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Complex of α-Chymotrypsin and *N*-Acetyl-L-leucyl-L-phenylalanyl Trifluoromethyl Ketone: Structural Studies with NMR Spectroscopy†

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ABSTRACT: A dipeptidyl trifluoromethyl ketone, *N*-acetyl-L-leucyl-L-[1-^{13}C]phenylalanyl trifluoromethyl ketone, was synthesized. This compound inhibits chymotrypsin with K_i = 1.2 μM [Imperiali B., & Abeles, R. H. (1986) *Biochemistry 25*, 3760–3767]. The complex formed between this inhibitor and α-chymotrypsin was examined with ^{1}H, ^{13}C, and ^{19}F NMR spectroscopy to establish its structure in solution. The keto group of the trifluoro ketone is present as an ionized hemiketal group as deduced from the comparison of its ^{13}C chemical shift with those of model hemiketals. The pK_a of the hemiketal hydroxyl in the complex is approximately 4.9, which is about 4.2 units lower than the pK_a of model hemiketals. This observation provides direct evidence that serine proteases are able to stabilize the oxyanions of tetrahedral adducts. Evidence is also presented for the presence of an Asp–His H bond and protonation of the imidazole group of His-57 in the tetrahedral adduct. The pK_a of His-57 is higher than 10. This observation directly indicates that the pK_a of His-57 is elevated in a complex containing a tetrahedral adduct.

N-Acetyl-L-leucyl-L-phenylalanyl trifluoromethyl ketone (**1**) is a slow binding inhibitor of chymotrypsin (K_i = 1.2 μM; Imperiali & Abeles, 1986). It has been proposed, on the basis of analogy with aldehydic peptides, that peptidyl trifluoromethyl ketones react with the active site serine to form a hemiketal. The resemblance of this hemiketal to the tetrahedral adduct formed during the catalytic reaction is probably a major factor contributing to the tight binding. In order to obtain direct information concerning the structure of the chymotrypsin–inhibitor adduct, we have carried out NMR studies of the enzyme–inhibitor complex.

Materials and Methods

α-Chymotrypsin (type II, 3× crystallized, from bovine pancreas) obtained from Sigma Chemical Co. was used without further purification. Trifluoroacetic acid silver salt and (2-bromoethyl)benzene were purchased from Aldrich Chemical Co. Barium [^{13}C]carbonate (99 atom % ^{13}C) was obtained from Cambridge Isotope Laboratories (Woburn, MA).

Synthesis of 4-Phenyl-1,1,1-trifluoro[2-^{13}C]butan-2-ol. This trifluoromethyl alcohol was synthesized from [1-^{13}C]-hydrocinnamaldehyde according to the procedure of Kitazume and Ishikawa (1981). The labeled hydrocinnamaldehyde was prepared as follows: (2-phenylethyl)magnesium bromide was prepared from (2-bromoethyl)benzene and magnesium turnings in dry ether. To this ether solution at 0 °C was added $^{13}CO_2$ generated from $Ba^{13}CO_3$ and sulfuric acid (Ott, 1981). [1-^{13}C]Hydrocinnamic acid thus obtained was reduced with excess lithium aluminum hydride in ether at 0 °C to afford [1-^{13}C]hydrocinnamyl alcohol, which was then oxidized to give [1-^{13}C]hydrocinnamaldehyde with pyridinium chlorochromate (Corey & Suggs, 1975).

Synthesis of 4-Phenyl-1,1,1-trifluoro[2-^{13}C]butan-2-one. A solution of the trifluoromethyl alcohol (2.0 g), obtained from the previous reaction, in 10 mL of dioxane was stirred in a cold-water bath. To this solution was added 33 mL of 0.3 N $KMnO_4$ solution containing 0.5 N NaOH. The reaction was quenched after 5 min by addition of solid sodium bisulfite and dilute HCl solution. Ethyl acetate (30 mL) was added to the resultant solution. The aqueous layer was separated and extracted with ethyl acetate (20 mL two times). The combined organic extracts were washed with water (30 mL), washed with brine (20 mL), dried ($MgSO_4$), and evaporated to give 1.7 g of product. ^{1}H NMR[1] ($CDCl_3$) δ: 7.31–7.15 (m, 5 H) and 2.97 (m, 4 H). ^{13}C NMR ($CDCl_3$) δ: 190.68 (q, J_{CF} = 35 Hz).

Synthesis of 3-Bromo-4-phenyl-1,1,1-trifluoro[2-^{13}C]butan-2-one. The trifluoromethyl ketone obtained from the previous reaction was converted into the corresponding silyl enol ether following a literature procedure (House et al., 1969). This silyl enol ether was brominated in $CHCl_3$ at −78 °C according to the procedure of Reuss and Hassner (1974).

