studies of the catalytic mechanism and specificity of these enzymes.

There is, however, an ambiguity in the ^{13}C NMR studies in that ionization of the imidazole ring of His57 cannot be ruled out as the cause of the pH dependence of the hemiketal ^{13}C NMR signals. In view of the important implications of the conclusions of the ^{13}C NMR work as outlined above it seems highly desirable to remove this uncertainty. Our previous work has demonstrated that the imidazole ring ^{15}N NMR signals are remarkably informative about the chemistry of His57 in α-lytic protease (Bachovchin, 1986; Bachovchin & Roberts, 1978; Bachovchin et al., 1988). They are especially unambiguous in identifying the location of protons on the imidazole ring and its state of protonation, which is the information needed to resolve the question at hand. Here we describe the behavior of the ^{15}N signals of His57 in a cmk complex of α-lytic protease. We have also reinvestigated the behavior of the ^{1}H signal arising from the proton in the Asp-His H-bond in cmk complexes and show it can be used diagnostically on other cmk−serine protease complexes to reveal whether or not the catalytic histidine titrates and to determine the pK_a of the hemiketal hydroxyl group.

MATERIALS AND METHODS

Bovine pancreatic α-chymotrypsin (EC 3.4.21.1) (type II), porcine pancreatic elastase (EC 3.4.21.36) (type II-A), porcine pancreatic trypsin (EC 3.4.21.4) (type IX), and inhibitor TLCK were purchased from Sigma Chemical Co. (St Louis, MO). Ala-Ala-Phe−cmk and Ala-Ala-Pro-Val−cmk were purchased from Bachem Inc. (Torrance, CA).

Z-Val−cmk was synthesized using the following procedure. The diazoketone Z-ValCHN_2 was prepared from Z-Val and diazomethane using the mixed anhydride method (Penke et al., 1970). Anhydrous HCl was then bubbled through a solution of Z-ValCHN_2 in ether−tetrahydrofuran at 5 °C until it turned colorless. The solvents were evaporated and the residue was dissolved in ethyl acetate, washed with citric acid and then with a $NaHCO_3$ solution, and dried over $MgSO_4$. Evaporation of the solvent gave an oil that solidified upon standing. It was purified on a silica gel column (15 cm × 5 cm) (Aldrich, Milwaukee, WI) and eluted with chloroform to give Z-Val−cmk.

^{15}N-labeled His-57 α-lytic protease (EC 3.4.21.12) was purified from a histidine auxotroph mutant of *Lysobacter enzymogenes* as previously described (Bachovchin & Roberts, 1978). The enzyme was further purified using a S-200 Sepharose column (Pharmacia; Piscataway, NJ) equilibrated in 0.1 M KCl.

The ^{15}N NMR samples of α-lytic protease were prepared by concentrating the enzyme from the S-200 column on a stirred cell (Amicon, Beverly, MA) to about 2 mM in 1.6 mL. 10% D_2O was added for lock. 0.05 M Tris buffer was added to the ^{15}N−histidine-labeled samples to stabilize the pH at high pH value. The ^{1}H NMR samples of α-lytic protease were prepared by concentrating the enzyme to about 2 mM in 400 μL and adding 10% D_2O to provide an internal lock. The ^{1}H NMR samples of chymotrypsin and trypsin were prepared by dissolving the lyophilyzed powders from Sigma in about 400 μL of 0.1 M KCl. 10% D_2O was added for lock. The different chloromethyl ketone inhibitors were added directly to the NMR samples in about a 5−15-fold molar excess. The pH of the samples was varied by addition of 0.25 M NaOH or HCl.

^{15}N NMR spectra were recorded at 40.55 MHz on a Bruker AM-400 wide-bore NMR spectrometer equipped with an Aspect 3000 computer and a 10-mm single-frequency ^{15}N probe. Spectra were acquired with a 90° pulse, a spectral width of 15 000 Hz, 8K real data points and a recycling time of 0.8 s. Spectra were referenced to HNO_3 with positive shifts being upfield.

^{1}H NMR spectra were recorded at 400.13 MHz on the same spectrometer using a 5 mm single-frequency ^{1}H probe. The experiments were run at low temperature (278 K) using the Bruker variable-temperature accessory. Spectra were acquired using the "Hard 1−1" pulse sequence (Clore et al., 1983) with oversampling (spectral width of 40 000 Hz, 32K data points) (Delsuc & Lallemand, 1986). The ^{15}N and ^{1}H NMR data were processed on a Sun Sparc Station computer (Sun Microsystems, Fremont, CA) using Felix 2.0 (Biosym Technologies, Inc., Parsippany, NJ).

Data analysis of the pH titrations was performed by fitting data to the appropriate equation using the BMDP routine AR (derivative free nonlinear regression) (BMDP Statistical Software Inc., Los Angeles, CA). The appropriate equation for a two-state pH titration for an intensive observable (i.e., concentration-independent) is:

$$\delta\ (\text{ppm}) = \frac{[10^{-\text{p}K_a} \times \delta(\text{ppm})B] + [10^{-\text{pH}} \times \delta(\text{ppm})A]}{10^{-\text{pH}} + 10^{-\text{p}K_a}}$$

