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Mechanism of Slow-Binding Inhibition of Human Leukocyte Elastase by Trifluoromethyl Ketones

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ABSTRACT: Kinetics of inhibition have been determined for the interaction of human leukocyte elastase (HLE) with two series of peptide trifluoromethyl ketones (TFMKs): X-Val-CF_3, X-Pro-Val-CF_3, X-Val-Pro-Val-CF_3, and X-Lys(Z)-Val-Pro-Val-CF_3, where X is MeOSuc or Z. These compounds are "slow-binding" inhibitors of HLE and, thus, allow the determination of K_i, the dissociation constant for the stable complex of inhibitor and enzyme, as well as k_{on} and k_{off}, the rate constants for formation and decomposition of this complex. Maximal potency is reached with Z-Lys(Z)-Val-Pro-Val-CF_3, which displays a $K_i < 0.1$ nM. Upon binding to HLE, these compounds undergo addition by the hydroxyl of the active site serine to form a hemiketal. The evidence supporting a hemiketal intermediate includes (i) K_i values of 1.6 and 80000 nM for Z-Val-Pro-Val-CF_3 and its alcohol analogue, (ii) linear free energy correlations between inhibitory potency and catalytic efficiency for structurally related TFMKs and substrates, and (iii) the pH dependence of k_{on} for the inhibition of HLE by Z-Val-Pro-Val-CF_3, which is sigmoidal and displays a pK_a of 6.9. Hemiketal formation is probably not rate limiting, however. Kinetic solvent isotope effects of unity suggest that k_{on} cannot be rate limited by a reaction step, like hemiketal formation, that is subject to protolytic catalysis. A general mechanism that is consistent with these results is one in which formation of the hemiketal is rapid and is followed or preceded by a slow step that rate limits k_{on}. This step must be insensitive to both the isotopic composition of the solvent and the pH and may be a conformational change of one of the enzyme–inhibitor complexes preceding the final complex.

Fluorine-substituted ketones are known inhibitors of a variety of hydrolytic enzymes, including acetylcholine esterase (Brodeck et al., 1979; Gelb et al., 1985), juvenile hormone esterase (Prestwich et al., 1984), carboxypeptidase A (Gelb et al., 1985), angiotensin converting enzyme (Gelb et al., 1985), pepsin (Gelb et al., 1985), phospholipase A_2 (Gelb, 1986), porcine pancreatic elastase (Imperiali & Abeles, 1986), and chymotrypsin (Imperiali & Abeles, 1986). For serine hydrolases, such as AChE[1] and PPE, trifluoromethyl ketones resembling specific substrates have been prepared and shown to be potent inhibitors. TFMKs are thought to inhibit these enzymes by combining with the active site serine to form a hemiketal that resembles the tetrahedral intermediate or transition states that occur during serine acylation by peptide substrates.

In this paper we explore the mechanism of inhibition of the serine protease human leukocyte elastase (Stein et al., 1985) by TFMKs. Our studies suggest a mechanism in which for-

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[1] Abbreviations: HLE, human leukocyte elastase; PPE, porcine pancreatic elastase; AChE, acetylcholine esterase; TFMK, trifluoromethyl ketone; MeOSuc, *N*-(methoxysuccinyl); pNA, *p*-nitroanilide; Z, *N*-(carbobenzoxy); ONP, *p*-nitrophenyl ester; Tris, tris(hydroxymethyl)-aminomethane; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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mation of the hemiketal is rapid and is followed or preceded by a slow step that rate limits the overall association process. This step may be a conformational isomerization of an enzyme–inhibitor complex that precedes the final complex.

Materials and Methods

Materials. MeOSuc-Ala-Ala-Pro-Val-pNA and MeOSuc-Ala-Pro-Ala-pNA were available from previous studies (Stein, 1983; Stein et al., 1987a). TFMKs were synthesized according to published methods (Trainor et al., 1986) and exist as *RS* racemic mixtures at P_1.[2] HLE was prepared as previously described (Stein, 1985a; Viscarello et al., 1983). Sephadex G-25M was purchased as prepackaged, disposable PD-10 "desalting" columns from Pharmacia Fine Chemicals. Buffer salts and Me_2SO were of analytical grade from several sources. D_2O was from Sigma Chemical Co.

Kinetic Assay. Reaction progress was measured spectrophotometrically by monitoring the release of *p*-nitroaniline at 410 nm during the hydrolysis of MeOSuc-Ala-Ala-Pro-Val-pNA or MeOSuc-Ala-Pro-Ala-pNA. In a typical experiment, a cuvette containing 2.89 mL of buffer and 50 μL each of Me_2SO solutions of inhibitor and substrate was brought to thermal equilibrium (5–10 min) in a jacketed holder in the cell compartment of a Cary-210 spectrophotometer. The temperature was maintained by water circulated from a Lauda K-RD bath. Injection of 10 μL of enzyme solution initiated the reaction. Absorbances were continuously measured, digitized, and stored in a Digital Electronics Corp. PDP 11/73 minicomputer. Progress curves were composed of from 600 to 1000 {absorbance, time} pairs.

Reversal of Inhibition and Determination of k_{off}. The first-order rate constant for the dissociation of the stable complex of HLE and MeOSuc-Val-Pro-Val-CF_3 was determined as follows: 2 mL of a solution of 1 μM HLE and 10 μM inhibitor was allowed to incubate at room temperature for 5 min, put on ice for 15 min, and then layered on top of a Sephadex G-25 column (1.5 × 5.1 cm) that had been equilibrated at 4 °C with a pH 7.6 buffer composed of 0.1 M sodium phosphate and 0.5 M NaCl. The column was eluted at 4 °C with this same buffer, and fractions containing the isolated HLE–TFMK complex were pooled and put on ice; 15 μL of the pooled fractions was then diluted into 2.85 mL of a 1.0 mM solution of MeOSuc-Ala-Ala-Pro-Val-pNA ([S] = $18K_m$) at 25 °C and the release of *p*-nitroaniline monitored as described above. Instantaneous velocities were determined at 15–25 time points along the progress curve, and these data were fit to the expression of eq 1 (Williams & Morrison, 1979; Morrison, 1982), where v_t is the observed velocity at time t, v_f is the final velocity attained when the enzyme–inhibitor complex is completely dissociated, v_i is the initial, near-zero velocity, and k_{off} is the first-order rate constant for the dissociation of the enzyme–inhibitor complex.

$$v_t = v_f[1 - \exp(-k_{off}t)] + v_i \quad (1)$$

Results

Kinetics of Inhibition. Biphasic reaction progress curves were observed during the inhibition of HLE by peptide trifluoromethyl ketones, as illustrated in Figure 1 for MeOSuc-Val-Pro-Val-CF_3, and indicate that these compounds are slow-binding inhibitors of HLE (Morrison, 1982; Williams & Morrison, 1979). Progress curves for slow-binding inhibitors are described by the expression of eq 2, where P is the product

$$P = v_s t + (v_0 - v_s)[1 - \exp(-k_{obsd}t)]/k_{obsd} + d \quad (2)$$

concentration (in this case, related to the absorbance by an extinction coefficient of 8800), v_0 is the reaction velocity at $t = 0$, v_s is the final steady-state velocity, k_{obsd} is the observed first-order rate constant for the approach to steady-state, and d is the displacement of P from zero at $t = 0$. Equation 2 is a general expression for any mechanism of inhibition in which the steady-state is reached by a first-order process.