Synthesis of 3-Amino-4-phenyl-1,1,1-trifluoro[2-^{13}C]butan-2-ol. The trifluoromethyl bromo ketone (0.7 g) from the previous reaction was dissolved in 5 mL of dioxane and added to an aqueous solution (5 mL) containing 0.35 g of NaN_3. The solution was stirred at room temperature for 2 h. At this time,

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[1] Abbreviations: NMR, nuclear magnetic resonance; ZLCK, 1-chloro-3-(carbobenzyloxyamino)-7-aminoheptan-2-one; FID, free induction decay; DMSO, dimethyl sulfoxide.

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ethyl acetate (20 mL) and water (10 mL) were added. The aqueous layer was separated and extracted with 20 mL of ethyl acetate. The combined organic solution was then dried with $MgSO_4$ and evaporated on a Rotavap. The oil residue was reduced with excess $LiAlH_4$ in ether to give 0.14 g of diastereomeric amino alcohols after purification on a silica gel column (ethyl acetate; R_f on TLC, 0.1 and 0.2). ^{13}C NMR ($CDCl_3$) δ: 69.1 and 68.2 (q, J_{CF} = 30 Hz).

Synthesis of N-Acetyl-L-leucyl-L-[1-^{13}C]phenylalanyl Trifluoromethyl Ketone. The amino alcohol obtained from the above reaction was coupled with *N*-acetyl-L-leucine according to the procedure of Anderson et al. (1967). The resultant peptide alcohols were oxidized with alkaline permanganate solution according to the above-mentioned procedure. This offered diastereomeric ketones, which were separated on a preparative TLC plate to give 8 mg of desired product. This compound has the same R_f as the authentic compound (R_f 0.2 in EtOAc/$CHCl_3$, 1:1) and a similar NMR spectrum: ^1H NMR (acetone-d_6) δ 7.62 (d, J = 7.0 Hz, 1 H, amide H), 7.36 (d, J = 7.0 Hz, 1 H, amide H), 7.22 (m, 5 H, Ar H), 4.3 (m, 2 H, α-H and α-H), 3.24 (dd, J = 3.0 Hz, J = 14.0 Hz, 1 H, benzylic H), 2.9 (dd, J = 14.0 Hz, J = 2.0 Hz, 1 H, benzylic H), 1.87 (s, 3 H, acetyl H), 1.53 (m, 1 H, methine H), 1.34 (dd, J = 7.3 Hz, J = 7.0 Hz, 2 H, methylene H), 0.83 (d, J = 6.6 Hz, 3 H, methyl), and 0.79 (d, J = 6.6 Hz, 3 H, methyl); ^{13}C NMR ($CDCl_3$) δ 94.5 (q, J_{CF} = 30.6 Hz).

NMR Spectra. ^1H NMR spectra of enzyme and enzyme–inhibitor complex in ^1H_2O were recorded at 5 °C on a 500-MHz spectrometer equipped with an Oxford magnet. Water signal suppression was achieved by employing a Redfield 2–1–4 pulse (Redfield et al., 1975). Weak convolution difference functions were applied to FID's before transformation to correct wavy base lines. Spectral conditions were 8K-Hz spectral width and 2K time-domain data points. All ^{13}C and ^{19}F NMR spectra were recorded on a Varian XL-300 spectrometer. ^{13}C NMR spectra of enzyme–inhibitor complex were obtained with 2.0-mL samples in 10-mm NMR tubes at ambient temperature. Spectral conditions were 30K time-domain data points, 20-μs pulse width (30 μs = 90° pulse), 0.9-s acquisition time, ~210 ppm spectral width, 10-Hz line broadening, and low-power proton noise decoupling. ^{19}F NMR spectra were obtained with 0.5–1.0-mL samples in 5-mm NMR tubes at ambient temperature. Spectral conditions were 3K time-domain data points, 20-μs pulse width (34.5 μs = 90° pulse), 0.3-s acquisition time, 5000-Hz spectral width, 5-Hz line broadening, and no proton decoupling.

