pH Dependence of the Inhibition of Chymotrypsin by a Peptidyl Trifluoromethyl Ketone†

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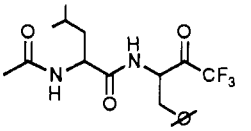
Received May 2, 1989; Revised Manuscript Received July 17, 1989

ABSTRACT: The effects of pH on the kinetics of association and dissociation of chymotrypsin and the dipeptidyl trifluoromethyl ketone (TFK) *N*-acetyl-L-leucyl-L-phenylalanyltrifluoromethane (**1**) were examined through the pH range 4–9.5. The pH dependence of the association rate (k_{on}) is similar to that of k_{cat}/K_m for ester and peptide substrates and is dependent on two pK's at 7.0 and 8.9. We assign these pK's to the active site His and to the amino group of the N-terminal isoleucine residue. K_i for the complex of **1** and chymotrypsin has a pH dependence very similar to that of k_{on}, and we conclude that the same ionizable groups which determine the pH dependence of k_{on} are involved. The dissociation constant of the enzyme–inhibitor complex (k_{off}) shows no pH dependence between pH 4 and pH 9.5. The data indicate that the inhibitor reacts with a form of the enzyme in which His 57 is unprotonated, and the resulting complex contains no groups which ionize between pH 4 and pH 9.5. This is consistent with conclusions previously reached from NMR data (Liang & Abeles, 1987). These experiments led to the conclusion that **1** reacts with chymotrypsin to form a tetrahedral complex in which His 57 is protonated (pK >9.5) and the OH group of serine 195 has added to the carbonyl group of **1** to form an ionized hemiketal (pK <4.9). The pK of His 57 is increased by >3 units over that in the free enzyme, and the pK of the hemiketal decreased by >4 units compared to the pK in solution. We attribute the pK shifts to Coulombic interactions between the hemiketal oxyanion and the positively charged His, as well as to stabilization of the oxyanion by hydrogen-bond formation.

In the past decade, peptidyl aldehydes (Thompson, 1973), boronic acids (Kettner & Shenvi, 1984), and trifluoromethyl ketones (Imperiali & Abeles, 1986) have been investigated as inhibitors of serine proteases. All three classes of inhibitor react with the nucleophilic hydroxyl of serine proteases to form covalent, nearly tetrahedral adducts which mimic the transition state of the normal catalytic reaction (Delbaere & Brayer, 1985; Bone et al., 1987; Takahashi et al., 1988). All three classes of inhibitors exhibit "slow-binding" kinetics (Morrison, 1982). The affinity of these inhibitors for their respective proteases is a function of the electrophilic center (i.e., TFK,[1] aldehyde, or boronate), the P1 specificity of the inhibitor, and the number and nature of "extended" peptidyl contacts in either the N-terminal (P) or C-terminal (P′) directions (Kettner et al., 1988; Imperiali & Abeles, 1987; Stein et al., 1987).

The complexes formed by serine proteases (chymotrypsin, α-lytic protease) with peptidyl boronic acids and TFKs have been studied by NMR spectroscopy (Bachovchin et al., 1988; Liang & Abeles, 1987) over the pH range 4–10. The complex of α-lytic protease with substrate-like boronic acids is stable over this entire pH range, and the NMR chemical shifts are consistent with a zwitterionic complex consisting of a protonated imidazole and an anionic tetrahedral boron atom.

The complex of chymotrypsin with *N*-acetyl-L-leucyl-L-phenylalanyltrifluoromethane (**1**) was reported to be zwitterionic over the pH range 4.9 to >10, bearing a protonated imidazole and a hemiketal oxyanion (Liang & Abeles, 1987).