To investigate the kinetic mechanism of inhibition of HLE by MeOSuc-Val-Pro-Val-CF_3, we determined progress curves at several concentrations of inhibitor and substrate. These curves were successfully fit to eq 2 by nonlinear least-squares

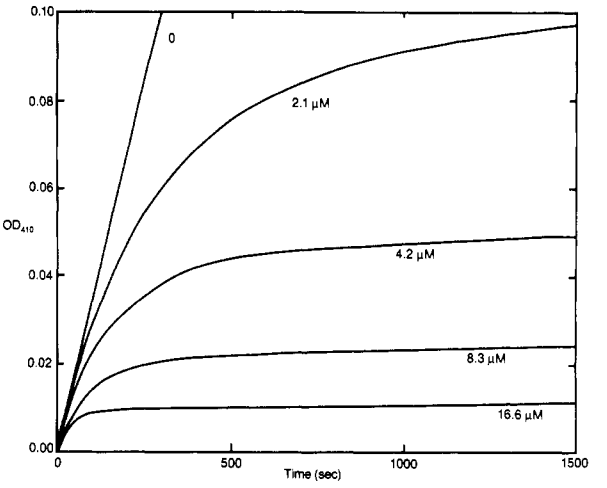


FIGURE 1: Progress curves for the inhibition of HLE by MeOSuc-Val-Pro-Val-CF_3. Absorbance at 410 nm was recorded for reaction solutions containing 7 nM HLE, 160 μM MeOSuc-Ala-Ala-Pro-Val-pNA, and the indicated concentration of inhibitor in a pH 7.6 buffer composed of 0.1 M sodium phosphate, 0.5 M NaCl, and 3.3% Me_2SO. Temperature was maintained at 25 ± 0.1 °C, and reactions were initiated by the addition of enzyme.

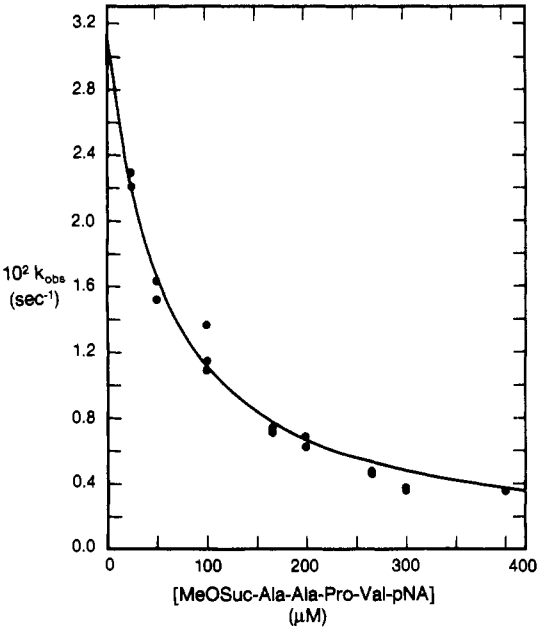


FIGURE 2: Dependence of the first-order rate constant for the approach to steady state on concentration of MeOSuc-Ala-Ala-Pro-Val-pNA. Values of k_{obsd} were determined at various concentrations of substrate in solutions containing 7 nM HLE and 5.4 μM MeOSuc-Val-Pro-Val-CF_3. The solid line is the nonlinear best fit to the data according to eq 5 for competitive inhibition and was constructed with the parameters k_{on} = 5800 M^{-1} s^{-1}, K_m = 54 μM, and k_{off} = 0.

[2] The nomenclature for the amino acid residues of substrate or inhibitor (P_1, P_2, P_3, ..., P_n) and the corresponding protease subsites to which they bind (S_1, S_2, S_3, ..., S_n) is that of Schechter and Berger (1967).

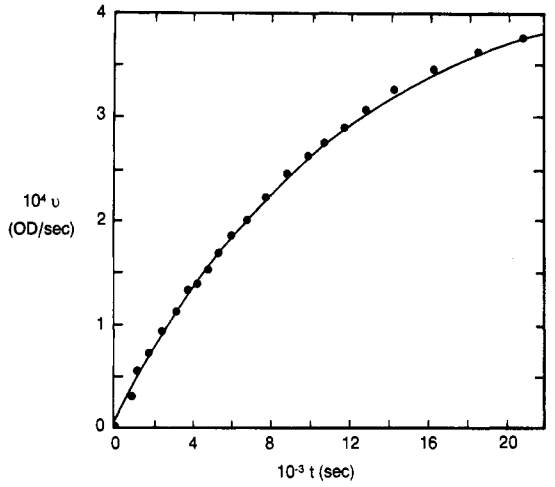


FIGURE 3: Reversal of inhibition of HLE by MeOSuc-Val-Pro-Val-CF_3. See text for details.

analysis and provided values for the three empirical parameters that interest us, v_0, v_s, and k_{obsd}.

Figure 2 contains a plot of k_{obsd} vs. [S] determined at a MeOSuc-Val-Pro-Val-CF_3 concentration of 5.4 μM. k_{obsd} decreases with increasing substrate concentration and suggests the simple competitive mechanism of Scheme I (Williams & Morrison, 1979; Morrison, 1982; Cha, 1976). The associated rate expressions for this mechanism are given in eq 3–5. Nonlinear least-squares fit of the data of Figure 2 to eq 5 yields a value for k_{on} of 5800 ± 250 M^{-1} s^{-1}. In this analysis, K_m was constrained to 54 μM (Stein, 1983) and k_{off} was set to zero (see below for justification of this assumption).

