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^{13}C NMR Study of the Ionizations within a Trypsin–Chloromethyl Ketone Inhibitor Complex†

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Received November 15, 1984

ABSTRACT: ^{13}C NMR is used to detect ionizations within a trypsin–chloromethyl ketone inhibitor complex. The pK_a values observed are compared with those predicted by free-energy relationships. For the denatured/autolyzed inhibitor complex, a $pK_a = 5.26$ is observed, which is assigned to the ionization of the imidazole of histidine-57. For the intact inhibitor complex a $pK_a = 7.88$ is determined. This pK_a is assigned to the ionization of the hemiketal hydroxyl ($pK_a = 7.88$–8.1) and provides the first direct evidence that the serine proteases are able to stabilize the oxyanion of tetrahedral adducts. Indirect evidence is adduced that the imidazole pK_1 of histidine-57 is ≥ 8.1. Line-broadening studies suggest that there may be extra fast exchange line broadening, which could result from rapid tautomeric exchange between neutral and zwitterionic species within the inhibitor complex. The significance of these results for the catalytic mechanism of serine proteases is discussed.

It is generally assumed that proteolysis reactions catalyzed by the serine proteases proceed via tetrahedral addition compounds formed prior and subsequent to the acylation and deacylation reactions. Although a tetrahedral intermediate (THI)[1] should not accumulate during catalysis (Mackenzie et al., 1984), its stabilization could account for much of the catalytic efficiency of proteases. Indeed, recent kinetic studies suggest that transition-state stabilization of the oxyanion of a THI is essential for the serine proteases (Asboth & Polgar, 1983). Considerable effort has therefore been exerted in the development of transition-state analogues that will mimic such intermediates in the hope that such studies will provide evidence of how enzymic stabilization of the THI is achieved.

Hydrogen bonding of the oxyanion of the THI was one of the first mechanisms to be proposed (Henderson, 1970). Stabilization of the oxyanion by interaction with cationic histidine-57 was first discussed by Caplow (Caplow, 1969; Lucas et al., 1973), who suggested that such an interaction would lower the pK_a of histidine-57. Considerable evidence

† This work was supported by the National Institutes of Health (GM32596).
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[1] Abbreviations: Z-Lys-CMK, 1-chloro-3-(carbobenzyloxyamino)-7-aminoheptan-2-one; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; LEFS, uniform-field linear electric field shift; LB, line broadening; FID, free-induction decay; NMR, nuclear magnetic resonance; T, tesla; THI, tetrahedral intermediate.

0006-2960/85/0424-3478$01.50/0

has now accumulated to show that negatively charged species bound at serine-195 raise the pK_a of histidine-57 (Robillard & Shulman, 1974a,b; Markley et al., 1980; Kossiakoff & Spencer, 1981). It has been suggested that catalysis cannot proceed via a THI if the histidine pK_a is not raised (Komiyama & Bender, 1979).

^{1}H NMR has provided evidence that specific aldehyde inhibitors form stable hemiacetals with serine proteases (Wyeth et al., 1980; Lowe & Nurse, 1977; Chen et al., 1979), while ^{13}C NMR has enabled the direct observation of both hemiketals (Malthouse et al., 1983) and hemiacetals (Shah et al., 1984) with trypsin and chymotrypsin, respectively, when these enzymes are inhibited with site-specific carbonyl reagents.

However, all of these inhibitors readily form stable sp^3-hybridized hydrates in solution. Fastrez (1983) has used free-energy relationships to calculate that the pK_a values of such enzyme hemiacetals will be essentially the same as expected for aqueous hydrates. Since it is the oxyanion and not its conjugate acid that should be the catalytically relevant species (see Discussion), it would appear that the studies to date may have minimal relevance to the catalytic process per se.

Chloromethyl ketone inhibitors of specific substrates are potent irreversible inhibitors of serine proteases, alkylating N-3 of the imidazole of histidine-57 (Coggins et al., 1974; Shaw & Springhorn, 1967). In an earlier paper (Malthouse et al., 1983), it was shown that a tetrahedral adduct formed in a trypsin–Z-Lys-CMK inhibitor complex could be observed directly in a ^{13}C NMR experiment.

In the present study, we examine the ionizations within this inhibitor complex for both intact and denatured/autolyzed inhibitor complex. We provide the first direct evidence that trypsin can stabilize the formation of an anionic hemiketal and offer indirect evidence that the pK_a of the imidazole of His-57 is raised in the inhibitor complex. The pK_a values observed are compared with those predicted by free-energy relationships, thereby revealing the extent to which these pK_as are perturbed within the enzyme–inhibitor complex.

Materials and Methods

Materials. Trypsin (type III, twice crystallized, salt free, from bovine pancreas) was obtained from Sigma Chemical Co. Chromatographic analysis (Schroeder & Shaw, 1968) determined that the trypsin used (lot 103F 8075) was 85% β-trypsin (92% fully active enzyme) and 15% α-trypsin (92% fully active enzyme). Commercial trypsin (lot 103F 8075, ≈82% fully active enzyme) was used without further purification.

SP-Sephadex C-50 and Sephadex G-25 were obtained from Pharmacia. [1-^{13}C]-L-lysine hydrochloride (99 atom %) was obtained from Merck Sharp & Dohme (lot 2320-H).

Trypsin Solutions. Protein concentrations were determined by using ϵ_{280}^{1cm} = 36960 M^{-1} cm^{-1} (calculated using $\epsilon_{1\%}^{1cm}$ = 15.4 at 280 nm and M_r 24 000; Keil, 1971). The concentration of fully active enzyme was determined by titration with *p*-nitrophenyl *p*-guanidobenzoate (Chase & Shaw, 1967).

Inhibition of Trypsin by [2-^{13}C]Z-Lys-CMK. [2-^{13}C]Z-Lys-CMK was added to trypsin at pH 7.0 in 20 mM phosphate buffer. The extent of inhibition was determined by removing aliquots and assaying with N^{α}-benzoyl-DL-arginine-*p*-nitroanilide at pH 7.0 and 0.1 M phosphate buffer as described by Malthouse & Brocklehurst (1976). Low molecular weight components were then removed by gel filtration using Sephadex G-25 and 2 mM HCl as eluant. The inhibited trypsin retained less than 1% of its activity toward N^{α}-benzoyl-DL-arginine-*p*-nitroanilide.

For NMR experiments the inhibited trypsin solutions were concentrated by ultrafiltration using an Amicon PM10 membrane. It was assumed that the extinction at 280 nm was the same as that for native trypsin. Distilled, deionized water was used in all cases, and the enzyme–inhibitor complex was in all cases subjected to gel filtration to remove any low molecular weight contaminants.

pH Titrations and pH Measurement. pH measurements were made either with a Radiometer combination electrode (GK 2401C) or by using a Beckman combination electrode (No. 39522). pH titrations were carried out with Hamilton syringes with micrometer attachments, which allowed small volumes (less than 10 μL) of acid (0.875 M HCl, 12.5% v/v D_2O) to be added slowly and accurately with continuous stirring. pH changes during measurement of chemical shifts and line widths were ≤0.02 pH unit. The line widths of buffer resonances were used as internal references and were constant (±1 Hz) during pH titrations, confirming that changes in line width were not due to changes in sample homogeneity. pH data were fitted to the appropriate equation by a computerized nonlinear least-squares regression program (Wilkinson, 1961; Cleland, 1967).

NMR Spectra. Spectra at 7.045 T were recorded on a Bruker WM300 wide-bore spectrometer (sample size 8–10 mL) while spectra at 1.868 T were recorded on a Varian FT-80 spectrometer (sample size 1.2–2 mL). Chemical shifts are reported relative to tetramethylsilane at 0 ppm. For samples that did not contain D_2O, a concentric 5-mm tube containing D_2O was used to obtain the deuterium lock signal. Spectral conditions at 7.045 T were 8000 time-domain data points, 22-μs pulse width (112 μs = 90° pulse), ~0.25-s acquisition time and ~220 ppm spectral width, 10–20-Hz exponential weighting factor (line broadening), and low-power noise decoupling (0.4 W). At 1.868 T, spectral conditions were 4K data points, 9-μs pulse width (12.5 μs = 90° pulse), 0.5-s acquisition time and 200 ppm spectral width, 2–10-Hz exponential weighting factor, and 1.0-W decoupler power.