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The boronic acid and TFK complexes are both remarkable in their abilities to shift the pK of the active site imidazoles to >10 and that of the hemiketal of the serine–TFK adduct to <4.9. This represents an increase in the pK of the imidazole of >3 units as compared to that of free enzymes and a decrease in the pK of the hemiketal of >4 units compared to the pK of the TFK–methanol adduct (Liang & Abeles, 1987). This remarkable and unexpected shift in pK prompted us to seek further support for the pH shifts through investigation of the pH dependence of K_i, k_{on}, and k_{off} of the reaction of **1** with chymotrypsin. A study of the pH dependence of the reaction of a TFK with HLE has been reported previously (Stein et al., 1987). It was reported that the dissociation rate constant (k_{off}) for the inhibitor from the HLE–inhibitor complex shows an inflection at pH 6.5, which was attributed to ionization of the active site histidine. This conclusion is inconsistent with the NMR data cited above. It is noted that the NMR data were not obtained with HLE, although it seems likely that the

† Publication No. 1693 of the Graduate Department of Biochemistry, Brandeis University. This research is supported by NIH Grant GM 12633-25 to R.H.A.

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[1] Abbreviations: TFK, trifluoromethyl ketone; NMR, nuclear magnetic resonance; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; BOC, *tert*-butyloxycarbonyl; SAAPFpNA, *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide.

0006-2960/89/0428-9066$01.50/0

mechanism of reaction with TFKs is similar for all serine proteases.

Materials and Methods

Materials. [^{3}H]Acetic anhydride (5 mCi, 8.3 mCi/mmol) was obtained from New England Nuclear. Sephadexes G-15 and G-25 were purchased from Bio-Rad. *N*-BOC-L-leucine, BTEE, and *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (SAAPFpNA) were from Sigma Chemical Co. All other reagents were as previously described (Liang & Abeles, 1987). All buffer salts were purchased from Fisher Scientific. Buffers used were 50 mM sodium formate (pH 4.0), 50 mM sodium acetate (pH 4.5–5.5), 50 mM potassium phosphate (pH 6.0–7.5), 50 mM Tris (pH 7.5–9.0), and 50 mM glycine (pH 9.0–10.0). The ionic strength of all buffers was adjusted to 0.2 M by addition of KCl.

Synthesis of N-[^{3}H]Acetyl-L-Leucyl-L-Phenylalanyltrifluoromethane. *N*-(*N*-BOC-L-leucyl)-3-amino-4-phenyl-1,1,1-trifluorobutan-2-ol (a mixture of 4 stereoisomers) was prepared as previously described (Liang & Abeles, 1987), except *N*-BOC-L-leucine was used instead of *N*-acetyl-L-leucine. Removal of the BOC group was accomplished by treating the peptide with trifluoroacetic acid in methylene chloride at room temperature for 2 h. Excess trifluoroacetic acid was removed by repetitive evaporation from carbon tetrachloride. The residue was then dried under vacuum for 12 h.

The deprotected peptide *N*-L-leucyl-3-amino-4-phenyl-1,1,1-trifluorobutan-2-ol (267 mg, 0.6 mmol; a mixture of four isomers) and 0.11 mL (1.0 mmol) of *N*-methylmorpholine were dissolved in 5 mL of tetrahydrofuran. This solution was then put in the upper portion of a sealed tube containing [^{3}H]acetic anhydride (5 mCi, 8.3 mCi/mmol). While the tube was cooled in an acetone–dry ice bath, the goose-neck seal was broken with strokes of a glass rod. The solution was sucked into the lower portion of the tube upon breakage. The solution was allowed to react at −78 °C for 30 min with occasional shaking. It was then allowed to warm up to room temperature and stand for 1 h. Acetic anhydride (0.01 mL, 0.1 mmol) was added, and the reaction progressed for another 2 h. The solution was then transferred to a beaker and the solvent removed under a stream of nitrogen. The residue was taken up in 5 mL of dioxane and oxidized with alkaline potassium permaganate as described (Liang & Abeles, 1987).

The radioactive inhibitor was purified on preparative silica plates first with ethyl acetate and chloroform (1:1, R_f = 0.2) and then with chloroform and 2-propanol (9:1, R_f = 0.6). This afforded 55 mg (0.15 mmol) of labeled inhibitor with a specific activity of 8.1 mCi/mmol after recrystallization from ether/petroleum ether. The radioactivity comigrated with the standard compound in three different TLC systems (ethyl acetate/chloroform, 1:1, R_f = 0.2; chloroform/2-propanol, 9:1, R_f = 0.6; neat ethyl acetate, R_f = 0.5).