Scheme I

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} E{:}S \xrightarrow{k_c} E + P$$

$$k_{on}[I] \Big\Downarrow k_{off}$$

$$E{:}I$$

$$v_s = \frac{k_c[E][S]}{K_m(1 + [I]/K_i) + [S]} \quad (3)$$

$$K_i = k_{off}/k_{on} \quad (4)$$

$$k_{obsd} = k_{on}[I]/(1 + [S]/K_m) + k_{off} \quad (5)$$

k_{on} can also be calculated from the dependence of k_{obsd} on inhibitor concentration. When K_m and k_{off} are set to 54 μM and zero ([S] = 160 μM), respectively (see above), linear least-squares analysis of data generated in such an experiment yielded a value for k_{on} of 5450 ± 150 M^{-1} s^{-1} (data not shown). This is essentially identical with the value of k_{on} calculated from the dependence of k_{obsd} on substrate concentration and supports competitive inhibition of HLE by MeOSuc-Val-Pro-Val-CF_3. Competitive inhibition is also supported by the inability of kinetic models for noncompetitive, uncompetitive, and mixed inhibition to account for the data of Figure 2.

The analyses discussed above both assume that k_{off} is zero or, rather, $k_{off} \ll k_{on}[I]/(1 + [S]/K_m)$. We were able to justify this assumption in separate experiments (see Figure 3 and Materials and Methods) where k_{off}, in three separate experiments, was determined to be (7.8 ± 0.6) × 10^{-5} s^{-1}. This value is much smaller than typical values for the term $k_{on}[I]/(1 + [S]/K_m)$, which range from 50 × 10^{-5} to 500 × 10^{-5} s^{-1}.

The dissociation constant K_i for the inhibition of HLE by MeOSuc-Val-Pro-Val-CF_3 was calculated according to eq 6,

$$K_i = \frac{[I]}{v_c/v_s - 1} \Big/ (1 + [S]/K_m) \quad (6)$$

Table I: Dependence of Inhibition Potency on Peptide Chain Length[a]

	K_i (nM)	k_{on} (M^{-1} s^{-1})	$10^5 k_{off}$ (s^{-1})[b]
MeOSuc-Val-CF_3	53 000		
MeOSuc-Pro-Val-CF_3	3 200	2 100	670
MeOSuc-Val-Pro-Val-CF_3	13	5 600	7
MeOSuc-Lys(Z)-Val-Pro-Val-CF_3	<0.3	45 000	<1
Z-Val-CF_3	13 000		
Z-Pro-Val-CF_3	1 800		
Z-Val-Pro-Val-CF_3	1.6	25 000	4
Z-Lys(Z)-Val-Pro-Val-CF_3	<0.1	80 000	<1

[a] Conditions: 0.1 M sodium phosphate, 0.5 M NaCl, pH 7.6, 3.3% Me_2SO, 25 °C, [MeOSuc-Ala-Ala-Pro-Val-pNA] = 160 μM, and [HLE] = 7 nM. Replicate determinations indicate standard deviations for the kinetic parameters of 25% or less. [b] $k_{off} = k_{on}K_i$.

where v_c is the control velocity in the absence of inhibitor and v_s is the final, steady-state inhibited velocity derived from progress curve analysis as described above. From replicate experiments, we calculate K_i = 13 ± 3 nM. Note that the product of K_i and k_{on} should equal k_{off} (see eq 4). This product is (13 × 10^{-9} M)(5600 M^{-1} s^{-1}) or 7.3 × 10^{-5} s^{-1} and is equal to the experimentally determined value, 7.8 × 10^{-5} s^{-1}.

Reaction velocities at time zero, v_0, also derived from progress curve analysis, were found to be independent of MeOSuc-Val-Pro-Val-CF_3 concentration and suggest that any complex that forms prior to the slow step of k_{on} has a dissociation constant much greater than the highest inhibitor concentration and, thus, does not accumulate to any significant concentration. This is supported by the linear dependence of k_{obsd} on [I] mentioned above. If a complex were to accumulate prior to the rate-limiting step of k_{on}, k_{obsd} would have a hyperbolic dependence on [I], demonstrating saturation kinetics (Morrison, 1982; Kettner & Shenvi, 1984; Shapiro & Riordan, 1984; Bull et al., 1985). Keep in mind, however, that this does not preclude formation of a complex prior to the slow step of k_{on}, only that, if such a complex does form, it must have an unfavorable apparent dissociation constant, probably greater than 100 μM.

Finally, values of k_{on} and K_i were determined for the inhibition of HLE by two series of TFMKs: X-Val-CF_3, X-Pro-Val-CF_3, X-Val-Pro-Val-CF_3, and X-Lys(Z)-Val-Pro-Val-CF_3, where X = MeOSuc or Z. These values, along with k_{off} ($=k_{on}K_i$), are summarized in Table I and indicate a marked dependence of inhibitory potency on peptide chain length. This relationship between peptide chain length and potency is similar to the dependence of catalytic efficiency on chain length that is observed during substrate hydrolysis by HLE (Stein et al., 1987a).

pH Dependence of the Inhibition of HLE by Z-Val-Pro-Val-CF_3. With the methods outlined above, the kinetic parameters k_{on} and K_i were determined for the inhibition of HLE by Z-Val-Pro-Val-CF_3 at pH values between 6 and 8 and are collected, along with the pH dependence of k_{off} ($=k_{on}K_i$), in Table II. In this study we used a three-component buffer system composed of 0.10 M Tris, 0.05 M CAPS, and 0.05 M CHES with the ionic strength maintained at 0.50 M with NaCl. At each pH, the concentration of substrate, MeO-Suc-Ala-Ala-Pro-Val-pNA, was 3 times its K_m. The pH dependence of K_m for the reaction of this substrate with HLE was taken from a previous study (Stein, 1983).