Synthesis of [2-^{13}C]Z-Lys-CMK. The bis-protected derivative of [1-^{13}C]lysine hydrochloride, Z-(Boc)[1-^{13}C]Lys (Mackenzie et al., 1985), was converted to [2-^{13}C]Z-Lys-CMK following literature procedures (Coggins et al., 1974).

Results

pH Titration. For a single ionizing group (AH)

$$AH \overset{K_a}{\rightleftharpoons} A^- + H^+$$

the pH-dependent changes in the chemical shift are given by eq 1 where P_{AH} and P_{A^-} are the populations of AH and A$^-$,

$$\delta_{obsd} = P_{AH}S_1 + P_{A^-}S_2 \quad (1)$$

respectively, at a given pH. S_1 and S_2 are the chemical shifts of species AH and A$^-$, respectively.

For singly ionizing systems, chemical shift data were fitted to eq 2, which is derived from eq 1. This equation is valid for

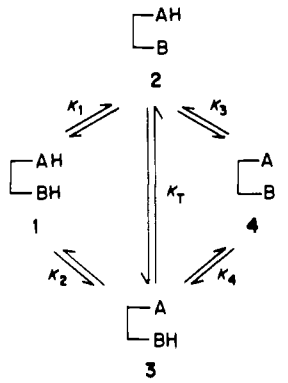
$$\delta_{obsd} = \frac{S_1 + S_2K_a/[H]}{1 + K_a/[H]} \quad (2)$$

fast exchange provided broadening does not exceed $\Delta\nu_{AB}/6$ (where $\Delta\nu_{AB}$ = frequency difference between S_1 and S_2; Sudmeier et al., 1980).

For a doubly ionizing system as shown in Scheme I, pH-dependent changes in chemical shift are related to the macroscopic ionization constants K_I and K_{II} by eq 3. S_1 and S_3

$$\delta_{obsd} = \frac{S_1[H]^2 + S_2K_I[H] + S_3K_IK_{II}}{[H]^2 + K_I[H] + K_IK_{II}} \quad (3)$$

Scheme I[a]



[a] $K_T = [2]/[3] = K_1/K_2; K_I = K_1 + K_2; K_{II} = K_4K_3/(K_4 + K_3)$.

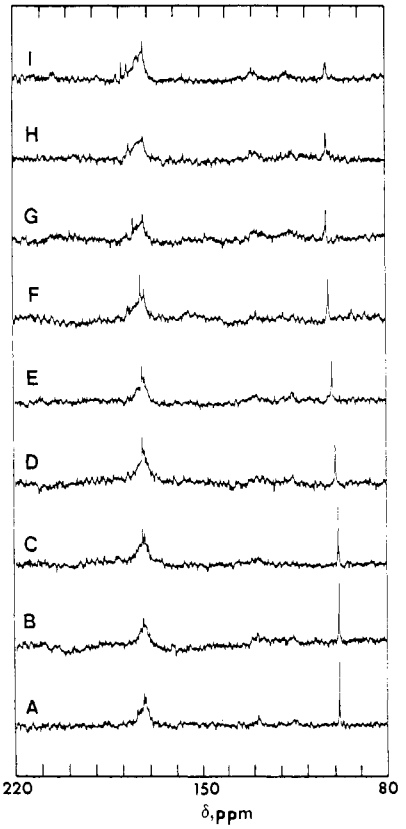


FIGURE 1: Effect of pH on the ^{13}C NMR spectra (80–220 ppm) of the intact [2-^{13}C]Z-Lys-CMK–trypsin inhibitor complex. Conditions: concentration of inhibited trypsin 0.53 mM, activity toward BAPA 0.4%, volume 8 mL, 20 mM glycine, and 20 mM calcium chloride. Sample contained no D_2O. Spectrometer was locked on D_2O (0.5 mL) contained in a 5-mm insert. Number of accumulations = 5000. pHs were 3.75, 5.62, 6.77, 7.51, 7.95, 8.6, 9.53, 9.98 and 10.47 for spectra A–I, respectively.

are the chemical shifts of species **1** and **4**, respectively, while S_2 is the average chemical shift of species **2** and **3**. Again this assumes that the system is undergoing fast exchange.

pH Titration of the Intact Z-Lys-CMK–Trypsin Inhibitor Complex. On increasing the pH the signal at ≈98 ppm moved downfield and decreased in intensity (Figure 1). Spectra A, C, E, G, and I were obtained consecutively on raising the pH while spectra H, F, D, and B were obtained consecutively on lowering the pH. This demonstrates that the pH-dependent changes in chemical shift and peak intensity are reversible and are not due to irreversible denaturation. The decreased intensity of the signal due to the ^{13}C-enriched carbon was due to its increase in line width with increasing pH. Similar pH-dependent changes in line width and chemical shift were observed when 50 mM Tris was substituted for 20 mM glycine (Figure 2). In the absence of 20 mM $CaCl_2$, both the chemical shift and intensity underwent similar pH-dependent changes. However, the pH titration was only partially reversible with a new resonance at 205.6 ppm appearing at pH values >7 and remaining when the pH was lowered below 7, while the intensity of the resonance at ≈98 ppm was decreased in intensity. Although alkylation of trypsin by Z-Lys-CMK reduced the activity by at least 99%, the remaining activity (0.3–0.8%) was sufficient to cause significant autolysis and consequent irreversible denaturation at alkaline pHs. Calcium chloride reduces autolysis at alkaline pHs (Nureddin & Inagami, 1969), and its incorporation into samples prevented significant autolysis during pH titrations (Figure 1).

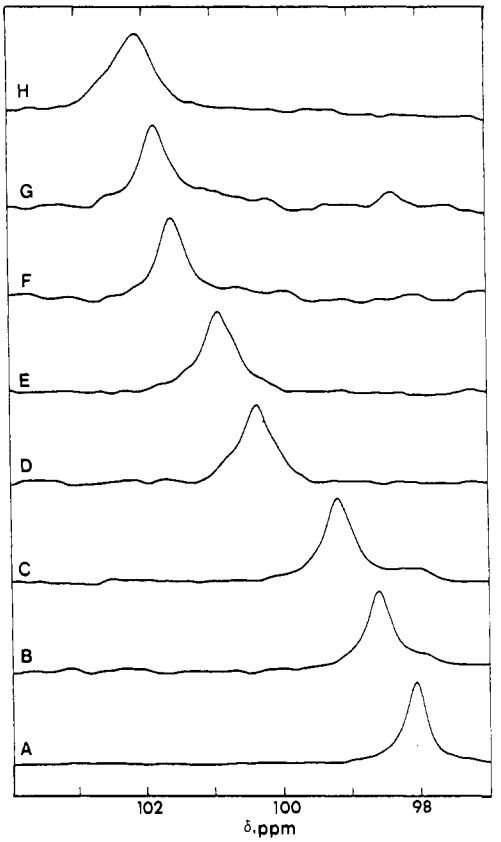


FIGURE 2: ^{13}C NMR spectra (97–104 ppm) of the pH titration shift of the ^{13}C-enriched carbon in the intact [2-^{13}C]Z-Lys-CMK–trypsin inhibitor complex. Conditions: concentration of inhibited trypsin 0.97 mM, activity toward BAPA 0.8%, volume 8 mL, 50 mM Tris, 20 mM calcium chloride, 12.5% v/v D_2O. Number of accumulations: B–G, 10 000; A, 50 000; H, 60 000. pHs were 6.44, 7.19, 7.47, 8.03, 8.29, 8.77, 9.14, and 9.86 for spectra A–H, respectively.