RESULTS

^{13}C NMR Studies of Enzyme–Inhibitor Complex. *N*-Acetyl-L-leucyl-L-[1-^{13}C]phenylalanyl trifluoromethyl ketone ([^{13}C]-**1**) has a ^{13}C chemical shift of 96.0 ppm in ^2H_2O due to the hydrate formation (Figure 1A). Incubation of this inhibitor (2.0 mM) with 2.0 mM α-chymotrypsin in 0.5 M potassium phosphate buffer, pH 6.8, yields a new signal at 104.0 ppm (Figure 1C). Both the free inhibitor signal at 96.0 ppm and the new signal at 104.0 ppm appear as quartets due to the fluorine couplings ($^1J_{CF}$ = 30 Hz) (Figure 1B). The new signal at 104.0 ppm has a broad line width (approximately 20 Hz) and an asymmetric quartet appearance. The broad line width is indicative of a macromolecule-associated signal, which we assign to that of an ionized hemiketal formed between the inhibitor and enzyme on the basis of the comparison of its chemical shift with those of model hemiketals[2] (vide infra). The asymmetric appearance of this enzyme-bound signal probably results from overlapping of two signals. Two signals were also detected with an aldehyde inhibitor–chymotrypsin complex (Shah et al., 1984).

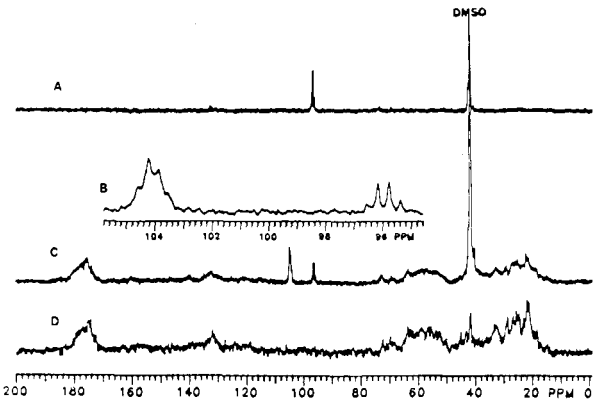

FIGURE 1: ^{13}C NMR spectra (75.4 MHz) of the complex of α-chymotrypsin and [2-^{13}C]-**1**. Spectral conditions were 16000-Hz spectral width, 30K time-domain data points, zero filled to 64 K before Fourier transformation, noise proton decoupling, and ambient temperature (~20 °C). (A) [2-^{13}C]-**1**, 0.5 mM in 0.5 mL of ^2H_2O with ca. 0.5% v/v DMSO. The spectrum was obtained with 30° pulse and 0.9-s acquisition time (3000 scans and 2-Hz line broadening). (B) Expanded region (105.8–94.6 ppm) of spectrum C. Spectra CD were obtained with 2.0-mL samples in 0.5 M phosphate buffer, pH 6.8, containing 10% v/v ^2H_2O. Both spectra were obtained with 60° pulse and 0.9-s acquisition time. (C) α-Chymotrypsin (2.0 mM) and [^{13}C]-**1** (2.0 mM), 40000 scans and 10-Hz line broadening. (D) α-Chymotrypsin (2.0 mM), 10000 scans and 10-Hz line broadening.

The signal of the enzyme–inhibitor complex remains constant at 104.0 ppm from pH 4.8 to pH 9.9 (data not shown). Above pH 10, the signal shifts to 102.5 ppm and broadens (data not shown). This small ($\Delta\delta$ = 1.5 ppm) upfield shift is not due to the ionization of the hemiketal hydroxyl group, because the same ionization gives a 4 ppm downfield (not upfield) shift with the model hemiketals. Instead, this small upfield shift at pH above 10 is most likely a result of conformational changes of the protein. Scott and co-workers (Malthouse et al., 1985) also observed conformational changes in this pH range with a ZLCK-inactivated trypsin. At pH 4.8, we observe a decrease in intensity of the enzyme-bound hemiketal signal, though the chemical shift of this signal remains the same (104.0 ppm, data not shown). The change in signal intensity is further investigated with relatively sensitive ^{19}F NMR spectroscopy and will be discussed later.

In order to assign this new signal of the enzyme–inhibitor complex at 104.0 ppm, we carry out ^{13}C NMR studies on model hemiketals. When **1** is dissolved in 80% (v/v) [$^2H_4$]-methanol and 20% (v/v) ^2H_2O, three signals are detected for the ketonic carbon (data not shown). The signal at 96.5 ppm is assigned to that of the hydrate on the basis of the similar chemical shift (96.0 ppm) observed when this compound is dissolved in ^2H_2O. Two signals at 98.8 and 98.5 ppm are assigned to diastereomeric hemiketals formed between this ketone and [$^2H_4$]methanol. Figure 2 shows the pH dependence of these three signals. The signal of hydrate moves from 96.5 ppm at low pH to 99.9 ppm at high pH ($\Delta\delta$ = 3.4 ppm) with

[2] The possibility that the imidazole of His-57 rather than the serine hydroxyl adds to the trifluoromethyl ketone was considered and ruled out for the following reasons: (1) The adduct of 1,1,1-trifluoroacetone and imidazole has a ^{13}C chemical shift at 74 ppm in $CDCl_3$, which is quite different from that (104 ppm) observed with the complex of **1** and chymotrypsin. (2) The X-ray crystallographic result indicates a covalent bond is formed between serine hydroxyl and the carbonyl of **1** (Brady et al., unpublished results).