RESULTS

Cmk-Induced Changes in ^{15}N Shifts of His57 α-Lytic Protease. Figure 1 shows ^{15}N NMR spectra of α-lytic protease inhibited with Ala-Ala-Pro-Val−cmk (spectra A−D) and compares these spectra with one of uninhibited enzyme (spectrum E). All spectra shown in Figure 1 are of doubly ^{15}N ($N^{\delta 1}$ and $N^{\epsilon 2}$) His57-labeled enzyme at pH 9. Comparison of spectra 1A−D with 1E shows that formation of the cmk complex induces substantial changes in the ^{15}N chemical shifts of His57. The signals from resting enzyme at ∼140 and ∼200 ppm (Figure 1E) disappear, and two new, closely spaced, signals with shifts of ∼193.5 and ∼194.2 ppm appear (Figure 1A). The signal at 193.5 ppm becomes a doublet with ^{1}J of ∼90 Hz in the absence of ^{1}H decoupling while the higher field signal remains a singlet. This can be seen in spectrum 1B where one-half of the doublet lies under the singlet at 194.2 ppm, but it can be more clearly seen in spectrum 1D, where owing to a small temperature dependence of the shifts the doublet and singlet become more clearly resolved.

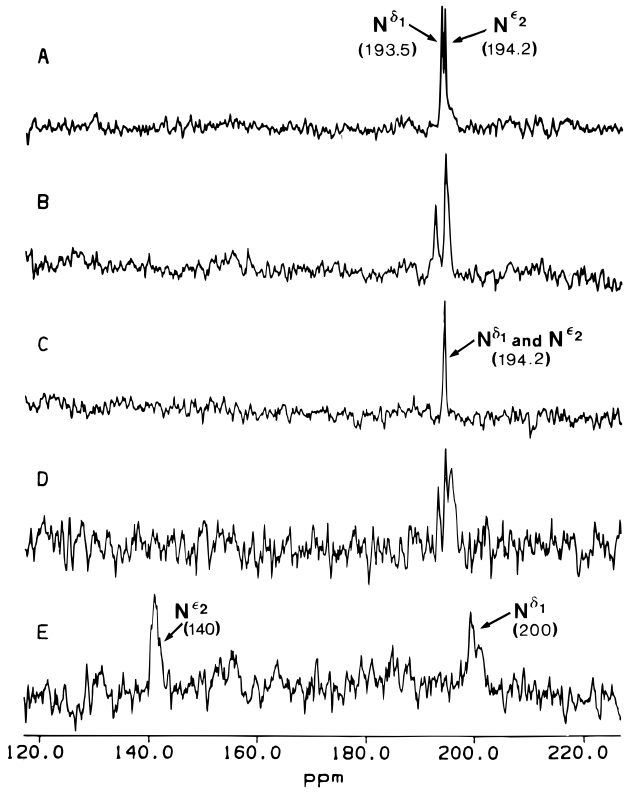


FIGURE 1: ^{15}N NMR spectra of ^{15}N-labeled His57 α-lytic protease (99% ^{15}N, both ring nitrogens) inhibited with Ala-Ala-Pro-Val−cmk at pH 9. (A) ^{1}H-decoupled, 298 K; (B) ^{1}H-coupled, 298 K; (C) ^{1}H-decoupled, 278 K; (D) ^{1}H-coupled, 278 K; (E) uninhibited resting enzyme, ^{1}H-coupled, 298 K.

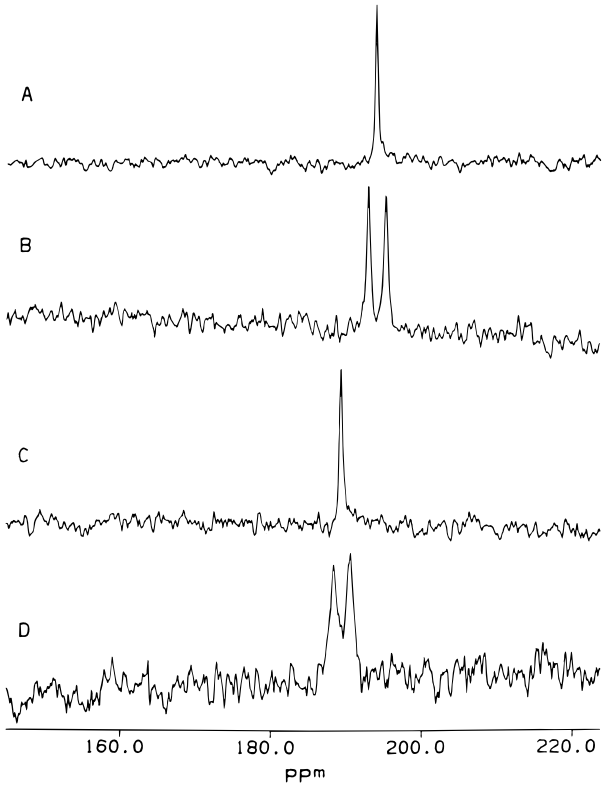


FIGURE 2: ^{15}N NMR spectra of ^{15}N-labeled α-lytic protease (99% ^{15}N at $N^{\delta 1}$ of His57) at 298 K inhibited with Ala-Ala-Pro-Val−cmk. (A) pH 9.2, ^{1}H-decoupled; (B) pH 9.2, ^{1}H-coupled; (C) pH 5, ^{1}H-decoupled; (D) pH 5, ^{1}H-coupled.

Spectra of singly $^{15}N^{\delta 1}$ His-labeled enzyme, Figure 2, allow assignment of the doublet at 193.5 ppm to $N^{\delta 1}$ and the singlet at 194.2 ppm to $N^{\epsilon 2}$ (Figure 2A,B). The changes induced by cmk inactivation and the behavior of the ^{15}N signals in the complex are easily interpreted and immediately provide a clear picture of the chemistry of His57 in the complex as explained below.