Enzyme Activity Assays. In the pH range 6.0–9.5 chymotrypsin was assayed by monitoring the hydrolysis of BTEE via the absorbance at 256 nm at 25 °C. The kinetic constants V_{max} and K_m for hydrolysis of BTEE by chymotrypsin were determined for each pH value and buffer. BTEE (20 mM in CH_3CN) was added to a 1.0-mL reaction to a final concentration of 100 or 40 μM. Enzyme was added to a concentration of 30 nM, and A_{256nm} was monitored until hydrolysis was complete. The value of $\Delta\epsilon$ for BTEE hydrolysis was calculated from the total change in absorbance at each pH, and a Basic program (PROGSUB) was used to convert the trace of absorbance vs time to velocity vs [S]. Agreement observed between curves derived from differing starting substrate concentrations demonstrated that product inhibition was negligible. Velocity vs [S] curves were fit by nonlinear regression to the equation $V = V_{max}[S]/([S] + K_m)$.

Enzyme–Inhibitor Association Kinetics. Pseudo-first-order incubations were used to measure the second-order association rate between enzyme and inhibitor. At pH <6.0, where the association rates are relatively slow, these incubations were performed in the absence of competing substrate. At pH >6.0, incubations were performed in the presence of BTEE, which competes for free enzyme and slows down the apparent association kinetics.

For reactions at pH >6.0 BTEE (25 μL of a 20 mM stock in CH_3CN) was added to 1.0 mL of buffer, followed by 5 or 10 μL of an enzyme stock to a final enzyme concentration of 2–30 nM. After an incubation of approximately 1 min to ascertain normal progress of hydrolysis, inhibitor **1** was added from a 38 mM stock solution in dioxane to final concentrations ranging from 38 to 380 μM. Progress of BTEE hydrolysis was monitored by the change in absorbance at 256 nm, and reactions were terminated when the reaction rate had achieved a steady-state inhibited value. The amount of enzyme used in each progress curve was adjusted so that less than 30% of the total substrate was consumed during approach to equilibrium.

The second-order rate constant describing the reaction between **1** and chymotrypsin was derived by the method of Cha (1976). Formation of product is described by eq 1, where p_0

$$p = p_0 + v_s t + (v_0 - v_s)[1 - \exp(-k_{obs}t)]/k_{obs} \quad (1)$$

is the concentration of product at $t = 0$, v_s is the steady-state enzymatic activity, v_0 is the initial enzymatic activity, and k_{obs} is the pseudo-first-order rate constant for the change of enzymatic activities. The pseudo-first-order rate constant, k_{obs}, depends on the rates of complex formation (k_{on}) and dissociation (k_{off}) as described by eq 2. A plot of k_{obs} versus

$$k_{obs} = k_{off} + k_{on}[I]/(1 + [S]/K_m) \quad (2)$$

inhibitor concentration, [I], gives k_{off} as the ordinate intercept; k_{on} is evaluated from the slope, $k_{on}/(1 + [S]/K_m)$, by use of the known K_m and the average value of [S] during the assay.[2] K_i is determined directly from the initial and steady-state velocities as $K_i = [I]V_s/(1 + [S]/K_m)(V_0 - V_s)$.

For experiments at pH <6.0, enzyme was incubated with various amounts of inhibitor (1.0 mM to 5 μM) in an appropriate buffer at 25 °C. At least five different concentrations of inhibitor were used at each pH value. At intervals, aliquots were withdrawn from the reaction mixture, and the remaining enzymatic activity was measured by a standard assay (100 mM potassium phosphate buffer, pH 7.0, 0.4 mM BTEE). The pseudo-first-order decrease of enzymatic activity was described by eq 3, where $A(t)$, A_0, and A_s are activities at time t, time

$$A(t) = A_0 + (A_0 - A_s)e^{-kt} \quad (3)$$

0, and at equilibrium, respectively (Morrison, 1982). The apparent rate constant k is a function of k_{on}, k_{off}, and inhibitor concentration, [I], as described by eq 4. A plot of k versus

$$k = k_{off} + k_{on}[I] \quad (4)$$

[I] will yield k_{off} as the oridinate intercept and k_{on} as the slope. K_i was determined from A_0 and A_s as $K_i = A_s[I]/(A_0 - A_s)$, where [I] ≫ [E] at all times. The pseudo-first-order incubations of enzyme with inhibitor yield k_{off} from the ordinate intercept of the plots of k vs [I]. These experiments also yield

[2] In all cases plots of k_{obs} vs [I] were linear, indicating that the mechanism E + I = EI is consistent with kinetic data; i.e., a mechanism with a rapidly equilibrating enzyme–inhibitor complex prior to formation of EI is not required.