All the chemical shift data shown in Figure 3 were fitted to eq 2, which gave $pK_a = 7.882 \pm 0.014$, $S_1 = 97.95 \pm 0.01$ ppm, and $S_2 = 102.08 \pm 0.04$ ppm. The theoretical line (Figure 3) was generated from these parameters and eq 2. The experimental data therefore are consistent with a single ionizing group, $pK_a = 7.88$, affecting the chemical shift from pH 3.68 to pH 10.5. No change in chemical shift could be detected even at pH 2.8 (data not shown).

pH Titration of the Autolyzed/Denatured Z-Lys-CMK–Trypsin Inhibitor Complex. Trypsin partially inhibited (88%) by [2-^{13}C]Z-Lys-CMK was prepared and separated from low molecular weight materials as described under Materials and Methods. The sample that retained 12% activity toward BAPA was incubated at pH 9.11 for 11 h (20 °C). The intensity of the resonance at 101.8 ppm decreased, and two new resonances were detected, one at 205.6 ppm and the other

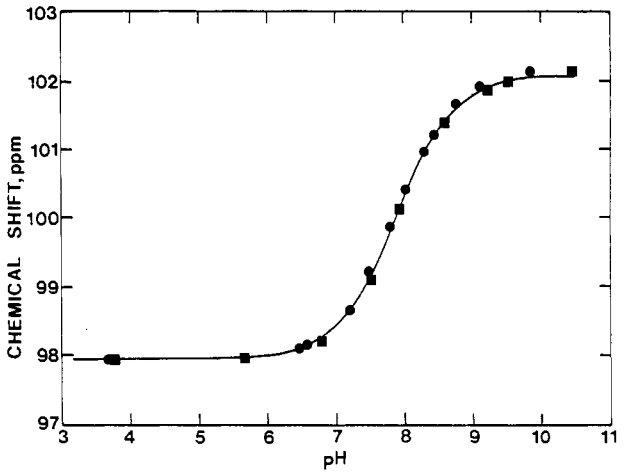


FIGURE 3: Effect of pH on the chemical shift of the ^{13}C-enriched carbon in the intact [2-^{13}C]Z-Lys-CMK–trypsin inhibitor complex. Conditions: (●) 20 mM $CaCl_2$, 50 mM Tris, and 12.5% v/v D_2O (see Figure 2 for further experimental details). (■) 20 mM $CaCl_2$ and 20 mM glycine (see Figure 1 for further experimental details). Experimental data were fitted to eq 2. The solid line was calculated from eq 2 and the fitted parameters $pK_a = 7.882 \pm 0.014$, $S_1 = 97.950 \pm 0.013$, and $S_2 = 102.081 \pm 0.039$.

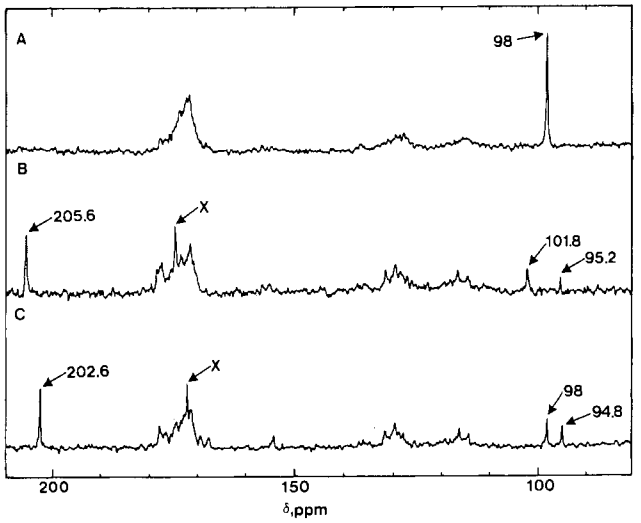


FIGURE 4: ^{13}C NMR spectra of the [2-^{13}C]Z-Lys-CMK–trypsin inhibitor complex ketone and after denaturation/autolysis: (A) protein concentration 0.36 mM, activity toward BAPA 12%, pH 3.24, TV = 10 mL, number of accumulations 161 615; (B) protein concentration 0.36 mM, pH 9.11, TV = 10.25 mL, number of accumulations 80 000 after 11 h incubation at pH 9.11; (C) protein concentration 0.35 mM, pH 3.75, TV = 10.55 mL, number of accumulations 190 000. Spectra A–C contained 12.5% v/v D_2O. The chemical shifts shown are in ppm. The resonance X is due to the carboxyl carbon of glycine.

at 95.2 ppm (Figure 4). The chemical shifts of all these resonances were pH dependent (Figures 4–6). The signal at 205.62 ppm moved upfield with decreasing pH to 202.64 ppm with a $pK_a = 5.26$ (Figure 7). A similar but much smaller upfield shift (95.2 to 94.8 ppm) was observed for the other resonance.

Model Compounds and Magnitude of Imidazole Titration Shifts. For amines, the ^{13}C titration shift of the β-carbon (β-C) is often large (4–5 ppm) for methylene groups and approximately 2 ppm for a β quaternary carbon (Batchelor et al., 1975). In order to assess whether the titration shifts observed for the enzyme adducts reflect ionization of imidazole-57, several model compounds were synthesized. The pK_a values and titration shifts of the β-carbons (^{13}C-7) of these N-substituted imidazoles along with the β-^{13}C titration shifts of some amine analogues are shown in Table I.

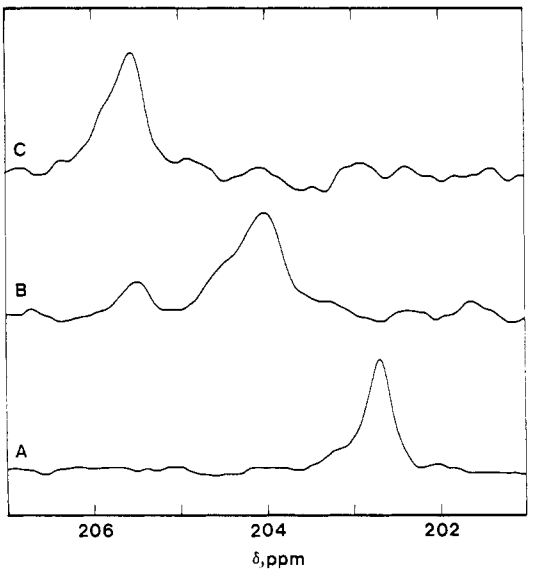


FIGURE 5: ^{13}C NMR spectra (201–207 ppm) of the denatured/autolyzed inhibitor complex (see Figure 4 for experimental details): (A) pH 3.43, number of accumulations 103 575; (B) pH 5.27, number of accumulations 240 000; (C) pH 9.10, number of accumulations 67 211.

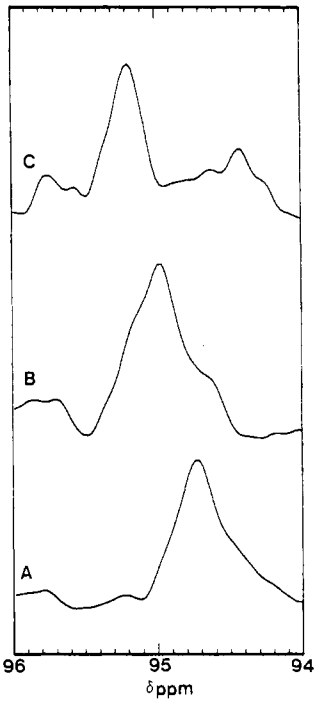


FIGURE 6: ^{13}C NMR spectra (94–96 ppm) of the denatured/autolyzed inhibitor complex (see Figure 5 for experimental details).

The carboxylate titration shifts of the β-C are similar in both N-substituted imidazoles and amines (e.g., compounds **4** and **8**, Table I), whereas the β-C titration shifts due to titration of the imidazole group are 3-fold (e.g., compounds **5** and **9**, Table I) to 5-fold (e.g., compounds **1** and **7**, Table I) smaller than those of the analogous amine. We predict therefore that the N-substituted imidazole analogue (compound **6**, Table I) of the neopentylamine (compound **10**, Table I) would, by simple analogy, be expected to have a titration shift of 0.3–0.6 ppm.