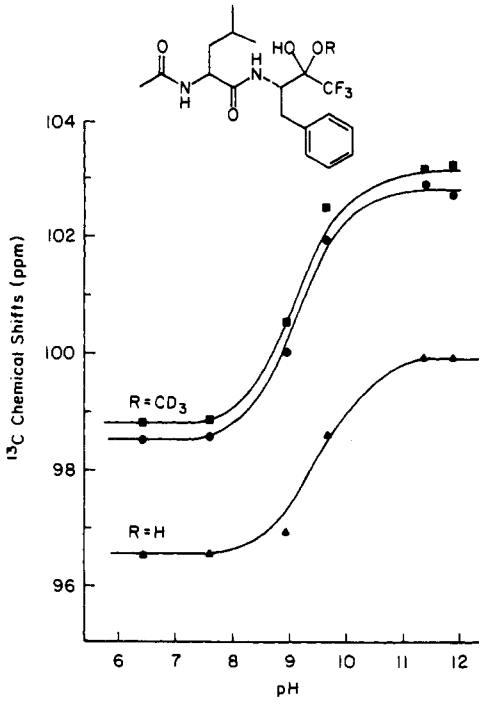

FIGURE 2: pH dependence of ^{13}C chemical shifts of **1** and its methyl hemiketals. ^{13}C NMR spectra were obtained with **1** dissolved in 80% v/v [$^2H_4$]methanol and 20% v/v ^2H_2O. This allowed simulataneous observation of signals derived from hydrate and methyl hemiketals. pH adjustments were made with addition of 0.1 N NaOH solution. pH values of samples were first measured with a pH electrode without corrections for isotope and solvent effects. These pH values gave a pK_a (10.1) of the hydrate 0.6 unit higher than that (9.5) obtained from a titration of the hydrate in 95% v/v H_2O and 5% v/v ^2H_2O. Therefore, the pH values shown in the figure were adjusted by 0.6 unit so that the pK_a of the hydrate was the same as that obtained in aqueous solution. Curves shown were fitted for $\delta_{obsd} = [\delta_{HA} + \delta_{A^-}(K_a/[H])]/(1 + K_a/[H])$ (Sudmeier et al., 1980).

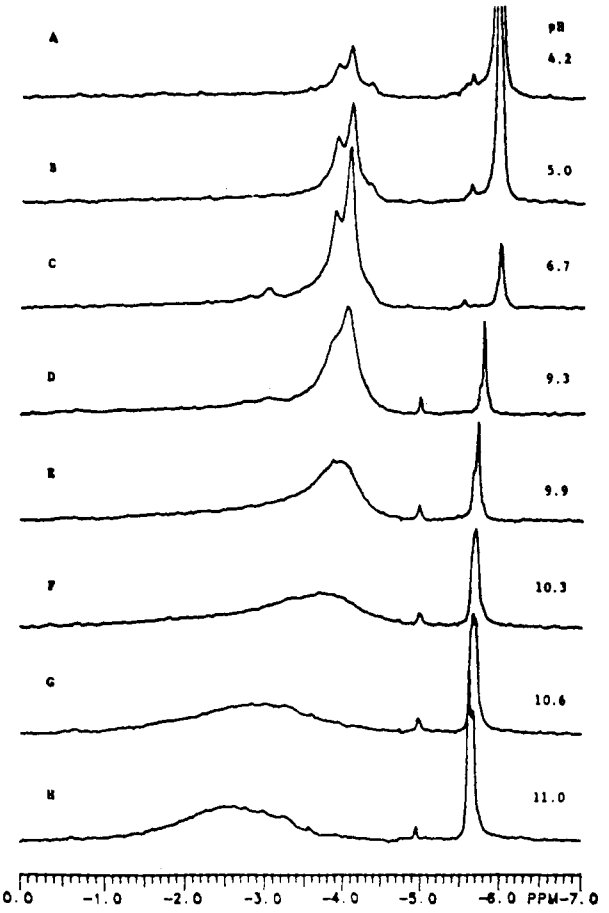

FIGURE 3: ^{19}F NMR spectra (282.2 MHz) of the enzyme–inhibitor complex at various pHs. Samples contained 2.0 mM α-chymotrypsin and 2.0 mM **1** in 0.5 M NaCl solution containing 10% v/v ^2H_2O. pH values of each sample were measured before and after acquisitions and found to be within 0.1 unit of each other. pH adjustments were made with addition of 1.0 N NaOH or 1.0 N HCl solution. The chemical shifts were referred to an external standard of sodium trifluoroacetate in ^2H_2O. Spectral conditions were 5000-Hz spectral width, 0.3-s acquisition time, 55° pulse, and 3000 scans for each spectrum.

a pK_a of 9.5. Both hemiketals are titrated with pK's of 9.1. Thus, the signal 98.5 ppm at low pH moves to 102.8 ppm ($\Delta\delta$ = 4.3 ppm) at high pH, while the signal at 98.8 ppm at low pH moves to 103.2 ppm at high pH ($\Delta\delta$ = 4.4 ppm).