Table I: pH Dependence of Kinetic Constants[a]

pH	buffer	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1} × 10^3)	K_i (μM)	method
4.0	acetate		0.5		C
4.0	acetate	2.8	1.2	446.0	A
4.5	acetate		0.47		C
4.5	acetate	7.70	0.45	150.0	A
5.0	phosphate	23.2	0.85	36.0	A
5.5	phosphate	54.5	0.74	13.6	A
6.0	phosphate	74.0	1.2	0.60	B
6.5	phosphate	228.0	0.38	0.51	B
7.0	phosphate	859.0	0.28	0.33	B
7.5	phosphate	1458.0	0.60	0.40	B
7.5	Tris	1218.0	0.60	0.23	B
8.0	Tris	1435.0	0.53	0.24	B
8.5	Tris	1342.0	0.14	0.29	B
9.0	Tris	713.0	0.71	0.36	B
9.0	Gly	978.0	0.45	0.46	B
9.5	Gly	451.0	1.70	0.49	B
9.5	Gly		0.24		C
10.0	Gly		0.26		C

[a] Methods: (A) pseudo-first-order incubation; (B) pseudo-first-order incubation with substrate; (C) displacement of radiolabeled inhibitor by excess "cold" inhibitor. Estimation of error associated with k_{on} and K_i is ±15%, on the basis of the observed reproductivity of the measurements.

values of K_i and k_{on}, which permit a second evaluation of k_{off} from $K_i k_{on}$.

Both pseudo-first-order methods rely on evaluation of enzyme activity as the indicator of the amount of EI complex present. *Direct* measurement of k_{off} by dissociation of enzyme–^{3}H-labeled inhibitor complex was used at the very high and low pH values to verify these methods. Enzyme (100 μM) and radiolabeled inhibitor **1** (100 μM) were incubated for 2 h in 1.0 mL of 5 mM potassium phosphate, pH 7.0, with the ionic strength adjusted to 200 mM with KCl. At t = 0, 200 μL of this mixture was added to 800 μL of buffer (pH 4.0, 4.5, 9,5, or 10.0, as described above) containing 1.0 mL unlabeled **1**. Aliquots (100 μL) of this mixture were removed at times from 1 min to 18 h and applied to a 0.5 × 5 cm Sephadex G-15 column. The column was spun in a table-top clinical centrifuge to achieve rapid separation of enzyme (plus bound inhibitor) and free inhibitor (Penefsky, 1979). The eluant fraction containing enzyme + bound ligand was dissolved in 2 mL of Amersham ACS scintillation fluid and counted in a Beckman LS100 scintillation counter. The dissociation rate of the E–I* complex was evaluated from a plot of log $[(C_t - C_i)/(C_0 - C_i)]$ vs t, where C_t, C_i, and C_0 are counts at time t, 18 h, and 0 h, respectively. All plots were linear, and at the minimum dissociation rate (2.6×10^{-4} s^{-1}), the final time point (18 h) represents 24 half-lives.

Results

pH Dependence of k_{cat}, K_m, and k_{cat}/K_m (BTEE). The Michaelis–Menton kinetic constants for chymotrypsin and BTEE are required for subsequent experiments. These constants were measured as a function of pH over the pH range 6.0–9.5. BTEE hydrolysis is dominated by the protonation states of two ionizable groups with pK = 6.87 and 9.01, in agreement with past studies of ester and amide substrates (Bender et al., 1964; Fersht & Renard, 1974). The ionization with pK's = 6.87 affects k_{cat}, the ionization with pK = 9.01 affects K_m, and both pK's affect the second-order rate constant k_{cat}/K_m. The pH-independent values for K_{cat}, K_m, and k_{cat}/K_m were 61 s^{-1}, 8.9 μM, and 6.0×10^6 m^{-1} s^{-1}.