Imidazole rings contain three canonical-type nitrogen atoms: pyridine-like (>N:); pyrrole-like (>N−H); and pyrrole-like within a protonated imidazole ring (+>N−H) (Bachovchin, 1986; Farr-Jones et al., 1993). In aqueous solutions the chemical shifts of >N: and >N−H types differ by more than 80 ppm with >N: nitrogens typically resonating at ∼128 ppm and >N−H nitrogens at ∼210 ppm. The third canonical nitrogen type is found in a protonated imidazole ring and designated +>N−H. This nitrogen type resembles the pyridine >N−H type nitrogen in both structure and in chemical shift, but is distinguished by occurring 8−10 ppm downfield from the >N−H type nitrogen. For example, protonation of a >N: type nitrogen of a neutral imidazole ring in a pure tautomeric form typically induces the signal from the other imidazole ring nitrogen, which must be an >N−H type nitrogen, to move about an 8−10 ppm downfield. Thus, +>N−H type nitrogens typically resonate at ∼ 200 ppm compared to ∼210 ppm for N−H type nitrogens.

The signals at ∼140 and ∼200 ppm in the spectrum of resting α-lytic protease are from $N^{\epsilon 2}$, and $N^{\delta 1}$ respectively (Figure 1E). We have previously shown that these shifts reflect a fully neutral imidazole in a single tautomeric form, the $N^{\delta 1}$−H tautomer, and that the anomalous chemical shift positions of both nitrogen atoms for a pure $N^{\delta 1}$−H tautomer are due to the H-bonding interactions with Asp102 and Ser195 (Bachovchin, 1986; Bachovchin & Roberts, 1978). The H-bond from Ser195 to $N^{\epsilon 2}$ causes it to resonate ∼10 ppm upfield from the shift expected for a canonical >N: type nitrogen while the H-bond to Asp102 causes $N^{\delta 1}$ to resonate ∼10 ppm downfield from that expected for a canonical >N−H type nitrogen as illustrated in Scheme 1.

The ^{15}N chemical shifts for an alkylated $N^{\epsilon 2}$ imidazole ring nitrogen in cmk−protease complexes or for closely related model systems have not yet been reported. However, imidazole ring nitrogens alkylated with methyl groups have been shown to have chemical shifts similar to those >N−H and +>N−H type nitrogens (Farr-Jones et al., 1993). There is no reason to believe that the chemical shift of an imidazole ring nitrogen alkylated with a methyl ketone moiety should differ greatly from one alkylated by a methyl group or therefore from one directly bonded to a proton. However, an alkylated nitrogen should differ from one directly bonded to a proton by not exhibiting direct one-bond N−H spin coupling. Thus, both the chemical shift of ∼194.2 ppm (Figure 1, spectra A−D) for $N^{\epsilon 2}$ and the absence of one-bond proton splitting are consistent with it having become alkylated as expected on cmk inactivation and illustrated in Scheme 1.

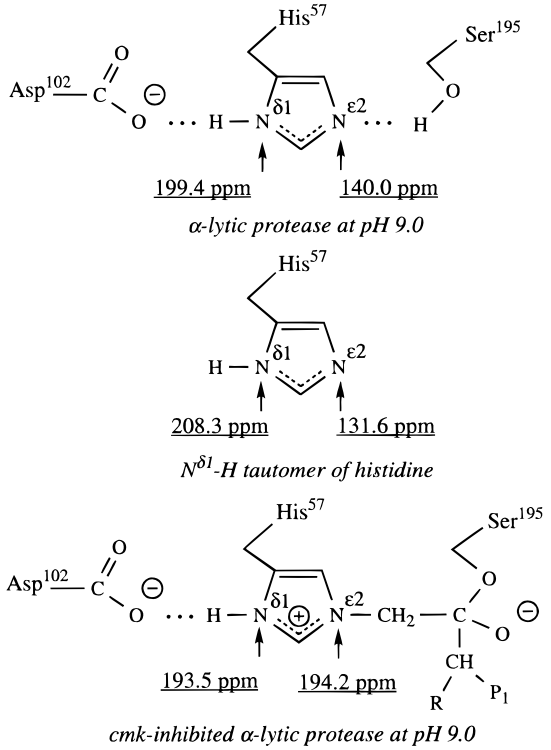
The chemical shift of $N^{\delta 1}$ as well as the observed one bond ^{1}H splitting (Figures 1 and 2) shows that $N^{\delta 1}$ retains its proton on cmk inactivation. The 8−10 ppm downfield movement of $N^{\delta 1}$ on cmk inactivation can be understood in terms $N^{\delta 1}$ being converted from an >N−H type to a +>N−H type nitrogen by alkylation at $N^{\epsilon 2}$, as the effect of N-alkylation on the imidazole ring is similar to a protonation. The unusually low-field position of $N^{\delta 1}$ for an +>N−H type nitrogen, ∼193.5 ppm, signals the presence of the H-bond

Table 1: Summary of Hemiketal pK_a's and Titration Shifts Observed by ^{1}H, ^{13}C, and ^{15}N NMR of cmk−Serine Proteinase Complexes

enzyme/inhibitor	hemiketal pK_a from ^{15}N of His57	hemiketal pK_a from ^{13}C of hemiketal	hemiketal pK_a from ^{1}H ($N^{\delta1}$ and His57)	titration shifts (ppm) low pH	titration shifts (ppm) high pH
chymotrypsin/					
Ala-Ala-Phe−cmk			8.65	17.59	16.72
TPCK		8.67[a]		99.08	103.44
trypsin/					
TLCK			7.80	16.91	16.14
ZLCK		7.88[b]		97.95	102.08
α-Lytic protease/					
Ala-Ala-Pro-Val−cmk			7.40	16.73	15.86
	$N^{\epsilon2}$ 7.44[c]			200.9[d]	194.1[d]
	$N^{\delta1}$ 7.52[c]			188.9[d]	193.7[d]
Z-Val−cmk			7.32	16.73	15.84

[a] From Finucane et al. (1989). [b] From Malthouse et al. (1985). [c] From a single experiment monitoring both ^{15}N resonances of the active site histidine. [d] ^{15}N shifts referenced to external 1 M HNO_3 with positive shifts being upfield.