The fact that ionized hemiketals appear at 102.8 and 103.2 ppm shows that the adduct formed between this inhibitor and the enzyme is an ionized hemiketal, which has a ^{13}C chemical shift of 104.0 ppm. Furthermore, both model hemiketals are shifted downfield with chemical shift changes of 4.3 and 4.4 ppm, respectively. The sizes (4.3 and 4.4 ppm) of the downfield titration shifts argue against the possibility that the change in chemical shift of the enzyme-bound hemiketal from 104.0 ppm (pH < 10) to 102.5 ppm (pH >10) ($\Delta\delta$ = 1.5 ppm, upfield shift) is due to the ionization of the hemiketal hydroxyl group.

Experiments with concentric NMR tubes containing ^2H_2O and H_2O in different compartments can be used to detect deuterium isotopic shifts of the ^{13}C signals (Pfeffer et al., 1979; Ho et al., 1978; Reuben, 1983). This technique can be employed to reveal the number of exchangeable protons on the functional groups attached to a particular carbon, since the deuterium isotopic shifts are additive. For example, Rich and co-workers (Schmidt et al., 1984) showed with this technique that a peptide containing pepstatone was bound as a hydrate rather than a ketone or covalent adduct in the active site of pepsin. When a similar experiment was carried out with the complex of chymotrypsin and **1**, no detectable deuterium isotopic shift was observed on the ^{13}C signal of hemiketal adduct at 104.0 ppm, while a 0.13 ppm (10 Hz for two deuterium atoms) upfield shift was observed for the free inhibitor signal in ^2H_2O (95.87 ppm) relative to that in H_2O (96.0 ppm). The lack of deuterium isotopic shift on the hemiketal signal is consistent with the existence of the hemiketal as an enzyme-stabilized deprotonated oxyanion. The hemiketal is, presumably, formed through the addition of active site serine to the carbonyl of **1**.

^{19}F NMR Studies on Enzyme–Inhibitor Complex. Due to the high sensitivity and lack of background interference from protein, ^{19}F NMR spectroscopy is especially suitable for investigating enzyme–inhibitor interactions (Gorenstein & Shah, 1982; Shah & Gorenstein, 1983).

The trifluoromethyl group of **1** appears at −5.9 ppm in ^2H_2O relative to an external standard trifluoroacetate in ^2H_2O (Figure 3). This signal is ascribed to that associated with the hydrate rather than the ketone form of the inhibitor on the basis of the structure deduced from a ^{13}C chemical shift of 96.0 ppm in water. The signal corresponding to the ketone form of the inhibitor is not detectable even after a long-term acquisition. We estimate that there is less than 1% of **1** in ketone form in aqueous solution.

Incubation of **1** (2.0 mM) with 2.0 mM α-chymotrypsin in 0.5 M NaCl, pH 4.2–9.3, results in two new signals at −3.9 and −4.15 ppm in addition to the free inhibitor signal (Figure 3A–D). These new signals are broad (line widths are approximately 50 Hz at −3.9 ppm and 30 Hz at −4.15 ppm) and

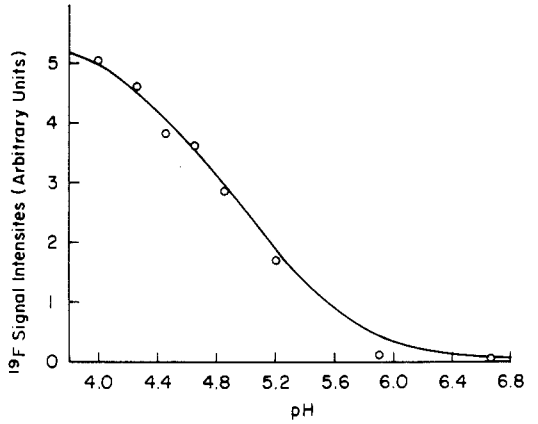

FIGURE 4: Relative ^{19}F signal intensities of unbound inhibitor at various pHs. Spectra were obtained with 1-mL samples in 0.2 M acetate buffers containing 5% ^2H_2O, 1.0 mM α-chymotrypsin, 1.0 mM **1**, and 0.15 mM sodium trifluoroacetate. The latter compound is added as an internal standard for intensity measurements. Each spectrum represents 2000 scans. Spectral conditions are 2000-Hz spectral width, 8K time-domain data points, 30° pulse (34.5 μs = 90° pulse), and 2-s acquisition time. Signal intensities (of unbound inhibitor at −5.9 ppm) are expressed in arbitrary units relative to that of the internal standard. The curve is fitted with a pK_a of 4.9 and an unbound-signal intensity of 5.6 (arbitrary unit) at extremely low pH by assuming the hypothetical ionization (K_a) HA (unbound) $\rightleftharpoons$ H^+ + A^- (bound).