*pH Dependence of k_{on}, k_{off}, and K_i (Inhibitor **1**).* The data are summarized in Table I. Above pH 6.0, enzyme–inhibitor association rates (k_{on}) were determined by pseudo-first-order incubations in the presence of substrate; below pH 6.0, k_{on} was obtained by incubating enzyme and inhibitor under pseudo-first-order conditions in the absence of substrate. The pH dependence of the rate of enzyme–inhibitor complex formation (k_{on}) is plotted by the method of Dixon (1979) and shown in Figure 1a. The pH dependence of k_{on} parallels that of k_{cat}/K_m for the catalytic reaction, and k'_{on} = 1920 ± 155 M^{-1} s^{-1}. The pK of 7.1 ± 0.1 is assigned to His 57 in the active site, and the pK of 8.9 ± 0.1 is that of N-terminal isoleucine. Thus, in a manner analogous to the hydrolysis of BTEE, the inhibitor reacts only with enzyme bearing an unprotonated imidazole in the active site and protonated N-terminal isoleucine.

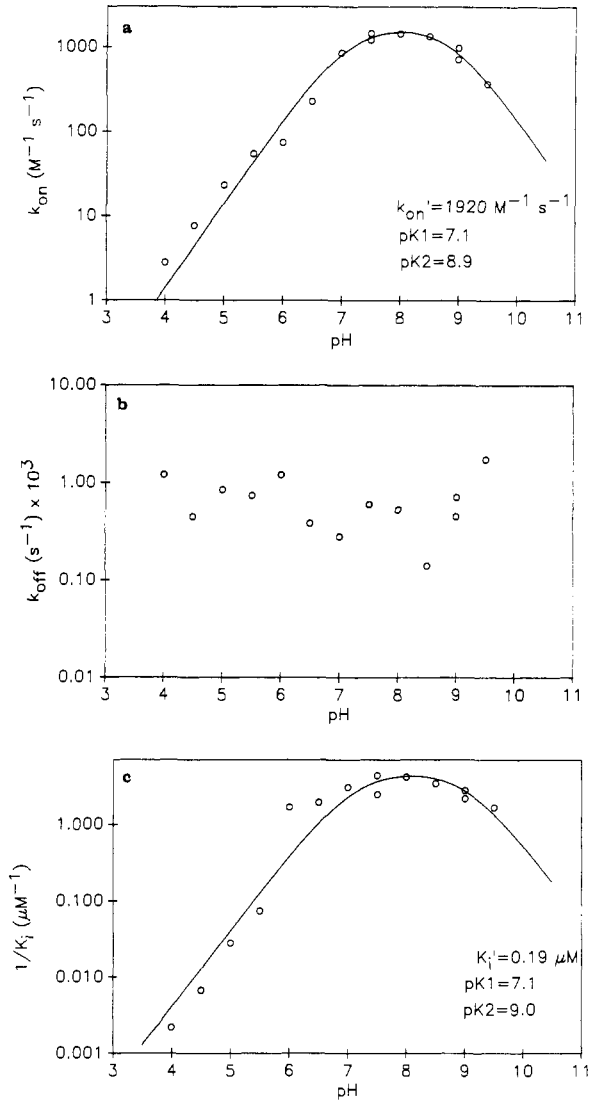
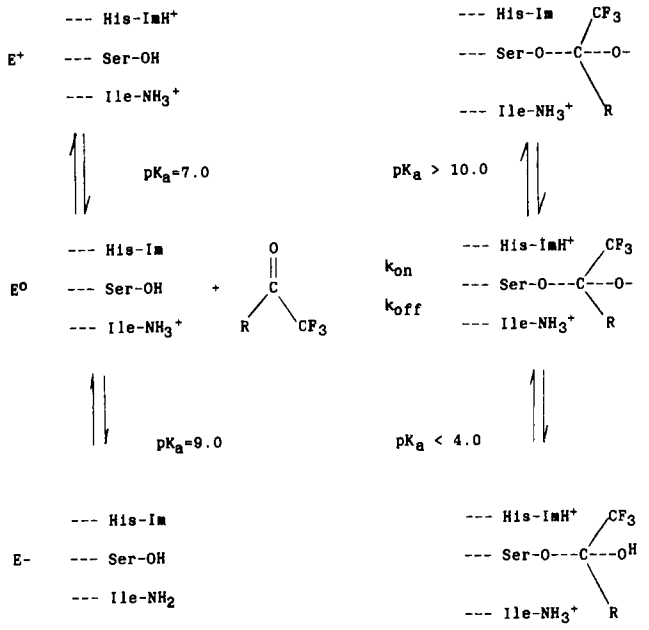