The pH dependence of k_{on} is shown in Figure 4A and was fit, by nonlinear least-squares analysis, to eq 7, where $(k_{on})_{limit}$

$$k_{on} = \frac{(k_{on})_{limit}}{1 + ([H^+]/K_a)} \quad (7)$$

Table II: pH Dependence of the Inhibition of HLE by Z-Val-Pro-Val-CF_3[a]

pH	$10^{-3}k_{on}$ (M^{-1} s^{-1})	$10^{5}k_{off}$ (s^{-1})[b]	K_i (nM)
6.0	3.2 ± 0.8	20 ± 7	61 ± 14
6.5	7.4 ± 0.4	16 ± 6	22 ± 8
7.0	17 ± 0.8	5.5 ± 0.8	3.2 ± 0.5
7.5	25 ± 1.0	4.0 ± 0.5	1.6 ± 0.2
8.0	28 ± 1.4	3.4 ± 0.9	1.2 ± 0.3

[a] A three-component buffer system composed of 0.10 M Tris, 0.05 M CAPS, and 0.05 M CHES was used in this study. Ionic strength was adjusted to 0.05 M with NaCl. [HLE] = 7 nM. Concentration of inhibitor ranged from 5 to 250 μM. [Me_2SO] = 3.3%. Temperature = 25 ± 0.1 °C. [b] $k_{off} = k_{on}K_i$.

is the limiting value of the association rate constant at high pH and K_a is an acid dissociation constant (Fersht, 1985b). This analysis provided the values $(k_{on})_{limit}$ = 30 000 ± 1000 M^{-1} s^{-1} and pK_a = 6.93 ± 0.04.

The pH dependence of k_{off} (Table II, Figure 4C) indicates that dissociation of the enzyme–inhibitor complex is accelerated at low pH. Despite the large errors associated with these k_{off} values, we were able to fit these data to the expression of eq 8, where $(k_{off})_{limit}$ is the limiting value of k_{off} at low pH

$$k_{off} = \frac{(k_{off})_{limit}}{1 + (K_a/[H^+])} \quad (8)$$

and K_a is an acid dissociation constant. The parameter estimates arrived at are $(k_{off})_{limit} = (25 \pm 4) \times 10^{-5}$ s^{-1} and pK_a = 6.7 ± 0.2. Significantly, this pK_a value is identical, within experimental error, with the pK_a derived from the pH dependence of k_{on} and suggests that both the formation and decomposition of the stable complex of enzyme and inhibitor depend on the ionization of the same amino acid residue, most probably the active site histidine.

Given the pH dependencies for k_{off} and k_{on}, the pH dependence of K_i can be calculated as the ratio of eq 8 and 7: $K_i = (K_i)_{limit}([H^+]/K_a)$. In Figure 4C is shown the pH dependence of K_i with $(K_i)_{limit}$ = 10 nM and $K_a = 1.6 \times 10^{-7}$ M (pK_a = 6.8).

Correlation of the Free Energy of Activation for Catalysis with the Free Energy of Association for Inhibition. We want to establish whether there exists a correlation between inhibitory potency, as reflected in K_i, and catalytic efficiency, as reflected in k_c/K_m, for series of structurally related TFMK inhibitors and *p*-nitroanilide substrates. To test for this correlation, two types of free energy differences had to be calculated: the energy of association of enzyme and inhibitor, ΔG_{inhib}, and the activation energy for the reaction of enzyme and substrate, $\Delta G^{\ddagger}_{cat}$. Values of ΔG_{inhib} are calculated from the reciprocal of the dissociation constant, K_i, and a standard-state inhibitor concentration according to eq 9, while values

$$\Delta G_{inhib} = -RT \ln ([I]/K_i) \quad (9)$$

of $\Delta G^{\ddagger}_{cat}$ are calculated from k_c/K_m and a standard-state enzyme concentration according to eq 10. The substrates and

$$\Delta G^{\ddagger}_{cat} = -RT \ln \{([E]k_c/K_m)(h/kT)\} \quad (10)$$

corresponding k_c/K_m values used in this correlation are as follows: MeOSuc-Val-pNA, 75 M^{-1} s^{-1}; MeOSuc-Pro-Val-pNA, 575 M^{-1} s^{-1}; MeOSuc-Ala-Pro-Val-pNA, 56 000 M^{-1} s^{-1}; MeOSuc-Lys(Z)-Ala-Pro-Val-pNA, 1 000 000 M^{-1} s^{-1}. The data for the first three substrates are from a study from this laboratory (Stein et al., 1987a) while k_c/K_m for the tetrapeptide is from the work of Yasutaka and Powers (1981). k_c/K_m for the latter substrate was corrected from a reported value of 710 000 M^{-1} s^{-1} ([Me_2SO] = 10%) to reflect the lower concentration of Me_2SO used in this study.