Replacement of a proton by a hydroxyl group (cf. compounds **1** and **2**, Table I) produced only a small increase (0.2 ppm) in titration shift. Therefore, both hydrates and hemiketals formed at C-7 of N-substituted imidazoles should also

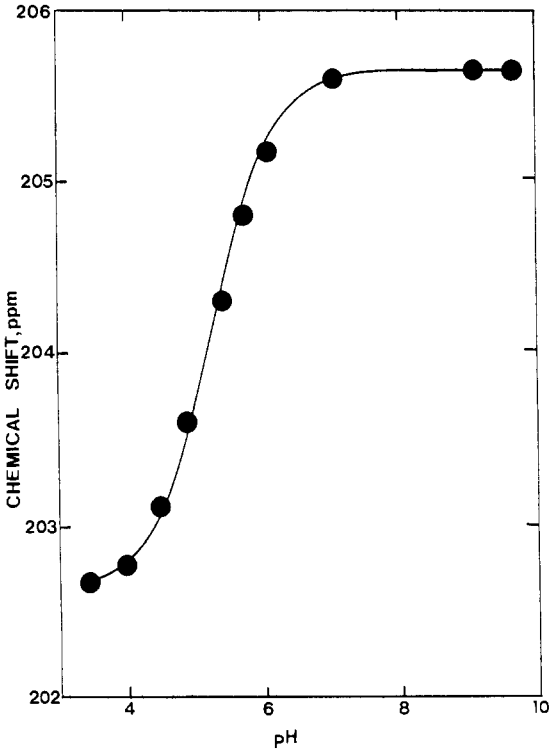


FIGURE 7: Effect of pH on the chemical shift of the ^{13}C-enriched carbon in the denatured/autolyzed [2-^{13}C]Z-Lys-CMK–trypsin inhibitor complex (see Figures 4 and 5 for experimental details). Experimental data were fitted to eq 2, and the solid line was calculated from eq 2 and the fitted parameters $pK_a = 5.258 \pm 0.004$, $S_1 = 202.639 \pm 0.002$, and $S_2 = 205.623 \pm 0.007$.

Table I: Model Compounds

R	no.	pK_a	Δppm,[a] β-C	no.	Δppm,[a] β-C[b]
		R—$C^{\alpha}H_2$—N(+)H			$^{+}H_3N$–$C^{\alpha}H_2R$
CH_3	**1**	7.3[c]	0.96 ± 0.06[h]	**7**	5.18[b,d]
CH_2OH	**2**	6.87 ± 0.08	1.16 ± 0.07[h]		
$COCH_3$	**3**	6.13 ± 0.06	3.32 ± 0.14[h]		
$COOH$[g]	**4**	2.06 ± 0.05	2.43 ± 0.07[g]	**8**	2.52[e,g]
COO^-[h]	**5**	6.92 ± 0.02[f]	3.23 ± 0.5[h]	**9**	9.68[b,e]
CH_3–C(CH_3)$_2$–CH_3	**6**			**10**	1.82

[a] Upfield shifts positive. [b] Titration shift due to conjugate acid of amine. [c] Elguero et al., 1968. [d] Batchelor et al., 1975. [e] Quirt et al., 1974. [f] pK_a determined from C-4 titration shift and pK_a from C-7 titration shift, 6.85 ± 0.15. [g] Titration of carboxylic acid. [h] Titration of imidazolium cation. [i] Except where specified, pK_a values and titration shifts were determined as described under Materials and Methods.

have small titration shifts (0.3–0.6 ppm).

Batchelor et al. (1975) have shown that ^{13}C titration shifts can be interpreted in terms of two components, the uniform-field linear electric field shift (LEFS) and the residual titration shift. A quaternary carbon with perfect tetrahedral symmetry shows no LEFS contribution to the observed titration shift. Therefore, subtraction of the residual titration shift obtained from a tetrahedral carbon with no directly bonded protons from that of the analogous compound with directly bonded protons gives a direct estimate of the LEFS contribution to the titration shift.

Positive (upfield) ^{13}C LEFS titration shifts are expected for all α-carbon (α-C) and β-C methyl or methylene resonances (Batchelor et al., 1975). While the α-C of amines often show large negative residual titration shifts, the β-C of both amines

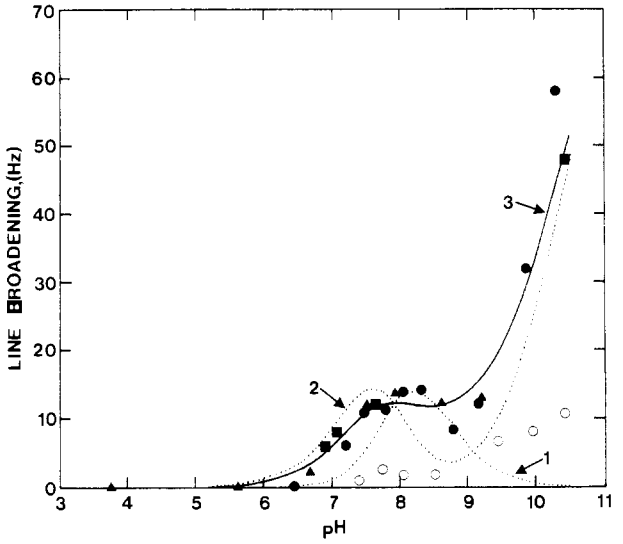


FIGURE 8: pH-dependent line broadening within the intact inhibitor complex at 7.045 T (●, ▲, ■) and at 1.868 T (○): (●) 20 mM $CaCl_2$, 50 mM Tris, 12.5% v/v D_2O, volume 8 mL, concentration of inhibited trypsin 0.97 mM, field strength 7.045 T; (▲) 20 mM $CaCl_2$, 20 mM glycine, 0% v/v D_2O, volume 8 mL, concentration of inhibited trypsin 0.53 mM, and field strength = 7.045 T; (■) 20 mM sodium phosphate, 0% v/v D_2O, volume 8 mL, concentration of inhibited trypsin 0.35 mM, and field strength 7.045 T; (○) 20 mM $CaCl_2$, 20 mM glycine, 12.5% v/v D_2O, volume 1.3 mL, concentration of inhibited trypsin 2.15 mM, and field strength 1.868 T. (Line 1) $\Delta\omega = P_A{}^2P_B{}^2 4\pi(\Delta\nu_{AB})^2/k_{ON}^{H^+}K_a)$, $k_{ON}^{H^+} = 9.54 \times 10^{11}$ M^{-1} s^{-1}, and $pK_a = 7.88$; (line 2) $\Delta\omega = P_A{}^2P_B 4\pi(\Delta\nu_{AB})^2/[K_B k_{ON}^{OH} + \omega_c/(1 + [H]/K_c)]$, $k_{ON}^{OH} = 1.66 \times 10^{10}$ M^{-1} s^{-1}, $pK_B = 6.12$, $pK_C = 10.2$, and $\omega_c = 70$ Hz; (line 3) $\Delta\omega = P_A{}^2P_B 4\pi(\Delta\nu_{AB})^2/[K_B k_{ON}^{OH} + P_B X + \omega_c/(1 + [H]/K_C)]$, $k_{ON}^{OH} = 3 \times 10^{10}$ M^{-1} s^{-1}, $pK_B = 6.12$, $pK_C = 10.2$, $\omega_c = 60$ Hz, and $X = P_2P_3 4\pi(\Delta\nu_{AB})^2/k_E{}^* = 12$ Hz, where P_2 and P_3 are the populations of the neutral and zwitterionic species of Scheme IV, and $k_E{}^* = k_T + k_{-T}$, and $P_B = P_2 + P_3$.

and amino acids have positive residual titration shifts. Therefore, although a negative residual titration shift at the β-C of N-substituted imidazoles is possible, it appears unlikely.

We conclude therefore that, for a hydrate or hemiketal at C-7 (β-C) of an N-substituted imidazole, the imidazole titration shift will be equal to or less than approximately 0.6 ppm and titration shifts greater than 0.6 ppm cannot be ascribed solely to the ionization of the imidazole group.

pH-Dependent Changes in Line Width. The increases in line width with increasing pH at 20 MHz and 75 MHz are shown in Figure 8. Since fast exchange broadening is directly proportional to the square of the field strength, then the line broadening below pH 8.5 is predominantly due to fast exchange broadening. At higher pHs there is a 5–6-fold increase in line width at both field strengths that cannot be attributed to fast exchange broadening. Since the increases in line width are reversible (Figure 1), they cannot be attributed to irreversible denaturation. A 5–6-fold increase in rotational correlation time at high pH can be excluded by the fact that the line width of the C-1 methylene (Primrose, 1984) of the inhibitor increased from 50 to 70 Hz at pH 9.5 and not from 50 to ≈300 Hz (line width $\propto \tau_R$). Slow exchange broadening is field independent and can be also excluded at high pH as equal line broadening would be expected at both field strengths. Conformational changes in trypsin resulting from titration of the aspartic acid-195–N-terminal isoleucine ion pair ($pK_a \simeq$ 10.1; Spomer & Wooton, 1971; Wang & Carpenter, 1968; Huber & Bode, 1978) have been observed at high pHs while increases in K_m values at high pHs have been attributed to deprotonation of lysine substrates. These effects would result in different conformers, each having slightly different chemical

shifts producing line broadening directly proportional to the field strength. The increase in line width at pHs > 8.5 is consistent with a p$K_a \approx 10.2$, although the rate of increase with pH does appear greater than expected for a single ionizing group (Figure 8, curves 2 and 3) as might be expected for a positively cooperative conformational change.