Scheme 1: Comparison of the ^{15}N Shifts of His57 of α-Lytic Protease at High pH with Those of the $N^{\delta1}$−H Tautomer of Histidine and with His57 in cmk-Inhibited Enzyme



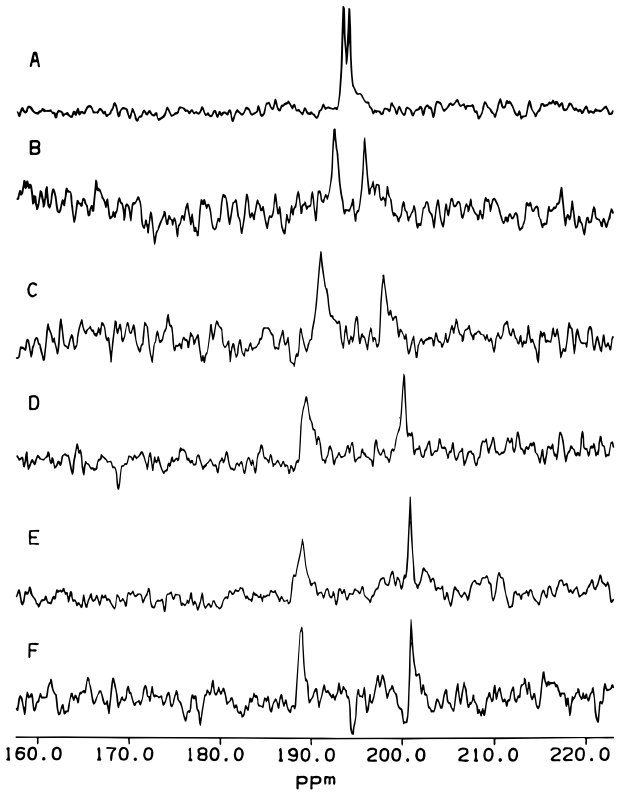


FIGURE 3: ^{15}N NMR spectra of ^{15}N-labeled His57 α-lytic protease (99% ^{15}N, both ring nitrogens) inhibited with Ala-Ala-Pro-Val−cmk, proton-decoupled, 20 °C at (A) pH 9.0; (B) pH 8.0; (C) pH 7.4; (D) pH 6.5; (E) pH 5.7; (F) pH 3.5.

to Asp102. Thus the behavior of the ^{15}N signals confirms that the inhibitor has alkylated His57 as expected and further shows that in the complex at pH 9.0 His57 remains fully protonated and positively charged, and strongly H-bonded to Asp102 through $N^{\delta1}$−H as an H-bond donor.

pH Dependence of $N^{\delta1}$ and $N^{\epsilon2}$ of His57 in cmk Complexes. Figure 3 illustrates the pH dependence of the ^{15}N signals from His57 of α-lytic protease in the Ala-Ala-Pro-Val−cmk complex. The spectra show that both the $N^{\delta1}$ and $N^{\epsilon2}$ resonances depend on pH. $N^{\delta1}$ moves from ∼193.5 ppm at pH 9 (Figure 3A) to ∼188.8 ppm at pH 3.5 (Figure 3F). $N^{\epsilon2}$ moves in the opposite direction going from ∼194.2 ppm at pH 9 (Figure 3A) to ∼201.1 ppm at pH 3.5 (Figure 3F). Both signals delineate a titration curve with a pK_a of ∼7.5 (Table 1; Figure 4). Nevertheless, the chemical shift behavior of $N^{\delta1}$ shows that it remains directly bonded to a proton, while that of $N^{\epsilon2}$ shows it remains alkylated. His57 therefore remains protonated over the entire pH range. Thus, although both ^{15}N signals sense the ionization of a functional group titrating with a pK_a near 7.5, the chemical shifts positions show that the imidazole ring itself is not the titrating species. The similarity in the magnitude of the pK_a sensed by the ^{15}N signals to those sensed by the hemiketal carbon atom in cmk complexes of trypsin and chymotrypsin indicate the ^{15}N and ^{13}C signals are responding to the same ionizing group (Table 1). This group must be the hemiketal oxygen as originally proposed by Malthouse and co-workers (1983, 1985) as the present results show that it cannot be His57.