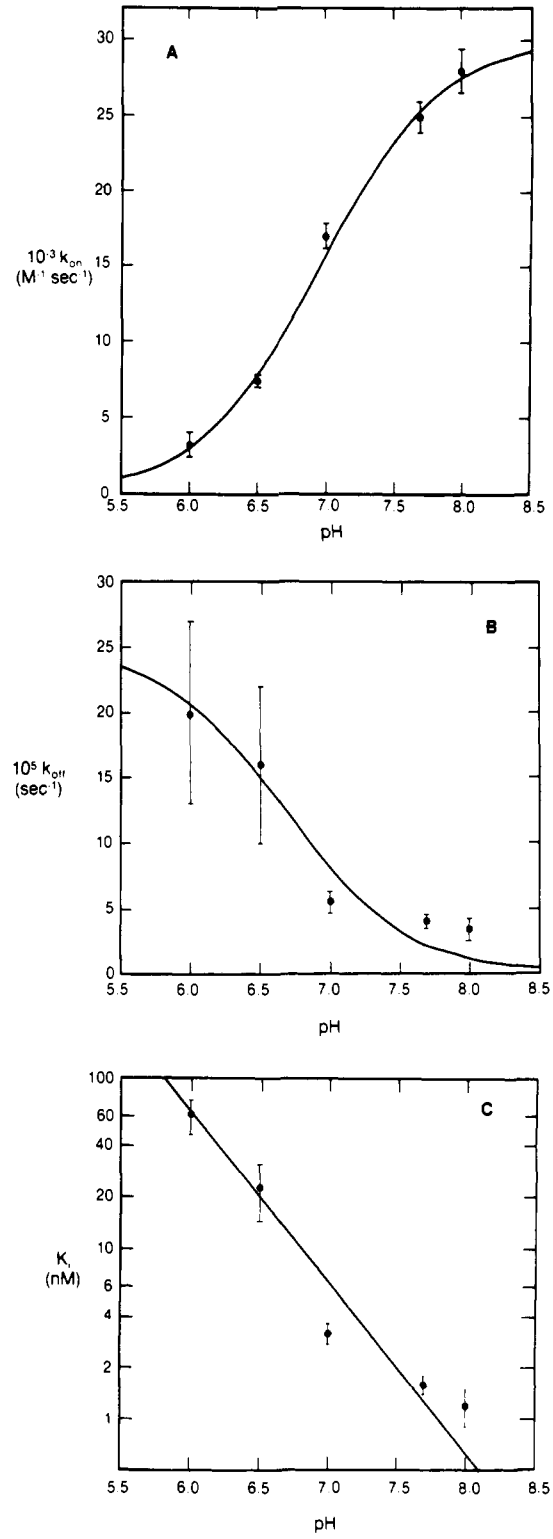


FIGURE 4: pH dependence of the inhibition of HLE by Z-Val-Pro-Val-CF_3. A three-component buffer system composed of 0.10 M Tris, 0.05 M CAPS, and 0.05 M CHES was used in this study. Ionic strength = 0.50 M (NaCl); [HLE] = 7 nM; [Me_2SO] = 3.3%; temperature = 25 ± 0.1 °C. (A) pH dependence of k_{on}. The solid line is the nonlinear best fit to the data according to eq 7 and was drawn with the parameters $(k_{on})_{limit}$ = 30 000 M^{-1} s^{-1} and pK_a = 6.9. (B) pH dependence of k_{off}. The solid line is the nonlinear best fit to the data according to eq 8 and was drawn with the parameters $(k_{off})_{limit} = 25 \times 10^{-5}$ s^{-1} and pK_a = 6.7. (C) pH dependence of K_i. The solid line was drawn with the ratio of eq 7 and 8 and the parameters $(K_i)_{limit}$ = 10 nM and pK_a = 6.8.

Figure 5 contains the plots of $\Delta G^{\ddagger}_{cat}$ ([HLE] = 10^{-8} M) for this series of substrates vs. ΔG_{inhib} ([I] = 10^{-6} M) for the MeOSuc-blocked and Z-blocked TFMKs of Table I. Both

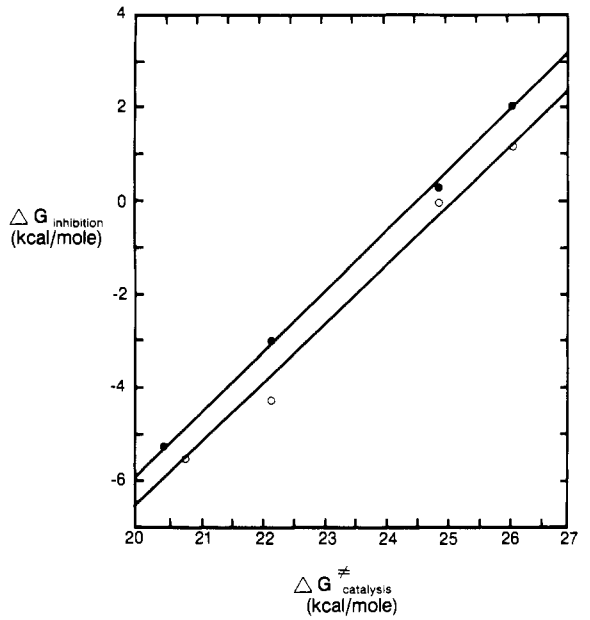


FIGURE 5: Free energy correlation between inhibitory potency and efficiency. Activation and association free energies were calculated as described in the text for a series of structurally related *p*-nitroanilide substrates and TFMKs and their correlation plotted as ΔG_{inhib} vs. $\Delta G^{\ddagger}_{cat}$ (○) Z-blocked TFMKs; (●) MeOSuc-blocked TFMKs.

Table III: Solvent Deuterium Isotope Effects for k_{on}[a]

inhibitor	k_{on} (M^{-1} s^{-1})	$^{D}k_{on}$
Suc-Val-Pro-Val-CF_3	1000	1.17 ± 0.07
MeOSuc-Pro-Val-CF_3	2100	1.20 ± 0.34
Suc-nLeu-Val-Pro-Val-CF_3	8000	0.99 ± 0.12
Z-Val-Pro-Val-CF_3	25000	0.92 ± 0.09
MeOSuc-Lys(Z)-Val-Pro-Val-CF_3	45000	1.05 ± 0.06

[a] Conditions: 0.10 M HEPES, 0.50 M NaCl, pH 7.65 and pD equivalent, 3.3% Me_2SO, and 25 ± 0.1 °C. [HLE] = 7 nM. Inhibitor concentrations were between 1 and 10 μM.

correlations are linear with slopes of 1.26 ± 0.02 and 1.29 ± 0.08, for the MeOSuc and Z series, respectively.

Solvent Deuterium Isotope Effects for k_{on}. To probe the catalytic mechanisms that operate during the association of HLE with TFMKs, solvent deuterium isotope effects were determined for k_{on} for several TFMKs. These studies were conducted with the substrate MeOSuc-Ala-Pro-Ala-pNA at a concentration of 0.3 mM (=K_m/5; Stein et al., 1987a). Under these conditions, eq 4 simplifies to $k_{obsd} = k_{on}[I]$ and facilitates determination of accurate values of isotope effects for k_{on}. Inhibition progress curves were recorded for reaction solutions containing 0.10 M HEPES and 0.50 M NaCl buffered at pH 7.65 and pD equivalent (Schowen & Schowen, 1982) and analyzed as outlined under Materials and Methods. Solvent deuterium isotope effects for k_{on} for the association of HLE with several TFMKs are summarized in Table III. Within their error limits, these values are all equal to 1 and suggest that the step that rate limits k_{on} does not involve proton transfer.

Inhibition by Z-Val-Pro-NH-CH(isopropyl)-CH(OH)-CF_3. The alcohol analogue of the Z-Val-Pro-Val-CF_3 was found to be a very poor inhibitor of HLE with a K_i value equal to 80 μM.

Discussion

In this study we have demonstrated that peptide trifluoromethyl ketones are competitive, slow-binding inhibitors of HLE. Three aspects of the inhibition of HLE by these compounds will now be considered: (i) evidence supporting an enzyme–hemiketal intermediate, (ii) kinetic mechanism of inhibition, and (iii) origin of slow-binding inhibition.

Evidence Supporting Formation of an Enzyme–Hemiketal Intermediate. Like aldehydes (Thompson & Bauer, 1979), peptide trifluoromethyl ketones are thought to form tetrahedral addition adducts with the active site serine of serine proteases (Imperiali & Abeles, 1986). Evidence supporting the formation of such adducts for trifluoromethyl ketones is outlined below.

First, alcohol analogues of trifluoromethyl ketones have greatly decreased potencies relative to the parent ketone inhibitor. This was first reported for the inhibition of chymotrypsin by Ac-Leu-Phe-CF_3 (Imperiali & Abeles, 1986). In this study, we observe a relative potency exceeding 50000 for the inhibition of HLE by Z-Val-Pro-Val-CF_3 and the corresponding secondary alcohol. Tight binding by the ketone can only be explained if it is able to develop additional, favorable interactions with HLE that are unavailable to the alcohol.