Sudmeier et al. (1980) have pointed out that in order to avoid buffer catalysis, exchange rates should be measured in the absence of buffers. This is, however, not practical as pH instability, especially at the pK_a, will produce large errors in line width; e.g., in the present study a pH shift of 0.1 pH unit at the pK_a gives an apparent line broadening of 20 Hz. In this work, three different buffer systems were used, and line broadening was independent of the type of buffer and its concentration (Figure 8), implying no significant buffer catalysis.

In the absence of buffer catalysis, there are two mechanisms, a and b, for deprotonation of an ionizing group:

mechanism a

$$AH + H_2O \underset{k_{ON}^{H^+}}{\overset{k_{OFF}^{H^+}}{\rightleftharpoons}} A^- + H_3O^+$$

mechanism b

$$AH + {}^-OH \underset{k_{OFF}^{-OH}}{\overset{k_{ON}^{-OH}}{\rightleftharpoons}} A^- + H_2O$$

For ionizing groups with pK_a values less than 7, mechanism a will normally predominate, while if the pK_a is greater than 7, mechanism b should dominate. If p$K_a \approx 7$, then both mechanisms can occur (cf. ionization of imidazole; Eigen, 1964).

The fast exchange broadening is given by eq 4,[2] where $\Delta\omega$

$$\Delta\omega = \frac{P_A P_B}{k_{OFF} + k_{ON}} 4\pi(\Delta\nu_{AB})^2 \qquad (4)$$

= fast exchange broadening (Hz), $P_A = [AH]/([A^-] + [AH])$, $P_B = [A^-]/([A^-] + [AH])$, $\Delta\nu_{AB}$ is the frequency difference (Hz) between the exchanging species, and k_{OFF} and k_{ON} are the first-order rate constants defined in mechanisms a and b. At the pK_a, $P_A = P_B$, $k_{OFF} = k_{ON}$, and $k_E = k_{ON} + k_{OFF}$. k_E can be calculated from the fast exchange broadening at the pK_a with eq 5. From the 12-Hz line broadening at the pK_a,

$$k_E = \pi(\Delta\nu_{AB})^2/\Delta\omega \qquad (5)$$

$k_E = 2.52 \times 10^4$ s^{-1}. If mechanism a predominates, $k_{OFF}^{H^+} = k_E/2 = 1.26 \times 10^4$ s^{-1} and $k_{ON}^{H^+} = k_{OFF}^{H^+}/K_a = 9.54 \times 10^{11}$ M^{-1} s^{-1}. If mechanism b predominates, then $k_{OFF}^{-OH} = 1.26 \times 10^4$ s^{-1} and $k_{ON}^{-OH} = k_{OFF}^{-OH}/K_B = 1.66 \times 10^{10}$ M^{-1} s^{-1} (pK_B = 14 − pK_a).

If mechanism a is dominant, maximum line broadening occurs when pH = pK_a + 0.3, while if mechanism b predominates, maximum line broadening occurs when pH = pK_a − 0.3. If both mechanisms a and b contribute equally, then maximum line broadening occurs when pH = pK_a. For the fast exchange process (Figure 8) maximum line broadening occurred at pH 8 to pH 8.3, suggesting that mechanism a predominates. As $k_{ON}^{H^+} = 9.54 \times 10^{11}$ M^{-1} s^{-1} and the diffusion-controlled limit is approximately 10^{10} M^{-1} s^{-1} (Eigen, 1964), then the exchange process could not be solvent mediated. The line broadening below pH 7.9 is significantly greater than expected if $k_{ON}^{H^+} = 9.54 \times 10^{11}$ M^{-1} s^{-1} and mechanism a is dominant (line 1, Figure 8). Line 2 (Figure 8) was calculated by assuming mechanism b is dominant and $k_{ON}^{-OH} = 1.66 \times 10^{10}$ M^{-1} s^{-1}. The extra term $\omega_H/(1 + [H]/K_c)$ assumes that the broadening at high pHs (>9) is due to a process that is controlled by a pK_a = 10.2 with maximum line broadening of 70 Hz (ω_c, see legend of Figure 8). Even allowing for this contribution to the line width, the observed line widths are much greater than predicted from pH 8 to pH 8.75. A poor fit is still obtained if ω_c is set to a larger value, e.g., 175 Hz (not shown).

There must therefore be an additional fast exchange process occurring below pH 8.5. Line 3 (Figure 8) gives a reasonable fit to the experimental data and assumes that an additional fast exchange process is occuring. The additional term introduced is XP_B, where X = 12 Hz (maximum additional line broadening), $= P_2P_3 4\pi(\Delta\nu_{AB})^2/k_E$, where k_E is a pH-independent rate constant and P_2 and P_3 are the populations of the species in fast exchange (note, the ratio P_2/P_3 is assumed to be pH independent). Line 3 also assumes mechanism b predominates as expected for a pK_a = 7.88. This scheme is discussed in greater detail under Discussion.

Although a detailed line-shape analysis was not attempted for the resonance at 202.64–205.62 ppm, there is a definite reversible increase in line width when pH = pK_a (Figure 5). Using the line widths of 25 Hz (pH ≫ pK_a), 42 Hz (pH = pK_a), and 11 Hz (pH ≪ pK_a), we estimate that $k_{ON}^{H^+} = (4.8–8.7) \times 10^8$ M^{-1} s^{-1}. These values are 1 order of magnitude less than expected for imidazole (Eigen, 1964) and for a freely accessible enzyme histidine (Bachovchin & Switzman, 1983), which suggests that for the denatured/autolyzed enzyme–inhibitor adduct N-1 of the imidazole ring is hydrogen bonded and/or not freely available to solvent.

Prediction of pK_a Values. Linear free-energy relationships have been successfully used to predict pK_as of both acids and bases (Clark & Perrin, 1964; Barlin & Perrin, 1966). Such relationships are especially useful for estimating the pK_a values of reactive intermediates whose pK_a values cannot be measured directly. Several workers [e.g., Sayer & Jencks (1973), Fox & Jencks (1974), and Fastrez (1977)] have used free-energy relationships for the prediction of the pK_a values of neutral, anionic, cationic, and zwitterionic tetrahedral intermediates.

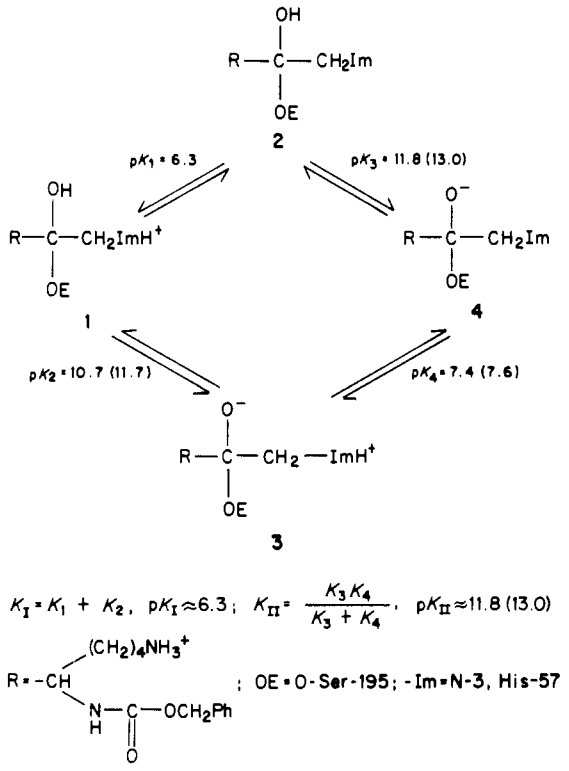
The pK_a values of ionizing groups on enzymes are often greatly perturbed relative to their values in aqueous solvents. Such perturbations can occur for many reasons, e.g., interaction with other ionizing groups, differences in dielectric constant, solvent accessibility, and changes in solvation. In order to assess the magnitude of such effects, the pK_a values of the groups must be estimated for the proposed species in fully aqueous media.