Behavior of the Asp-His Hydrogen-Bonded Proton at Low Field in cmk Complexes. The chemical shift behavior of the $^{15}N^{\delta1}$ signal shows that it is directly bonded to a proton and that the $N^{\delta1}$−H group is engaged in a strong H-bond, presumably to Asp102, in the cmk−α-lytic protease complex

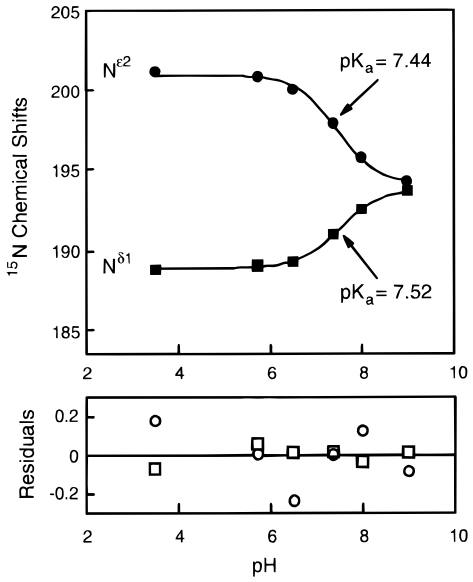


FIGURE 4: Effect of pH on the ^{15}N NMR chemical shifts of $N^{\delta 1}$ and $N^{\epsilon 2}$ nitrogens of ^{15}N-labeled His57 α-lytic protease inhibited with Ala-Ala-Pro-Val−cmk. Solid lines represent the best fit curves corresponding to values summarized in Table 1.

at all pH values. This indicates that the N−H proton should be directly observable at all pH values in the low-field region of the proton spectrum. Robillard and Shulman (1974) reported the presence of this signal in cmk complexes of chymotrypsin and observed that it did not titrate between pH 7.0 and 8.0. We have confirmed that this proton is visible in ^{1}H spectra of the Ala-Ala-Pro-Val−cmk−α-lytic protease complex at all pH values at 278 K. This behavior contrasts with that of this signal in resting enzyme where it is easily visible only at the extremes of pH, owing to acid−base exchange broadening near the pK_a (Bachovchin, 1985).

In the Ala-Ala-Pro-Val−cmk−α-lytic protease complex the Asp-His is hydrogen bonded. The ^{1}H NMR signal moves ∼0.8 ppm, going from ∼16.7 ppm at low pH to ∼15.9 ppm at high pH, responding to an apparent pK_a of ∼7.4 (Table 1), which agrees closely with the pK_a sensed by the ^{15}N signals. In resting enzyme this ^{1}H signal moves ∼3.0 ppm, going from ∼17.1 ppm at low pH to ∼13.8 ppm at high pH (Bachovchin, 1985). Thus, the low-field ^{1}H NMR signal not only responds to the same titrating group as the ^{15}N signals, like the ^{15}N signals it also moves over only a fraction of the distance it moves on ionization of His57 in resting enzyme. Notice also that the pH dependence of the ^{1}H signal is the same in the cmk complex compared to the resting enzyme (Bachovchin, 1986). In both the cmk complex and in the resting enzyme it moves upfield on increasing pH.

The above results show that the behavior of the Asp-His H-bond proton resonance in cmk complexes is quite distinctive and suggest that it should be possible to use this resonance diagnostically on other serine protease complexes to reveal whether or not the behavior of the active site His57 is conserved from one cmk complex to another, to verify whether or not His57 titrates, and to measure the pK_a of the hemiketal hydroxyl group. We have examined the behavior of this signal in Z-Val−cmk−α-lytic protease, TLCK-trypsin, and Ala-Ala-Phe−cmk−chymotrypsin, and the results are summarized in Table 1.

The behavior of the low-field ^{1}H signal in the α-lytic protease complex with Z-Val−cmk is essentially indistinguishable from its behavior in the Ala-Ala-Pro-Val−cmk complex (Table 1). This indicates that the additional interaction made by the Ala-Ala-Pro moiety in the peptide−cmk in the extended specificity subsites of the enzyme does not significantly affect the hemiketal hydroxyl group pK_a or the high- and low-pH chemical shift positions of the low-field proton.

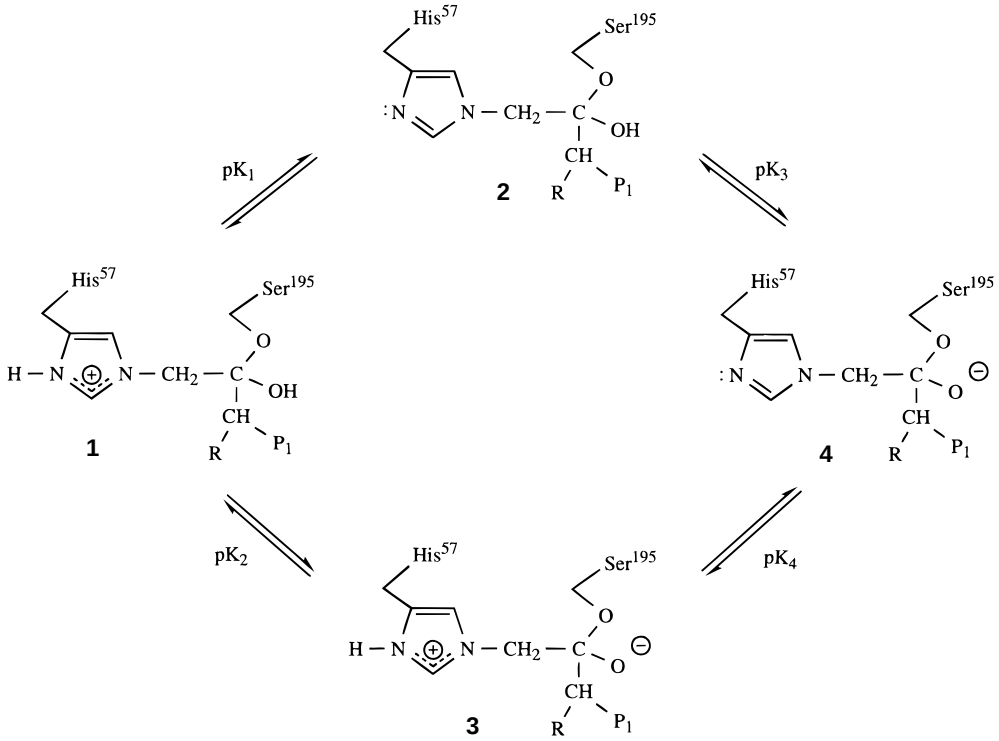
The ZLCK complex of trypsin was one of the subjects of the original ^{13}C NMR studies that led to the proposal that the enzyme-bound hemiketal ionizes (Malthouse et al., 1983, 1985). The ^{13}C NMR study assigned a pK_a of 7.88 to the hemiketal in this complex. The low-field ^{1}H in the TLCK−trypsin complex moves ∼0.77 ppm from 16.91 to 16.14 ppm on going from low to high pH, mapping out a pK_a of ∼7.8 (Table 1). For comparison, the low-field proton signal in resting trypsin moves ∼3.1 ppm, going from ∼14.5 ppm at pH 9 to ∼17.6 ppm at pH 4 mapping out a pK_a of ∼7.0 (Tsilikounas et al., 1992). Thus, the pK_a sensed by the low-field proton in TLCK−trypsin matches that reported in the ^{13}C NMR study, while the magnitude and direction of its movement matches that exhibited by the low-field ^{1}H signal in α-lytic protease−cmk complexes.