Second, we observe a linear correlation between association energies for TFMK inhibitors and activation energies for corresponding *p*-nitroanilide substrates (Figure 5). There is no reason to expect such a correlation unless the enzyme–TFMK interactions that lead to inhibition are similar to enzyme–anilide interactions that lead to substrate hydrolysis. A positive correlation is also observed between k_c/k_m and k_{on} and, again, can only be explained if the inhibition and catalytic mechanisms share essential features.

Third, the pH dependence of k_{on} (Figure 4) indicates a critical role for an amino acid residue having a pK_a of 7. This pH dependence is identical with the pH dependence of k_c/K_m observed during catalysis by HLE (Stein, 1983) and other serine proteases (Fersht, 1985b) and is consistent with the active site histidine serving as general-base catalyst during attack of the serine on the TFMK.

Finally, and certainly most convincingly, a recent X-ray crystallography study of the complex between PPE and a TFMK clearly indicates a covalent bond between the active site serine and the carbonyl carbon of the ketone (E. Meyer, personal communication).

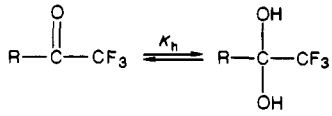
Kinetic Mechanism of Inhibition of HLE by Peptide Trifluoromethyl Ketones. In this section we will formulate a kinetic mechanism that describes the inhibition of HLE by TFMKs. We define the term "kinetic mechanism" in the broadest sense to include the number, sequence, and structure of all reaction intermediates, as well as rates and equilibria for their interconversion.

The starting point for our discussion is a consideration of the form of the inhibitor that is initially bound by the enzyme. Since in aqueous solution TFMKs are in equilibrium with their hydrate[3] (see Scheme II), HLE could conceivably bind either of these species. If it binds the ketone, formation of the hemiketal simply involves serine addition. However, if HLE binds the hydrate, it must first catalyze the dehydration of this species before serine addition can occur. On the basis of arguments advanced by others for the inhibition of serine proteases by aldehydes (Thompson & Bauer, 1979) and

[3] The mechanism advanced in this paper for the slow-binding inhibition of HLE is based on the assumption that the equilibrium of eq 11 is established rapidly relative to binding of the ketone to the enzyme. The validity of this assumption is supported by the following: (1) k_{obsd} is dependent on the inhibitor concentration as well as the substrate concentration; (2) k_{on} shows a sigmoidal pH dependence with an inflection at pH 6.9; (3) k_{on} is dependent on inhibitor structure of the form R-Pro-Val-CF_3; (4) k_{on} is generally much slower for porcine pancreatic elastase (unpublished results). None of these results can be explained by a slow hydration/dehydration equilibrium.

TFMKs (Imperiali & Abeles, 1986), we will assume in the remainder of our discussion that only the ketone form of the inhibitor binds to the enzyme.

Scheme II



$$K_h = [\text{hydrate}]/[\text{ketone}] \quad (11)$$

Our assumption that serine proteases bind only the carbonyl form of TFMKs requires that we correct the observed values of K_i and k_{on} for the fraction of total inhibitor that exists as the unreactive hydrate if these parameters are to reflect the interaction of enzyme and free ketone. For the inhibition constant, we define

$$(K_i)_{cor} = K_i/(1 + K_h) \quad (12)$$

This value reflects the equilibrium between the stable enzyme–inhibitor complex and free enzyme and ketone. Since K_h is about 100 for TFMKs (Imperiali & Abeles, 1986; Ritchie, 1984), values of $(K_i)_{cor}$ are about 100 times smaller than the experimentally observed K_i values of Table I. Likewise, for association rate constants, we define

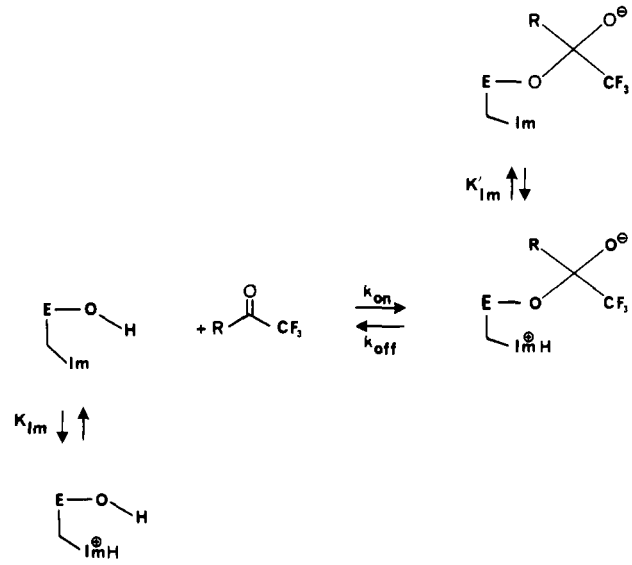
$$(k_{on})_{cor} = k_{on}(1 + K_h) \quad (13)$$

$(k_{on})_{cor}$ reflects the interaction of HLE and unhydrated ketone and is approximately equal to the observed value of k_{on}, as reported in Table I, multiplied by 100.

Our development of a kinetic mechanism for the inhibition of HLE by TFMKs starts with the pH dependence of inhibition and what it reveals about the structure of the hemiketal intermediate that accumulates. The pH dependence of k_{on} (Figure 4A) is sigmoidal and titrates with a pK_a of 6.9, indicating that a critical, ionizable amino acid residue in its basic form is required for inhibition. Significantly, this pK_a value is only slightly lower than pK_a values near 7.2 determined for k_c/K_m during HLE-catalyzed hydrolyses of peptide *p*-nitroanilides (Stein, 1983; R. L. Stein and A. M. Strimpler, unpublished results). It is reasonable to assume that the pK_a value determined for both TFMK inhibition and anilide hydrolysis is the dissociation constant of the active site histidine residue. A mechanism that is consistent with these observations involves general-base catalysis by histidine of serine addition to the carbonyl carbon of the ketone to form a tetrahedral adduct. The pH dependence of k_{off} is also sigmoidal and titrates with a dissociation constant of 6.7. In this case, however, activity depends on the acid form of the amino acid residue. Since k_{off} must be the microscopic reverse of k_{on}, the reaction governed by k_{off} is the general-acid catalyzed decomposition of the hemiketal intermediate with the imidazolium cation of the histidine residue serving as general acid. Finally, the pH dependence of K_i indicates that over the pH range investigated the most stable complex of enzyme and inhibitor has a charge of −1 relative to free enzyme and inhibitor (Dixon & Webb, 1979). An identical pH dependence was observed by Schultz and Cheerva (1975) for the inhibition of chymotrypsin by hydrocinnamaldehyde.