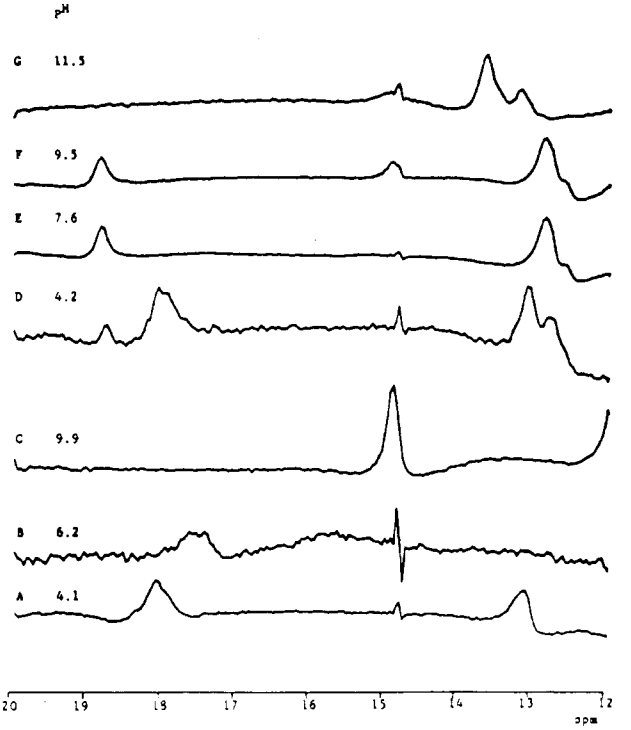

FIGURE 5: ^{1}H NMR spectra (500 MHz) of α-chymotrypsin and the enzyme–inhibitor complex. Spectra were obtained at 5 °C with 0.5-mL samples of 2.0 mM α-chymotrypsin with 2.0 mM inhibitor (D–G) or without inhibitor (A–C) in H_2O containing 5% ^2H_2O and 0.5 M NaCl. Water suppression was achieved with a Redfield 2–1–4 pulse. FID's (1000–6000 scans) were multiplied with weak convolution difference functions before transformation to correct wavy base lines. The spikes at 14.8 ppm are due to the carrier frequencies.

are assigned to those of enzyme-bound hemiketals on the basis of the results obtained with ^{13}C NMR spectroscopy under similar conditions. The presence of two enzyme-bound signals arises from polymerization of the enzyme. Upon dilution, the signal intensity at −3.9 ppm decreases relative to that at −4.15 ppm. This together with the fact that the signal at −3.9 ppm is broader (line width approximately 50 Hz) than that at −4.15 ppm (line width approximately 30 Hz) suggests that the signal at −3.9 ppm is derived from dimeric or trimeric enzyme, while that at −4.15 ppm is derived from monomeric enzyme. Chymotrypsin is known to form dimers and trimers at this concentration (Rao & Kegeles, 1958). However, we cannot rule out other possibilites (such as alternative binding modes) as the source of two enzyme-bound signals.

The chemical shifts of these enzyme-bound signals remain unchanged from pH 4.2 to pH 9.9 (Figure 3A–D). At pH values above 10 (Figure 3E–H), these signals move downfield and become broader, presumably, as a result of the above-mentioned conformational changes of the protein. It is interesting to note that even at pH 11.0 the covalent adduct stays intact, though the enzyme has little activity under this condition. At pH values below 5.5, intensities of the enzyme-bound signals decrease, while those of the unbound inhibitor signals increase (Figure 3A,B). The change of signal intensities can be correlated with a pK_a of 4.9 (Figure 4). The pK_a of 4.9 could be that of the enzyme-bound hemiketal hydroxyl or, less likely, a titratable group on the protein.