While substituent effects on the ionization of hydroxyl groups has been extensively studied [e.g., Ballinger & Long (1960), Takahashi et al. (1971), Fastrez (1977), and De Tar (1982)], relatively few studies have been made of N-substituted imidazoles. Collis & Edwards (1971) correlated pK_a with σ_I but used some compounds that would have significant resonance effects. Paiva et al. (1976) used dual-parameter fit and found resonance effects were small. In the structures we are examining, a methylene group is directly attached to the imidazole nitrogen, so that resonance effects can be neglected and correlations with σ_I used. pK_a values from Table I and other known pK_a values were correlated with σ_I (Figure 9). For *N*-acyl- and *N*-Phe-substituted imidazoles, pK_a values were corrected for resonance effects (see legend Figure 9). This gave the following correlation for the pK_a values of N-substituted imidazoles:

$$pK_a = -7.8\sigma_I + 6.9$$

[2] The basic equations and terms used to describe fast exchange broadening are described in more detail by Sudmeier et al. (1980).

Scheme II[a]



[a] (i) (pK_1) (a) $-CH(OH)_2$, $\sigma_I = 0.22$; $-CH(OR)_2$, $\sigma_I = 0.18$ (Exner, 1978); assume for $-CH(OH)(OR)$, $\sigma_I \approx 0.2$; assume for $CH_2CH(OH)(OR)$, $\sigma_I = 0.2/2.8$; $pK_1 = -7.8\sigma_I + 6.9$ (present work); $pK_1 = 6.3$. (b) OH, $\sigma_I = 0.25$; OR, $\sigma_I = 0.27$ (Exner, 1978); R, $\sigma_I = 0.13$ [Z-Lys, $pK_a^{COOH} = 3.27$ (present work)]; $ImCH_2CR_1R_2R_3$, $pK_1 = 0.997\Sigma\sigma_I + 7.14$ (present work); $pK_1 = 6.3$. (ii) (pK_2) (a) $-CH_2ImH^+$ [$pK_a^{COOH} = 2.06$ (present work)], $\sigma_I = 0.24$; $pK_2 = -7.39\Sigma\sigma_I + 15.472$ (De Tar, 1982); $pK_2 = 10.75$. (b) $pK_2 = 1.45\Sigma\sigma^* + 17.47$ (Fastrez, 1977); $\sigma^* = 6.23\sigma_I$; $pK_2 = 11.68$. (c) $pK_2 = -8.4\Sigma\sigma_I + 15.9$ (Fox & Jencks, 1974); $pK_2 = 10.52$. (iii) (pK_3) (a) Im, $\sigma^* = 1.6$ (Fastrez, 1977); $\sigma_I = 1.6/6.23$; $-CH_2Im$, $\sigma^* = 1.6/2.8$ and $\sigma_I = (1.6/6.23) \times 2.8$; $pK_3 = -7.39\Sigma\sigma_I + 15.472$ (De Tar, 1982); $pK_3 = 11.84$. (b) $pK_3 = -1.45\Sigma\sigma^* + 17.47$ (Fastrez, 1977); $pK_3 = 13.02$. (c) $pK_3 = -1.84\Sigma\sigma_I + 15.9$ (Fox & Jencks, 1974); $pK_3 = 11.77$. (iv) (pK_4) (a) $-O^-$, $\sigma_I = -0.16$ (Exner, 1978); $pK_4 = -0.997\Sigma\sigma_I + 7.14$; $pK_4 = 6.90$. (b) $pK_4 = pK_3 + pK_1 - pK_2$, using $pK_3 = 13.02$ and $pK_2 = 11.68$ (Fastrez, 1977); $pK_4 = 7.64$, using $pK_3 = 11.84$ and $pK_2 = 10.75$ (De Tar, 1982); $pK_4 = 7.39$. (v) pK_a values in parentheses were calculated from the free-energy relationship of Fastrez (1977); see (i). (vi) σ_I values were calculated from pK_a's of acetic acid derivatives with $\sigma_I + (4.712 - pK_a)/3.95$ (Charton, 1964) and a factor of 2.8 for the decrease in σ_I across a methylene group. For R, σ^* and σ_I were calculated from pK_a^{COOH} of Z-Lys with $\sigma^* = (4.644 - pK_a)/1.7$ (Takahashi, 1971); $\sigma_I = \sigma^*/6.23$.

This relationship and free-energy relationships for the hydroxyl group were used to calculate the expected pK_a values of the cationic (**1**), neutral (**2**), zwitterionic (**3**), and anionic (**4**) tetrahedral adducts formed within the trypsin–Z-Lys-CMK inhibitor adduct (Scheme II). These pK_a values are the values expected in the absence of perturbation by the enzyme. The microscopic and macroscopic pK_a values are shown in Scheme II, and the calculations are presented in detail in the legend to Scheme II.

Assignment of Resonances. (A) Denatured/Autolyzed Inhibitor Complex. The resonance at 202.64–205.62 ppm has a chemical shift characteristic of a ketone, and its titration shift (2.98 ppm) is similar to that (3.32 ppm) of the model compound ($CH_3COCH_2ImH^+$, compound **3**, Table I).

This resonance can therefore be unambiguously assigned to structures **5** and **8** (Scheme III), which are in fast exchange.

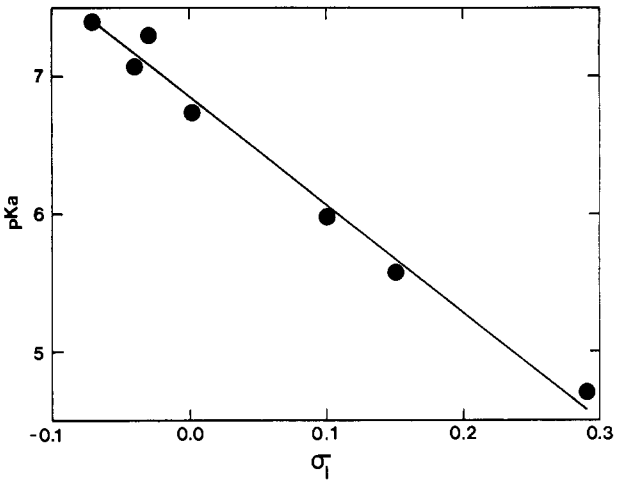
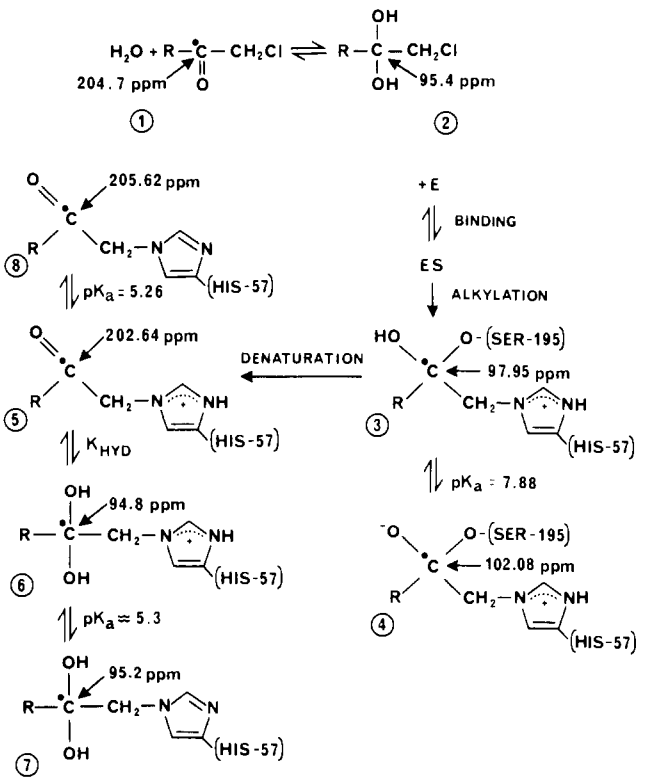