A second complex examined by Malthouse and co-workers was that of chymotrypsin with TPCK (Finucane et al., 1989). Here the behavior of the hemiketal carbon in ^{13}C NMR spectra indicated a pK_a of 8.67 for the hemiketal hydroxyl group, nearly one pK_a unit higher than in the trypsin−TLCK complex. The low-field proton in the complex of chymotrypsin with Ala-Ala-Phe−cmk responds to a pK_a of ∼8.65 (Table 1), which is in good agreement with the ^{13}C NMR results. Again, the low-field proton signal movement is characteristically small, ∼0.87 ppm, and has the same dependence on pH relative to this signal in resting enzyme, which goes from ∼18 ppm at low pH to ∼15 ppm at high pH (Robillard & Shulman, 1972; E. Tsilikounas, S. Farr-Jones, and W. W. Bachovchin, unpublished data). The above low-field proton results show that the active site chemistry observed in cmk complexes of α-lytic protease is strongly conserved in cmk complexes of trypsin and chymotrypsin and directly confirms that His57 is not the titrating group in the cmk−serine protease complexes that were the subjects of the original ^{13}C NMR studies.

DISCUSSION

Active Site Chemistry in cmk−Protease Complexes. The results presented here show that catalytic histidine residues in cmk complexes of α-lytic protease, trypsin, and chymotrypsin do not titrate. They remain essentially fully protonated, positively charged, and H-bonded to Asp102 over the entire pH range. This rules out ionization of His57 as the explanation for the pH dependence of the ^{13}C signal of the hemiketal carbon atom reported for cmk complexes of trypsin and chymotrypsin. The results also show that, although His 57 does not itself titrate, its ring nitrogen atoms and the proton in the Asp-His H-bond respond to a titrating functional group with a pK_a very similar or identical to that observed in the ^{13}C NMR studies. Thus, the present results strongly support the conclusion of Malthouse and co-workers that the hemiketal group in cmk−enzyme complexes ionizes to the oxyanion with pK_a values in the range of 7.0−9.0 (Finucane et al., 1989; Finucane & Malthouse, 1992; Malthouse et al., 1983; Malthouse et al., 1985; O'Connell & Malthouse, 1995; Primrose et al., 1985; Scott et al., 1986).

Scheme 2: Possible Ionization States and Microscopic pK Values for the Hemiketal Oxygen in cmk−Serine Proteinase Complexes



Scheme 2 shows the four possible ionization states and the corresponding microscopic pK_a's for a cmk−alkylated histidine. The pK_a values of 8.7 and 7.9 previously reported for the cmk complexes of chymotrypsin and trypsin and the pK_a value reported here of 7.5 for the cmk complexes of α-lytic protease correspond to pK_2 of Scheme 2, which is for the ionization of the hemiketal hydroxyl to the oxyanion in the presence of a protonated imidazole ring. On the basis of model system studies and free energy calculations, Finucane and Malthouse (1992) have estimated pK_2 to be ∼10.7 for the system free in aqueous solutions and thus in the absence of specific stabilizing interactions from an enzyme. This indicates that chymotrypsin, trypsin, and α-lytic protease lower pK_2 by ∼2, 2.8, and 3.3 pK_a units respectively, relative to water, which corresponds to stabilization energies of ∼2.7, 3.8, and 4.5 kcal/mol for the three enzymes, respectively.

What Stabilizes the Oxyanion in cmk Complexes? The first and most obvious answer to this question is the oxyanion hole. Since it was first noted in early crystallographic studies of serine proteases (Robertus et al., 1972), the oxyanion hole has been widely believed to contribute importantly to catalytic rate accelerations by stabilizing the oxyanion formed during catalysis.

Gerlt and Gassman (1993) have in fact recently proposed that the oxyanion hole by itself accounts for essentially all of the catalytic rate acceleration mediated by serine proteases. They have proposed that the hydrogen bonds formed between the NH groups of Ser195 and Gly193 and the oxyanion are examples of a newly discovered type of hydrogen bond termed a low-barrier hydrogen bond (LBHB). Such LBHBs have been proposed to be extraordinarily strong, ranging in energy from 12 to 24 kcal/mol. Thus, even a substantially suboptimal interaction between the oxyanion of a cmk-inhibited protease and the oxyanion hole could account for the magnitude of the depression of the pK_a if Gerlt and Gassman's proposal is correct.