FIGURE 1: Effect of pH on kinetic constants. Effect of pH on (a) association rate constant (k_{on}), (b) dissociation rate constant (k_{off}), and (c) equilibrium constant (K_i). Curves were fit by nonlinear regression to the expressions $k_{on} = k'_{on}/(1 + [H]/K_1 + K_2/[H])$ and $\log K_i = \log K_i' - \log (1 + [H]/K_1 + K_2/[H])$.

Dissociation rate constants, k_{off}, as determined from the [I] = 0 intercepts of pseudo-first-order incubations are plotted as a function of pH in Figure 1b. The small magnitude of this constant leads to considerable variability in the measured values. Within the scope of this variability, the plot of k_{off} vs pH is featureless. This indicates that there is no protonation or deprotonation associated with breakdown of the enzyme–inhibitor complex over the pH range 4.0–9.5. This observation is consistent with NMR results which show that the pK_a of the histidine residue is higher than 10.0 in the complex (Liang & Abeles, 1987).

Scheme I: Equilibria Governing Formation of Enzyme–Inhibitor Complex



The inhibition equilibrium constants (K_i) at each pH were determined from the steady-state inhibition achieved in the pseudo-first-order incubations (see above). Figure 1c shows pH transitions at 7.1 ± 0.15 and 9.0 ± 0.4, and K_i' = 0.19 ± 0.1 μM.

Discussion

In this study we have examined the kinetics of inhibition of chymotrypsin by the trifluoromethyl ketone **1** throughout the pH range 4.0–9.5. The inhibition constants (K_i) and the complex formation rate constants (k_{on}) exhibit two pK's at 7 and 9. The pH dependence of K_i reflects the effects of ionizable groups in the free enzyme, inhibitor, or enzyme–inhibitor complex. Since similar pK values are observed for the pH dependence of k_{on}, they must be due to the ionization of free inhibitor or free enzyme. Therefore, they are assigned to the pK's of the active site histidine and N-terminal isoleucine on the enzyme, respectively. It is significant that no additional pH transition is observed in the pH dependence of K_i. The lack of any additional pH transition indicates that there is no ionizable group in the enzyme–inhibitor complex between pH 4.0 and pH 9.5. Also, there is no pH dependence of the complex dissociation rate constants (k_{off}). The lack of a pH dependence of k_{off} further confirms the absence of an ionizable group in the enzyme–inhibitor complex between pH 4.0 and pH 9.5. Conclusions from these kinetic studies are summarized in Scheme I.

From NMR studies we reached the conclusion that the complex of chymotrypsin and **1** consists of an ionized hemiketal formed by addition of Ser 195 to the carbonyl group of **1**. The pK of the hemiketal is <4.9, and the pK of His is >9.5. The data presented here support these conclusions but require a reevaluation of the pK of the hemiketal to <4.0. This point will be discussed further below.

From NMR data (Liang & Abeles, 1987) it was concluded that the pK of the hemiketal was ~4.9. Kinetic data reported here provide no evidence for a pK at 4.9 and indicate that the pK of the hemiketal is less than 4.0. It is likely that we misinterpreted the NMR data obtained at low pH. Utilizing inhibitor **1** labeled with ^{13}C at the TFK carbonyl (Liang & Abeles, 1987), we observed a pH-dependent decrease in NMR signals of the covalent adduct and corresponding increase in the signal corresponding to the hydrated inhibitor. A pK_a of 4.9 correlated with the intensity change between these two resonances. This pK_a was assumed to derive from an ionizable group on the enzyme or the ionization of the hemiketal hydroxyl group. It is likely that the shift observed was instead due to a change in K_i resulting from enzyme polymerization at the high enzyme concentrations (2 mM) used in the NMR experiment (Faller & LaFond, 1971; Aune & Timasheff, 1971; Fersht & Renard, 1974). Anue and Timasheff report a pH-dependent transition in the dimerization of chymotrypsin involving an ionizable group with pK = 5.0. At an enzyme concentration of >100 μM, Fersht and Renard observe a concentration-dependent loss of activity of chymotrypsin at pH 5.6 and, on the basis of crystallographic data, report that their substrate is unable to bind productively to the dimer.