Scott and co-workers (Malthouse et al., 1985) reported a pK of 7.88–8.1 for the hemiketal hydroxyl of the trypsin–ZLCK complex. This pK is about 3 units lower than that calculated from free-energy prediction (De Tar, 1982; Fastrez, 1977; Fox & Jencks, 1974) for the corresponding hemiketal. This observation demonstrates the ability of the "oxyanion hole" to stabilize an oxyanion of a tetrahedral adduct. A pK of 6.1 would be expected for the hemiketal hydroxyl of the complex between **1** and chymotrypsin, if the active site stabilizes the oxyanion to the same extent, since the pK_a of the model hemiketals of **1** is 9.1. The fact that we do not see a titration at pH 6.1 suggests there are other factors that further stabilize the oxyanion of the hemiketal adduct of **1** and chymotrypsin. It is also possible that a direct comparison between our complex with a trypsin–ZLCK complex is not proper. In the latter complex, inhibitor alkylates the imidazole of His-57. As a result, the interaction between the oxyanion and the oxyanion hole may not be perfect.

^1H NMR Studies. Robillard and Shulman (1972, 1974a) observed a single low-field (15–18 ppm) proton resonance of chymotrypsin in ^1H_2O solution at low temperature. They assigned this signal to the N-1 proton of His-57. The abnormally low-field chemical shift of this proton and its slow exchange rate with ^1H_2O indicate the existence of the Asp–His H bond. This resonance appears at 18 ppm at low pH when His-57 is protonated and at 15 ppm at high pH when His-47 is neutral. A pK of 7.2 derived from the pH dependence of this low-field resonance is assigned to His-57.

Figure 5A–C essentially reproduces the work of Robillard and Shulman (1972, 1974a) by showing the presence of a low-field proton resonance in native chymotrypsin. In the complex of chymotrypsin inhibited with **1**, a new signal at 18.7 ppm appears (Figure 5D–F), which must be due to the same N-1 proton of His-57 of the enzyme–inhibitor adduct. In accord with this assignment, this signal at 18.7 ppm is only detected in ^1H_2O not in D_2O. (A property of an exchangeable proton such as an N-H proton.) The new signal at 18.7 ppm appears at the expense of the signal at 18 ppm. At pH 4.2, when formation of the hemiketal adduct is not complete, both signals at 18.7 and 18 ppm are observed (Figure 5D). At pH 7.6 and 9.5 (Figure 5E,F), the signal at 18.0 ppm disappears, and only that at 18.7 ppm is observed. At pH 11.5 (Figure 5G), both signals at 18.0 and 18.7 ppm are not detected. This observation is ascribed to the above-mentioned conformational changes that disrupt the Asp–His H bond at high pH (pH >10).

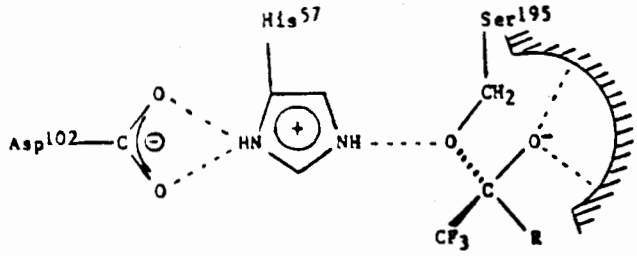

FIGURE 6: Structure of adduct formed between chymotrypsin and *N*-acetyl-L-leucyl-L-phenylalanyl trifluoromethyl ketone. R = $CH_3CONHCH[CH_2CH(CH_3)_2]CONHCH(CH_2Ph)$-. Striped area represents oxyanion hole; (---) H bonds to Ser-195 and Gly-193 in the oxyanion hole.

The abnormally low-field chemical shift (18.7 ppm) of this proton in the enzyme–inhibitor complex indicates the existence of the Asp–His H bond. In fact, a lower field shift ($\Delta\delta = 0.7$ ppm) of this proton in the complex (18.7 ppm) than in native enzyme (18.0 ppm) suggests that the Asp–His H bond is stronger in the complex. Furthermore, the chemical shift (18.7 ppm) indicates a protonated His-57 in the complex. The signal of this low-field proton stays at 18.7 ppm in the complex from pH 4.2 to pH 9.5 (Figure 5D–G). This is in contrast to the titration with a pK_a of 7.2 observed with native enzyme. This observation provides direct evidence that the pK_a of His-57 is raised in the tetrahedral adduct. A higher pK_a of His-57 has also been shown with negatively charged species (boronic acid phosphate derivatives and tryptophan) bound at Ser-195 (Robillard & Shulman, 1974b; Markley et al., 1980; Kossiakoff & Spencer, 1981) and with a ZLCK–trypsin complex (Malthouse et al., 1985; Primrose et al., 1985).

Possible causes for a higher pK_a of His-57 in the adduct of **1** and chymotrypsin include (1) ionic interaction between the oxyanion of the hemiketal adduct and the protonated imidazole (Kossiakoff & Spencer, 1981), (2) H-bond interaction between the imidazole N-3 proton and Ser-195, and (3) Possibly ion–dipole interaction between the trifluoromethyl group of the inhibitor and the imidazole (Street et al., 1986; Murray-Rust et al., 1983).