FIGURE 9: Plot of pK_a vs. σ_I for the ionization of N-substituted imidazolium ions. N-substituted imidazoles (substituent, pK_a, and σ_I): $-CH_3$, $pK_a = 7.06$ (Paiva et al., 1976), $\sigma_I = -0.04$ (Exner, 1978); $-C_2H_5$, $pK_a = 7.3$ (Elguero et al., 1968), $\sigma_I = -0.03$ (Exner, 1978); *tert*-butyl, $pK_a = 7.4$ (Elguero et al., 1968), $\sigma_I = -0.07$ (Charton, 1964); $-CH_2COCH_3$, $pK_a = 5.95$ (present work), $\sigma_I = 0.1$ (Exner, 1978); $-CH_2CH_2OH$, $pK_a = 6.74$ (present work), $\sigma_I = 0.05/2.8$ (Exner, 1978); $-C_6H_5$, $pK_a = 5.57$ [derived from $pK_a = 5.83$ (Elguero et al., 1968) using relationship of Paiva et al. (1976)], $\sigma_I = 0.15$ (Exner, 1978); Ac, $pK_a = 4.69$ [derived from $pK_a = 3.6$ (Goldberg et al., 1975) using relationship of Paiva et al. (1976)], $\sigma_I = 0.29$ (Exner, 1978). $pK_a = \rho\sigma_I + Q$, with fitted values $\rho = -7.82 \pm 0.44$ and $Q = 6.85 \pm 0.06$; correlation coefficient = 0.992. pK_a values were adjusted to zero ionic strength as described by Paiva (1976).

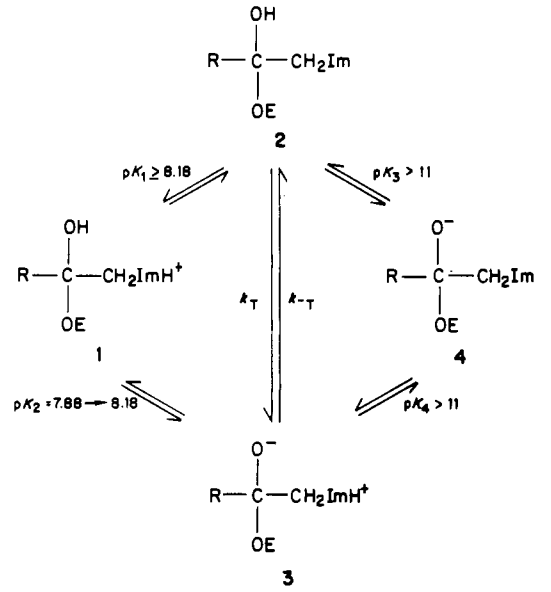
Scheme III



The pK_a value for the titration shift is assigned to the ionization of the imidazole ring of histidine-57. The pK_a value of 5.26 is lower than that of the model compound ($pK_a = 6.13$, compound **3**, Table I).

The resonance at 95 ± 0.2 ppm (species **6** and **7**, Scheme III) has a chemical shift very similar to the hydrates Z-Lys-CMK (species **2**, Scheme III). The pK_a of the hydrate (species **6** and **7**, Scheme III) is expected to be similar (+0.2 pH unit)

Scheme IV



to that of the nonhydrated species (species **5** and **8**, Scheme III); e.g., compare pK_a values of compound **3**, Table I, and the pK_1 of species **1**, scheme II. Although the small titration shift of the hydrate did not allow its pK_a to be determined accurately, the titration shift observed is consistent with a pK_a similar to that of the nonhydrated species. The small titration shift ($\leq$0.4 ppm) is also as predicted for hydrate (see Model Compounds under Results). Therefore, the resonances at 95 $\pm$ 0.2 ppm are assigned to structures **6** and **7** in Scheme III.

(*B*) *Intact Inhibitor Complex.* Only one resonance is detected (δ = 97.95–102.08 ppm) with no evidence for trigonal carbonyl geometry ($\delta \approx$ 205 ppm). This demonstrates that the ^{13}C-enriched carbon is stabilized as a tetrahedral species within the intact inhibitor complex.

It is not possible on the basis of chemical shift and pK_a values to discount the possibility that this resonance could result from aqueous hydration of the carbonyl of the covalently bound inhibitor. The X-ray crystallographic studies of subtilisin–chloromethyl ketone inhibitor complexes (Poulos et al., 1976) and the trypsin–Z-Lys-CMK inhibitor complex (Bode, private communication) however confirm that a tetrahedral hemiketal is formed between the hydroxyl of serine-195 and the inhibitor carbonyl. We shall hereafter assume a hemiketal structure (species **3** and **4**, Scheme III, and species **1**–**4**, Scheme IV). The assignment of the pK_a = 7.88 associated with this resonance is examined in detail under Discussion. It is important to point out that very similar pK_a values (within the 0.3 pH unit difference) resulting from the statistical factor of 2 are expected for the hydroxyl of the hemiketal or hydrate as the inductive effects at –OR and OH are essentially the same (OR, σ_I = 0.27; OH, σ_I = 0.25; Exner, 1978). If the pK_a = 7.88 is assigned to ionization of a hydroxyl directly bonded to the enriched carbon, then the pK_a of the anionic species must be lowered by 2–3 pH units in the enzyme adduct.

Discussion

The pK_a values of ionizing groups on enzymes are often perturbed relative to their values in low molecular weight compounds in aqueous solutions. These perturbations are difficult to assess and can arise from many factors, e.g., differences in solvation, dielectric constant, and entropic factors. In Scheme II we have used ab initio calculations to calculate both the macroscopic and microscopic pK_a values of the ionizing groups within the inhibitor complex in the absence of such perturbations. The calculations predict that the macroscopic pK_as, pK_I and pK_{II}, should be $\approx$6.3 and 11.8–13, respectively.

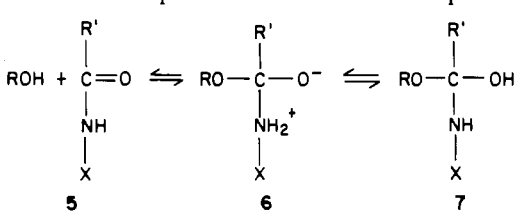
Since the experimentally observed pK_a = 7.88, it is clear that such calculations cannot accurately predict pK_a values within the inhibitor complex. They do, however, allow us to estimate the amount by which pK_a values are perturbed within the inhibitor complex.

Therefore, the experimentally observed pK_a = 7.88 could arise if pK_1 were raised $\approx$1.6 pH units and/or if pK_2 were lowered $\approx$2.8–3.8 pH units (Scheme II).

N-3 of the imidazole ring of histidine-57 is freely available to solvent (Steitz & Shulman, 1982). On alkylation of N-3, ionization of the imidazole would have to occur via the N-1 nitrogen of imidazole, which is less accessible to solvent. Therefore, it would be expected that the pK_a of the imidazole ring would be raised. Robillard & Shulman (1974a) observed that the pK_a of the His-57–Asp-102 H-bonded proton was raised to $\approx$8.4 in chymotrypsin–chloromethyl ketone complexes while in the presence of negatively charged boronic acid inhibitors the His–Asp H-bonded proton did not titrate in the pH range 6–9.5. There is therefore considerable evidence that the pK_a of the imidazole ring of histidine-57 can be raised in inhibitor complexes. Alkylation of N-3 raised the pK_a at least 2 pH units while with negatively charged inhibitors a further increase in pK_a of at least 1 pH unit is expected. We may conclude that pK_1 will be raised at least 2 pH units (to $\approx$8) and pK_4 raised to a value of at least 9. It would therefore appear that we could assign the pK_a = 7.88 to the ionization of the imidazole ring of histidine-57. As we have already discussed, however, this ionization alone should produce a titration of no more than 0.6 ppm. We therefore assign the pK_a of 7.88 to pK_2, i.e., to formation of the zwitterionic species **3** by deprotonation of the hemiketal hydroxyl (see Scheme IV). Since the macroscopic dissociation constant $K_I = K_1 + K_2$, the microscopic pK_a values can be defined; e.g., p$K_1 \geq$8.18 and pK_2 must lie in the range 7.88–8.18 (see Scheme IV). Since no additional titration shifts were observed up to pH 10.5, it is difficult to predict pK_{II}, but if pK_{II} > 10.5, then either pK_3 or pK_4 is greater than 10.5.