The remarkable conclusion derived from this and other studies is the shift in pK of the hemiketal OH group and the active site His. The methyl hemiketal of inhibitor **1** has a pK of 9.1 in aqueous solution (Liang & Abeles, 1987). The adduct of serine and inhibitor **1** is expected to have a similar pK. However, in the TFK–chymotrypsin complex the pK of the adduct is lowered by >5 pK units, which correspond to >7 kcal/mol of stabilization. We see two mechanisms through which this pH shift can occur: (1) Coulombic interaction between the positively charged imidazolium side chain of His 57 and the negatively charged hemiketal will decrease the pK of the hemiketal, and raise that of His. The increase in pK (>3 pK units) of His 57 is probably primarily due to electrostatic interaction. From this we conclude that electrostatic interaction can lower the pK of the hemiketal OH group by at least 3 pK units. The effect of the imidazolium on the pK of the oxyanion can be calculated from the relation of Kirkwood and Westheimer (1938):

$$2.3RT\Delta pK = e^2/DR$$

where D = dielectric constant of the medium, e = electronic charge, and R = distance between the charged groups (5.1 Å; Brady, 1989). From this relation, a dielectric constant of 16 is sufficient to create a ΔpK of 3 units induced by the imidazole on the oxyanion. The medium separating the imidazolium and the oxyanion is devoid of solvent and consists entirely of covalently linked C, O, and F atoms, and the dielectric across this medium may be as low as 2–3 (Gilson & Honig, 1987). (2) Two strong, well-oriented hydrogen bonds within the oxyanion hole will further stabilize the oxyanion. The oxyanion forms hydrogen bonds with the amide hydrogens of Gly 193 and Ser 195. In experiments with a genetically modified subtilisin in which one of the hydrogen-bond donors was removed, the strength of a single hydrogen bond between a negatively charged oxygen and an amide hydrogen was estimated at 3.7 kcal/mol (Bryan et al., 1986). However, the stabilization of the oxyanion of the chymotrypsin–**1** complex relative to that of the oxyanion of **1** in water will be less, since the oxyanion of **1** in water is hydrogen bonded by water.

An additional factor may stabilize the oxyanion of the chymotrypsin–**1** complex. van der Waals interactions between the leudine side chain of **1** and the active site histidine provide leverage transmitted along the peptide backbone of the inhibitor which pushes the hemiketal oxygen into the oxyanion hole (Brady 1989; Delbaere & Brayer, 1985). Once positioned in the oxyanion hole, deprotonation of the oxyanion will be unfavorable due to the increased stability of a hydrogen bond formed between a negatively and a positively charged entity.

In the catalytic process the transition state resembles the tetrahedral adduct formed by addition of the active site serine

to the carbonyl group of the substrate. This tetrahedral adduct may actually be a high-energy intermediate Fersht & Requena, 1971). The pK of the oxyanion which is transiently formed during the base-catalyzed hydrolysis of esters is approximately 14 (Sayer & Jencks, 1973; Fox & Jencks, 1974). Interactions at the active site must reduce the pK of this tetrahedral adduct. Stabilization of the oxyanion by hydrogen-bond interactions was first recognized by Henderson (1970); the electrostatic interactions between the imidazolium and the oxyanion have been considered by Caplow (1969), Lucas et al. (1973), and Kossiakoff and Spencer (1981). The experiments reported here directly show that at least 7 kcal/mol toward the stabilization of the oxyanion can be provided from Coulombic interaction and from placement of the oxyanion in the anionic hole.

Acknowledgments

We thank Dr. Anzi Wei of this laboratory for the X-ray structure information and Nyla Spawn for assistance in preparation of the manuscript.

Registry No. **1**, 113215-69-3; His, 71-00-1; chymotrypsin, 9004-07-3.

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