In addition to the N-1 proton of His-57 at 18–15 ppm, another proton resonating at 13.1 ppm is also detected in native chymotrypsin at low pH (pH 4.1) (Figure 5A). At higher pH, this signal at 13.1 ppm is no longer observable (Figure 5B,C). The identity of this proton is not known. Figure 5D–G shows that there are two protons resonating at 12.8–13.5 ppm in the enzyme–inhibitor complex. The identities of these two protons are not known, either.

Discussion

We have investigated the structure of an adduct formed with inhibitor **1** and chymotrypsin. Figure 6 depicts the structure of the enzyme–inhibitor complex as deduced from NMR studies. The salient features are as follows: (1) The inhibitor is bound as an ionized hemiketal. Formation of the ketal probably makes a major contribution to K_i. (2) The pK of His-57 ≥ 10. (3) The Asp–His bond is intact, with the proton primarily located on His. If **1** is a transition-state analogue or an analogue of a reaction intermediate, then the structure of this complex will also provide information about reaction intermediates in the normal catalytic process.

The pK_a of the hemiketal hydroxyl is ≤ 4.9, which is 4.2 units lower than the pK_a of model hemiketals. The lowering of the pK_a is most likely due to stabilization of the oxyanion by hydrogen-bond interactions with residues in the oxyanion hole, as pointed out by Henderson (1970), as well as electrostatic interactions between the oxyanion and imidazolium of histidine (Caplow, 1969; Lucas et al., 1973; Kossiakoff & Spencer, 1981). A similar result was reported by Scott and co-workers (Malthouse et al., 1985) with a ZLCK–trypsin complex. In their study, the pK_a of the hemiketal hydroxyl is 7.88–8.1, which is about 3 units lower than the pK_a estimated for the model hemiketal. In the complex with **1** the pK_a is lowered by 4.2 units. The difference can be ascribed to the structural differences between these complexes. In the ZLCK–trypsin complex, the inhibitor is covalently bound to histidine. This additional covalent bond may force the oxyanion out of an optimal position to form hydrogen bonds in the oxyanion hole.

The pK_a of His-57 in the enzyme–inhibitor complex is estimated to be higher than 10.0. The negative charge of the oxyanion is most probably a contributing factor to the high pK_a of His. A higher pK_a of His-57 has been shown with an enzyme inactivated by a chloromethyl ketone (Primrose et al., 1985; Malthouse et al., 1985) and enzymes complexed with boronic acid derivatives and tryptophan (Robillard & Shulman, 1974b; Markley et al., 1980; Kossiakoff & Spencer, 1981).

The Asp–His hydrogen bond in the complex of α-chymotrypsin and a trifluoromethyl ketone inhibitor exists as revealed by the low-field chemical shift of the N-1 proton of His-57 (Figure 5). In fact, this hydrogen bond is stronger in the complex than in native enzyme as evidenced by a downfield shift of this proton in the complex (18.7 ppm, as compared to 18.0 ppm in native enzyme). The detection of the Asp–His hydrogen bond in this complex suggests that a proton is not transferred from His-57 to Asp-102 upon tetrahedral adduct formation. Therefore, the role of Asp-102 in the "catalytic triad" is to enhance the histidine residue's ability to act as a general base (Bachovchin & Roberts, 1978). This observation is consistent with results of earlier theoretical studies (Umeyama et al., 1981, 1984; Nakagawa & Umeyama, 1984; Kollman & Hayes, 1981; Hayes & Kollman, 1979).

It is generally agreed that in the hydrolysis of peptides or esters by chymotrypsin an anionic tetrahedral intermediate is formed through the addition of serine OH to the carbonyl of the substrate. Concomitant with the formation of the tetrahedral adduct, a proton is transferred to His-57. This mechanism implies that the interaction with the active site leads to substantial reduction of the pK_a of the OH group of the tetrahedral intermediate as well as to an increase in pK_a of the active site His to prevent loss of the proton after formation of the tetrahedral adduct. The retention of the proton on His is important for His to act as a general acid in assisting the departure of the leaving group (alcohol or amine). With substrates, direct evidence for the presence of an oxyanion or for a high pK_a of His-57 has not been obtained. We have now obtained such evidence for a complex consisting of chymotrypsin and a transition-state analogue. In this complex the pK_a of the hemiketal hydroxyl is lowered by at least 4 units. It is likely that with a substrate a more favorable conformation is achieved, which could result in greater reduction of the pK_a. Additionally, we have established that in this complex the pK_a of His is >10.

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