Fastrez (1983) has calculated that the pK_a of a hemiacetal adduct with serine proteases will be approximately 13.3 (cf. pK_3, Scheme II). He concludes that since the imidazole of the active site is neutral at pH 8.0 in the inhibitor complex (Hunkapiller et al., 1975), then a zwitterionic species in which hemiacetal pK_a is lowered must be excluded. It should be pointed out, however, that in enzyme–substrate tetrahedral adducts, the solvent accessibility of the N-1 nitrogen of the imidazole may well be reduced, raising its pK_a. We also believe that comparison of our experimental pK_a values with those calculated by ab initio methods (Scheme II) shows that such calculations cannot accurately predict pK_a values within enzyme adducts.

Jencks (Fox & Jencks, 1974; Sayer & Jencks, 1973) has calculated that the pK_a for the protonation of the oxyanion of the zwitterionic species **6** will be $\approx$8. This species would



be extremely unstable and its concentration at least 10^6 times

less than the neutral tautomer **7**. The zwitterionic tautomer **6** should be much more efficient than the neutral tautomer at expelling a leaving group. Jencks has proposed (Satterthwait & Jencks, 1974) and that pK_a of the amide nitrogen will be similar to that of the parent amine ($pK_a \approx 10$) when an ionic tetrahedral adduct is formed. Therefore, the amine would be a better leaving group than the serine hydroxyl ($pK_a \approx 15$), which would favor partitioning for product formation as opposed to starting materials. It is of interest to note that for the neutral species **7** expulsion of the nonprotonated amine ($pK_a \approx 30$) as opposed to the serine hydroxyl ($pK_a \approx 15$) is not favored and partitioning in favor of initial reactants is favored. Therefore, we would expect the zwitterionic intermediate to be the catalytically relevant species. If the enzyme can stabilize the oxyanion (i.e., lower its pK_a), then optimal catalysis will be achieved. Komiyama & Bender (1979) have argued that enzymatic cleavage of amides cannot proceed via a tetrahedral intermediate as the pK_a of the leaving amine (8–11) would exceed that of the imidazole ($pK_a \approx 7$). As pointed out by Kossiakoff & Spencer (1981), this argument is not valid if the imidazole pK_a is raised.

One possible explanation for the additional fast exchange broadening ($\simeq$12 Hz, Figure 8) is that the tautomers **2** and **3** (Scheme IV) are in fast exchange. K_T is pH independent; i.e., the ratio of the tautomers is pH independent, but the concentration of the tautomers will increase on titration (pK_I) of species **1** (see eq 3 and legend to Figure 8). Therefore, the fast exchange broadening due to this exchange should increase as the total population of the tautomers increases. If $pK_1 = pK_2$, then Δppm observed ($\simeq$4 ppm) is an average value. Assuming that the chemical shifts of the enriched carbon in the neutral **2** and cationic species **1** (Scheme IV) are similar ($\simeq$98 ppm) while that of the zwitterionic species **3** is different ($\simeq$102 ppm), then Δppm $\approx$8 ppm and $k_E \approx 10^5$ s^{-1}; while if $pK_2 = K_1 + 1.6$ (i.e., $K_2 = 40K_1$, $K_T = 0.025$), then $k_E \approx 2.4 \times 10^3$ s^{-1} and Δppm $\approx$ 4 ppm. This is therefore a plausible explanation of the additional line broadening observed.

In the present study we have provided the first direct evidence that trypsin can stabilize the oxyanion of an enzymic hemiketal and that its pK_a can be lowered to $\approx$8 in the inhibitor complex. We also show indirectly that the imidazole pK_a is raised and that the primary reason for the perturbation of these pK_as is a direct electrostatic interaction between the imidazole ring of histidine-57 and the oxyanion.

Registry No. **1**, 96481-83-3; **2**, 96481-84-4; **3**, 96481-85-5; **4**, 96481-86-6; **5**, 22884-10-2; **6**, 96502-28-2; **7**, 16999-99-8; **8**, 20813-04-1; **9**, 56-40-6; **10**, 59867-00-4; Z-Lys-CMK, 52780-79-7.

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Structure of Metal–Nucleotide Complexes Bound to Creatine Kinase: ^{31}P NMR Measurements Using Mn(II) and Co(II)†

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ABSTRACT: The structures of metal–nucleotide complexes bound to rabbit muscle creatine kinase have been studied by making measurements of paramagnetic effects of two dissimilar activating paramagnetic cations, Mn(II) and Co(II), on the spin-relaxation rates of the ^{31}P nuclei of ATP and ADP in these complexes. The experiments were performed on enzyme-bound complexes, thereby limiting the contributions to the observed relaxation rate to two exchanging complexes (with and without the cation). Measurements were made as a function of temperature in the range 5–35 °C and at three ^{31}P NMR frequencies, 81, 121.5, and 190.2 MHz, in order to determine the effect of exchange on the observed relaxation rates. The relaxation rates in E·MnADP and E·MnATP are independent of frequency, and their temperature variation yields activation energies (ΔE) in the range 5–8 kcal/mol; in the transition-state analogue complex E·MnADP·NO_3^-·Cre (Cre is creatine), ΔE is increased to 17.3 kcal/mol. These results demonstrate that the relaxation rates in the Mn(II) complexes are exchange limited and are incapable of providing structural data. It is shown further that use of line-width measurements to estimate the lifetime of the paramagnetic complex leads to incorrect results. The relaxation rates in E·CoADP and E·CoATP exhibit frequency dependence and ΔE values in the range 1–3 kcal/mol; i.e., these rates depend on the Co(II)–^{31}P distances, whereas those in the E·CoADP·NO_3^-·Cre complex have $\Delta E \sim 18$ kcal/mol and are significantly contributed by exchange. Difficulties involved in estimating the electron relaxation times in E·CoADP and E·CoATP restrict the calculation of Co(II)–^{31}P distances in these complexes to lower and upper limits. These distances were all in the range 2.4–4.3 Å, similar to those for free complexes and appropriate for direct coordination between Co(II) and the phosphate groups. This conclusion is in agreement with that reached by using Mn(II) EPR on this enzyme [Leyh, T. S., Goodhart, P. J., Nguyen, A. C., Kenyon, G. L., & Reed, G. H. (1985) *Biochemistry 24*, 308–316] and is in contrast with the conclusion of second-hydration-sphere coordination for Mn(II)–nucleotide complexes of pyruvate kinase [Sloan, D. L., & Mildvan, A. S. (1976) *J. Biol. Chem. 251*, 2412–2420] reached by using ^{31}P relaxation measurements.

The role of obligatory divalent cations in enzymatic reactions utilizing ATP[1] has been a subject of continued interest. Since there are a variety of enzymes for which nucleotides are substrates, information on the structure of enzyme-bound nucleotide complexes under catalytic or exchange-inert conditions is of considerable value, not only because of its mechanistic implications but also because it affords a comparison of these structural features for different enzymes to gain insight into catalytic action. Several methods are being used for probing metal–nucleotide complexes at the active sites of enzymes in solution: (i) ^{17}O superhyperfine structure effects of selectively labeled ligands on Mn(II) EPR spectra (Reed & Leyh, 1980; Leyh et al., 1985); (ii) ^{17}O and ^{18}O isotope effects on ^{31}P NMR (Cohn, 1982; Tsai & Bruzik, 1983); (iii)

† This work was supported in part by grants from the NSF (PCM 83 09120) and the American Heart Association (Indiana affiliate). The NTC-300 NMR spectrometer at IUPUI was purchased with partial support from the NSF (PCM 80 18725), and the Purdue Biochemical Magnetic Resonance Laboratory is supported by NIH Grant RR01077.
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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; Cre, creatine; E·M·S, enzyme–metal–substrate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NMR, nuclear magnetic resonance.

0006-2960/85/0424-3487$01.50/0