Together, these dependencies support the minimal mechanism of Scheme III in which K_{Im} and K_{Im}' are the acid dissociation constants of the imidazole of the active site histidine residue in the free enzyme and hemiketal complex, respectively. Essential features of this mechanism are (i) the active site histidine of the free enzyme has to be in its basic form to allow combination of enzyme and TFMK, (ii) the histidine residue of the hemiketal complex must be protonated for the complex

Scheme III



to dissociate, (iii) K_{Im} and K_{Im}' are identical and approximately equal to 6.8 (see Figure 4A,B), and (iv) over the pH range investigated the hydroxyl of the hemiketal exists as the unprotonated oxyanion.

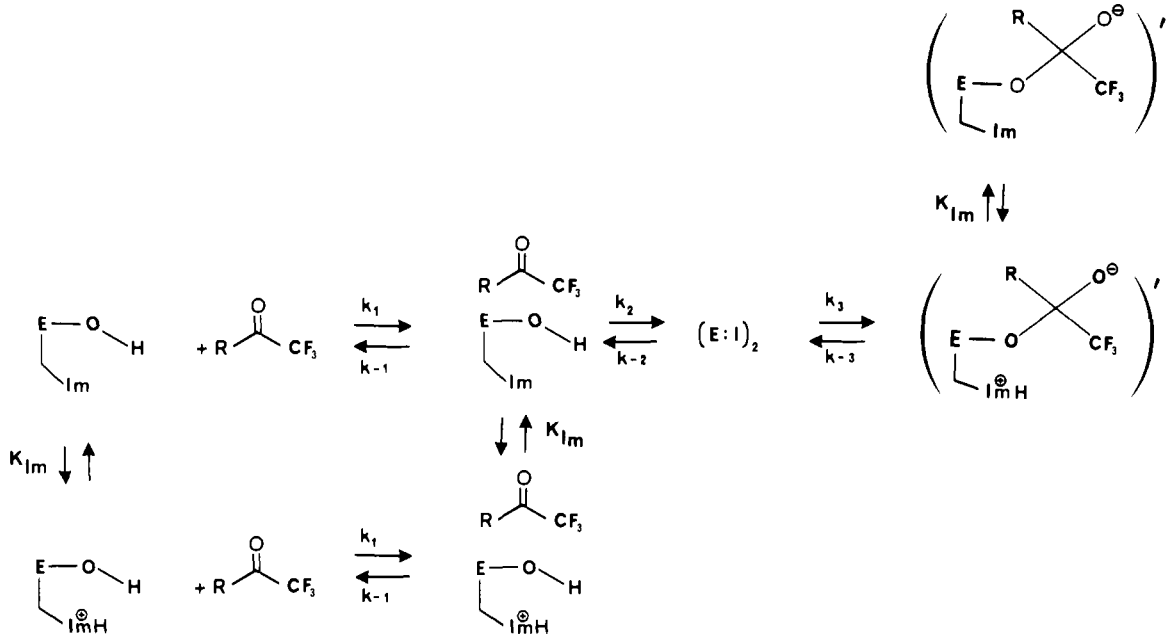
The minimal mechanism of Scheme III cannot explain all of the data, however. Scheme III indicates that k_{on} is rate-limited by serine addition to the carbonyl carbon on the inhibitor. The pH dependence of k_{on}, as well as experience in small molecule systems (Jencks, 1969), suggests that this reaction is subject to general-base catalysis by the imidazole of the histidine. Typically, reactions such as these generate solvent kinetic isotope effects between 2 and 4 (Bell, 1966; Stein, 1983; Stein & Strimpler, 1987; Stein et al., 1987b; Venkatasubban & Schowen, 1985). In this study, however, values of unity were determined for the interaction of HLE with five TFMKs and indicate that there is no proton transfer in the rate-limiting transition states for these reactions. We interpret these isotope effects to suggest that hemiketal formation does not rate limit k_{on} but rather that some other step, insensitive to the isotopic composition of the solvent, is rate limiting. To accommodate this view, we expand Scheme III to include another reaction step and thus another intermediate. The most general form for this mechanism is shown in Scheme IV. This mechanism also includes an initially formed encounter complex, analogous to the "Michaelis complex" formed during substrate hydrolysis. In Scheme IV, E is HLE, I is the ketone form of the inhibitor, $(E:I)_1$ is the encounter complex of enzyme and inhibitor, and $(E:I)_3$ is the final form of the hemiketal that accumulates. The identity of the central complex, $(E:I)_2$, depends on the mechanism.

Scheme IV

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (E:I)_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (E:I)_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} (E:I)_3$$

There are two principle mechanistic alternatives and two possible identities for $(E:I)_2$. According to the first, mechanism I, $(E:I)_2$ is a hemiketal formed from addition of serine within the Michaelis complex, $(E:I)_1$. If mechanism I obtains, hemiketal formation would have to be rapid and followed by a rate-limiting physical step, such as a conformational change of $(E:I)_2$. This conformational change would generate no isotope effect and would produce the final complex $(E:I)_3$, a "tightened" form of $(E:I)_2$ in which optimal interactions between enzyme and inhibitor have been achieved. Similar mechanisms have been proposed to account for the slow-

Scheme V



binding inhibition of several other enzymes (Baici & Gyger-Marazzi, 1982; Bull et al., 1985; Kettner & Shenvi, 1984; Rich & Sun, 1980; Shapiro & Riordan, 1984; Williams et al., 1980). According to the second alternative, mechanism II, the Michaelis complex, $(E:I)_1$, is followed by its slow and rate-limiting conversion to $(E:I)_2$, a complex in which the orientation of the inhibitor within the active site has been adjusted for efficient attack of the serine on the carbonyl carbon to form the hemiketal, $(E:I)_3$. This mechanism is similar to that advanced for substrate hydrolysis by HLE (Stein, 1983, 1985a; Stein et al., 1987a,b).

Unfortunately, these mechanisms cannot be distinguished by the results of this study. Both alternatives can be satisfied by the general mechanism of Scheme V.