Lower estimates of the stabilizing power of the oxyanion hole come from Warshel and co-workers (Warshel et al., 1989). They estimated, on the basis of theoretical calculations, that the oxyanion hole contributes ∼5−6 kcal/mol to the lowering of the activation energy on the basis of theoretical calculations. These estimates refer to energies over and above what would be supplied by solvent water. Warshel and co-workers argued that the oxyanion hole is more effective than solvent water in stabilizing the oxyanion because it acts as a prealigned dipole. In effect, the folding energy of the protein is used to pay the energetic cost of aligning the dipoles of the oxyanion hole. This then avoids energy losses owing to "solvent reorganization" associated with the polarization of solvent water molecules around newly formed charges. Warshel and co-workers' estimate of the stabilizing power of the oxyanion hole, although much less than that of Gerlt and Gassman's, is still sufficient to account for the pK_a reductions observed for the hemiketal hydroxyl group in cmk complexes.

Obtaining an estimate of the actual contribution of the oxyanion hole to catalysis experimentally is difficult for the trypsin family of proteolytic enzymes because in this family the oxyanion hole is composed of backbone N−H groups. However, in the subtilisin family the oxyanion hole is composed of the side chain NH_2 of Asn155,[3] and the OH of Thr220, in addition to the backbone NH of Ser221, making an experimental estimate of the contribution of at least Asn155 and Thr 220 possible through site-specific mutagenesis. Replacement of Asn155 reduces k_{cat} by factors ranging from ∼150 to ∼300 depending on the substitution (Bryan

[3] The subtilisin BPN′ numbering system is used when specifying the residues of the members of the subtilisin family.

et al., 1986; Carter & Wells, 1990). Substitution of Thr220 results in a ~20-fold reduction in k_{cat}, which is additive with Asn155 substitutions (Braxton & Wells, 1991). In both cases the K_m is not much affected, which supports the notion that these residues primarily function in stabilizing the transition state. Thus, oxyanion mutagenesis experiments indicate that the combined contribution of Asn155 and Thr220 to oxyanion stabilization in subtilisin is ~5−6 kcal/mol. This value seems to rule out the low-barrier hydrogen bond hypothesis (Gerlt & Gassman, 1993). However, it does agree well with Warshel and co-workers' theoretical calculations (1989), and roughly with the hemiketal hydroxyl group pK_a effects. The agreement with the latter indicates that the oxyanion hole is at least capable of providing the amount of stabilization reflected in the depressed hemiketal hydroxyl group pK_a but it does not prove that the oxyanion hole accounts for the depressed pK_a.

Role of the Asp-His Diad. Finucane and Malthouse have argued that the oxyanion hole does not contribute to lowering the hemiketal hydroxyl pK_a to a value less than would be achieved by water (Finucane et al., 1989; Finucane & Malthouse, 1992; Malthouse et al., 1985). They interpret the site-specific mutagenesis experiments as indicating that the stabilization provided by each oxyanion N−H is very similar to that expected for an ordinary hydrogen bond, not one involving a prealigned dipole or a low-barrier hydrogen bond. They propose instead that the oxyanion hole is responsible for lowering the oxyanion pK_a to a value similar to that expected in water and that the additional stabilization is provided by the electrostatic interaction between the protonated imidazolium ion of His57 and the oxyanion.

The nearby presence of a protonated imidazolium ion cannot by itself account for the stability of the hemiketal oxyanion. This is demonstrated by the model system studies which have shown that the pK_a for the ionization of the hemiketal hydroxyl in the presence of a protonated imidazolium ion (pK_2 of Scheme 2) in water is 10.7 (Finucane & Malthouse, 1992). Furthermore, these same model systems studies have shown that, in the presence of the hemiketal hydroxyl group, the imidazolium ion deprotonates with a pK_a of 6.7 (pK_1, Scheme 2), four pK_a units lower than for ionization of the hemiketal hydroxyl. Thus, in aqueous solutions, structure **2** of Scheme 2 predominates over structure **3** by 4 orders of magnitude. This contrasts sharply with the situation obtained within the confines of a serine protease active site where **3** greatly predominates over **2**. Clearly, α-lytic protease, trypsin, and chymotrypsin strongly stabilize the zwitterionic form **3** relative to the neutral form **2**. Finucane and Malthouse (1992) estimated these enzymes stabilize structure **3** over **2** by over 10 000-fold relative to water, which corresponds to 5.5 kcal/mol of stabilization energy.

Finucane and Malthouse (1992) noted that in order for the zwitterionic tetrahedral adduct, **3** (Scheme 2), to predominate over the neutral form, **2**, the pK_a of the imidazolium cation, of structure **1**, pK_1, must be higher than that of hemiketal hydroxyl group, pK_2. This means that the stability of the imidazolium ion cannot be attributed to electrostatic interaction with the negatively charged oxyanion as this species is already unusually stable in the presence of the neutral hydroxyl form. Thus, the enzyme itself must be conferring an unusual stability on the imidazolium ion in these complexes, and this in turn could underlie, at least in part, the seemingly unusual stability of the oxyanion. The most likely other source of imidazolium ion stabilization is the carboxylate of Asp102. Thus, the decrease in pK_2 in cmk−enzyme complexes may be attributable, indirectly, to Asp102.

Frey et al. (1994) have recently proposed that the Asp-His hydrogen bond becomes a low-barrier hydrogen bond on formation of the transition-state or tetrahedral intermediate during catalysis and that this hydrogen supplies much of the transition state stabilization in catalysis. Low-barrier hydrogen bonds, according to the current working hypothesis, are identified by chemical shifts of ~16 ppm or more for the proton in the hydrogen bond. The Asp-His proton resonates between ~17 and 18 ppm, depending on the protease, when His57 is protonated, and between ~13.8 and 15 ppm when His57 is neutral. An essential element of the proposal of Frey et al. is that the Asp-His hydrogen bond is not a low-barrier H-bond at catalytically active pH values, where His57 is neutral, but becomes one on protonation of the triad during catalysis, which is modeled by protonation of the triad on lowering the pH. The above working definition indicates that the Asp-His hydrogen bond may be low-barrier in cmk-inactivated complexes as the ^{1}H chemical shifts range from ~15.9 in α-lytic protease to ~16.7 in chymotrypsin (see Table 1). Thus, the formation of this low-barrier hydrogen bond should be considered as a potential contributor to the inhibitory potency of cmk's, and perhaps also to the depressed pK_a of the hemiketal oxyanion.

There are, however, several facts which are inconsistent with the idea that the Asp-His hydrogen bond becomes low-barrier on protonation of the triad. The first is that the ^{15}N chemical shifts of N$^{\delta}$1 and one-bond ^{1}H−^{15}N coupling constant, $^1J_{^{15}N^{\delta1}-^1H}$, of ~95 Hz in the protonated resting enzyme and in the cmk complex indicate the proton is essentially localized on N$^{\delta1}$ and not substantially shared between N$^{\delta1}$ of His57 and Asp102 as predicted by LBHB theory. Another is that these parameters do not indicate major differences in the character of the N$^{\delta1}$−H bond between the protonated and neutral forms of the triad in resting enzyme. Finally, the existence of a LBHB for the protonated form of the Asp-His diad but not for the neutral form suggests that the protonated Asp-His diad should be stabilized by ~12−24 kcal/mol relative to a free imidazolium ion and by ~5−18 kcal relative to an imidazolium ion stabilized by a normal hydrogen bond. The approximately "normal" pK_a of ~7.0 for the imidazolium ion of His57 seems inconsistent with such strong selective stabilization.

The ^{15}N$^{\delta1}$ chemical shift behavior of N$^{\delta1}$ of His57 indicates that the Asp-His H-bond though not a LBHB is nevertheless an unusually strong one as the H-bond induces an ~10 ppm downfield shift in the ^{15}N$^{\delta1}$ resonance position. That this interaction is important to catalysis is suppported by the ~10^4 decrease in activity observed for Asp102 mutants (Craik et al., 1987). It seems likely that this interaction contributes to cmk inhibition and to the depressed hemiketal pK_a. However, evaluating how much of the stabilizing effect of the Asp-His interaction should be assigned to hydrogen bonding versus electrostatics will be difficult.

Potential Usefulness of the Hemiketal pK_a. Finucane and Malthouse (1992) have proposed that the active sites of serine proteases have evolved to stabilize the zwitterionic form of tetrahedral intermediates and that such stabilization is an important factor contributing to catalysis. Whether the

oxyanion hole, the Asp-His diad, or both contribute to stabilizing this structure, the hemiketal hydroxyl group pK_a may provide some measure of the magnitude of this stabilization that could prove very useful in many types of mechanistic studies. For example, a well-known phenomenon is that elongation of a peptide substrate of a protease almost always results in an improved k_{cat} without much change in K_m (Fersht, 1985). This phenomenon has been interpreted as indicating that enzymes convert binding energy in the extended specificity subsites into catalytic power. The mechanism underlying the conversion of binding energy into transition-state stabilization is poorly understood. One possible mechanism is for occupancy of the specificity subsites to induce a conformational change in the enzyme which makes it better able to stabilize the tetrahedral oxyanion. The mechanism could involve a conformational change that enables the oxyanion hole to better stabilize the oxyanion or one that allows a better alignment of the Asp-His diad which then stabilizes the oxyanion.

If the hemiketal pK_a has the significance hypothesized, the above mechanism could be experimentally tested by determining how the hemiketal pK_a varies as the peptide chain of a cmk inhibitor is elongated. O'Connell and Malthouse (1995) have reported that the pK_a of the hemiketal is 0.5 pK_a units lower in a Z-Gly-Gly-Phe−cmk complex, than in the ZPCK complex of chymotrypsin, which suggests that such a mechanism may indeed be operating. On the other hand we have found essentially no difference in hemiketal pK_a between α-lytic protease complexes with a long and a short cmk (Table 1).

However, before using the hemiketal pK_a in such analyses it would be highly desirable to establish that it does indeed reflect enzyme-mediated transition-state stabilization as hypothesized. Fortunately, testing this hypothesis is now experimentally possible. Both the Asn155 mutant of subtilisin (R. M. Day, C. A. Kettner, and W. W. Bachovchin, unpublished observations) and the Asp102 mutants of subtilisin and trypsin family enzymes form complexes with cmk inhibitors (Craik et al., 1987). The behavior of the hemiketal hydroxyl pK_a in these mutant enzyme complexes should reveal the degree to which the hemiketal pK_a reflects stablization from the oxyanion hole versus from the Asp-His diad. Such experiments are now in progress.

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BI9513968