According to this mechanism, free enzyme and the Michaelis complex ionize similarly to the free enzyme of Scheme III, and the final complex that forms ionizes like the hemiketal of Scheme III. The key feature here is that $(E:I)_2$, regardless of its structure, does not ionize at all. Thus, for either mechanism I or mechanism II, the observed pH dependencies are predicted to be simple and governed by the ionization of the active site histidine regardless of which step is rate limiting.

A third, more complex mechanism should also be mentioned but will not be discussed in any detail. This combines important features of mechanisms I and II and involves conformational isomerizations of both the Michaelis complex, as in mechanism II, and the hemiketal, as in mechanism I. Further experimentation is clearly needed to allow us to choose the mechanistic proposal that most closely reflects reality.

Origins of Slow-Binding Inhibition of HLE by TFMKs. The discussion in the previous section outlined several mechanistic alternatives for the inhibition of HLE by TFMKs but made no attempt to explain why this inhibition is slow to develop. In this section, we will consider this feature of inhibition. We start from the kinetic premise that the origins of slow-binding inhibition are to be found in those factors that diminish the magnitudes of k_{on} and k_{off} relative to those values for structurally and mechanistically related classical inhibitors (Stein & Strimpler, 1987). For the inhibition of HLE by TFMKs, we will argue two points: (1) Although $(k_{on})_{cor}$ values are of magnitudes that, in and of themselves, would not contribute to the slow development of inhibition, the product of $(k_{on})_{cor}$ and the effective inhibitor concentration is small enough to be a factor in this phenomenon. (2) k_{off} is unusually small due to the pronounced stability of the final enzyme–inhibitor complex and reflects the structural analogy between this complex and transition states encountered during catalysis of substrate hydrolysis.

For the inhibitors of Table I, calculated values of $(k_{on})_{cor}$ range from approximately 200 000 to 8 000 000 M^{-1} s^{-1}. These values are similar in magnitude to rate constants for other associative reactions between enzymes and small ligands (Fersht, 1985a) and would not be expected to cause slow-binding inhibition. However, the important quantity to consider here is not $(k_{on})_{cor}$ but rather the pseudo-first-order association rate constant $(k_{on})_{cor}[I]$. Although the total concentration of inhibitor used in typical inhibition experiments with TFMKs is generally above 10^{-6} M, the effective inhibitor concentration (i.e., the concentration of ketone) is about 100 times less than this, or 10^{-8} M. This concentration of inhibitor leads to association half-times ranging from 10 to 350 s. On the time scale of our kinetic experiments, these half-times could certainly contribute to the slow development of inhibition.

k_{off} values for the more potent TFMKs are less than 10^{-4} s^{-1} and indicate a marked stability for the final complex, $(E:I)_3$. Two points are important here: (i) this stability is greater than expected for an enzyme-bound hemiketal derived from a trifluoromethyl ketone, and (ii) this stability is due to structural analogy of the complex with catalytic transition states.

We support the first point as follows. The dissociation constant associated with an enzyme-bound hemiketal can be estimated as the product of the dissociation constant for the initially formed encounter complex and a dissociation constant that reflects the equilibrium between the hemiketal and the first complex. The latter should be similar in magnitude to dissociation constants for TFMK hydrates (i.e., $1/K_h$, see Scheme II), which are approximately 10^{-2} (Imperiali & Abeles, 1986; Ritchie, 1984), while the former should be similar to dissociation constants for simple Michaelis complexes observed during substrate hydrolysis, approximately 10^{-4} M (Stein & Trainor, 1986; Stein et al., 1987a). The product of these values is 10^{-6} M and represents the inhibition constant for a hypothetical TFMK that does nothing more than bind to HLE and subsequently undergo addition by the serine.

Given that $(K_i)_{cor}$ values for the more potent TFMKs are less than 10 pM, this dissociation constant underestimates the potency of these inhibitors by at least 5 orders of magnitude! This clearly indicates that the final enzyme–inhibitor complex enjoys a stability that would not have been predicted simply on the basis of hemiketal formation. We believe that this enhanced stability originates in structural similarities between the final hemiketal complex and transition states for catalysis.

Evidence supporting the claim that these compounds are transition-state analogues is provided in the free energy correlations of Figure 5. The linearity and near unity slopes of these relationships indicate that changes in peptide structure that increase catalytic efficiency also increase inhibitory potency. Structural features that promote the stabilization of the transition state for k_c/K_m also cause a stabilization of the final enzyme–inhibitor complex. The realization of this relationship constitutes, by definition, transition-state analogue inhibition.

Summary

In this paper we reported that peptide trifluoromethyl ketones are slow-binding inhibitors of human leukocyte elastase. Slow-binding inhibition by trifluoromethyl ketones has also been observed for acetylcholine esterase (Gelb et al., 1985), porcine pancreatic elastase, and chymotrypsin (Imperiali & Abeles, 1986) and may be a general mechanistic feature of inhibition of serine hydrolases by trifluoromethyl ketones.

Inhibition of human leukocyte elastase by trifluoromethyl ketone involves the formation of an enzyme-bound hemiketal generated from the attack of the active site serine on the ketone carbonyl carbon of the inhibitor. The enzyme intermediate that ultimately accumulates is stabilized, relative to a simple hemiketal, from interactions of the peptide portion of the inhibitor with remote protease subsites. This additional increment of stabilization is greater than 7 kcal/mol and reflects the structural analogy between the final complex and transition states that occur during catalysis of substrate hydrolysis.

The mechanism by which this final complex forms involves the initial formation of a Michaelis complex followed by a rate-limiting conformational change of an enzyme–inhibitor complex. Finer resolution of the inhibitory mechanism is currently impossible. Two mechanistic alternatives are allowed by our data and differ in the identity of the reaction step that rate limits k_{on}. In one mechanism, k_{on} is rate limited by a conformational change of the initially formed Michaelis complex. This conformational change would presumably align the active site Ser for more favorable attack on the ketone. According to the second mechanism, k_{on} is rate limited by a conformational change of the enzyme-bound hemiketal intermediate. This conformational change would allow optimal interactions between enzyme and inhibitor to be realized and would result in the final, highly stabilized complex.

A mechanism similar to the latter has been proposed to explain the tight-binding inhibition of chymotrypsin, pancreatic elastase, and leukocyte elastase by peptide boronic acids (Kettner & Shenvi, 1985) and chymotrypsin by chymostatin (Stein & Strimpler, 1987). This final conformational isomerization may be a general requirement of serine hydrolase inhibitors that form tetrahedral adducts resembling catalytic transition states.

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