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Slow-Binding Inhibition of Chymotrypsin and Cathepsin G by the Peptide Aldehyde Chymostatin

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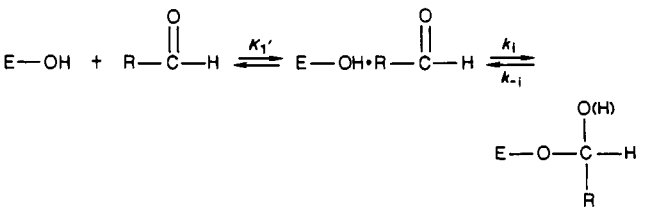
ABSTRACT: The microbial, peptide-derived aldehyde chymostatin is a potent, competitive inhibitor of chymotrypsin and cathepsin G: $K_i = 4 \times 10^{-10}$ and 1.5×10^{-7} M, respectively. It is a "slow-binding inhibitor" of both proteases and, as such, allows determination of rate constants for its association with and dissociation from these proteases. Inhibition kinetics indicate second-order rate constants for the association of chymostatin with chymotrypsin and cathepsin G of 360 000 and 2000 M^{-1} s^{-1}, respectively, and a first-order rate constant for the dissociation of both protease–chymostatin complexes of approximately 0.0002 s^{-1}. Thus, the extreme difference in potency of chymostatin as an inhibitor of chymotrypsin and cathepsin G originates entirely in k_{on}. Solvent deuterium isotope effects (SIE) were determined to probe the reaction step that rate limits k_{on}. For the reaction of chymotrypsin with chymostatin, the SIE for k_{on} is 1.6 ± 0.1, while for the reaction of chymotrypsin with the peptide substrates Ala-Ala-Phe-pNA and Suc-Ala-Ala-Pro-Phe-pNA, the SIE's for k_c/K_m are 2.8 ± 0.2 and 1.9 ± 0.1, respectively. These results suggest that k_{on} for the association of chymotrypsin with chymostatin is at least partially rate limited by a reaction step involving proton transfer. Combined with results for the inhibition of chymotrypsin by Bz-Phe-H [Kennedy, W. P., & Schultz, R. M. (1979) *Biochemistry 18*, 349–356], these data suggest a mechanism for inhibition by chymostatin involving the general-base-catalyzed formation of an enzyme-bound hemiacetal, followed by a conformational change of this intermediate that produces the final, stable complex of enzyme and inhibitor. For the inhibition of chymotrypsin, the transition states of these two reaction steps are of similar energy, and each partially rate limits the overall process governed by k_{on}.

Chymostatin is a microbial inhibitor of serine proteases (Umezawa et al., 1970; Tatsuta et al., 1973; Umezawa, 1976; Feinstein et al., 1976; Marossy et al., 1981; Bromme & Kleine, 1984; Galpin et al., 1984). It is isolated from various strains of *Streptomyces* as three forms, chymostatins A, B, and C, that differ only in the amino acid residue at the second position (Chart I). The major form is chymostatin A and typically accounts for greater than 80% of the mixture (Delbaere & Brayer, 1985). The Phe residue at the primary position predisposes the inhibitor for binding to chymotrypsin and other serine proteases having a primary specificity for Phe.

Scheme I



Chymostatin, like other peptide-derived aldehydes, is thought to inhibit serine proteases according to the minimal

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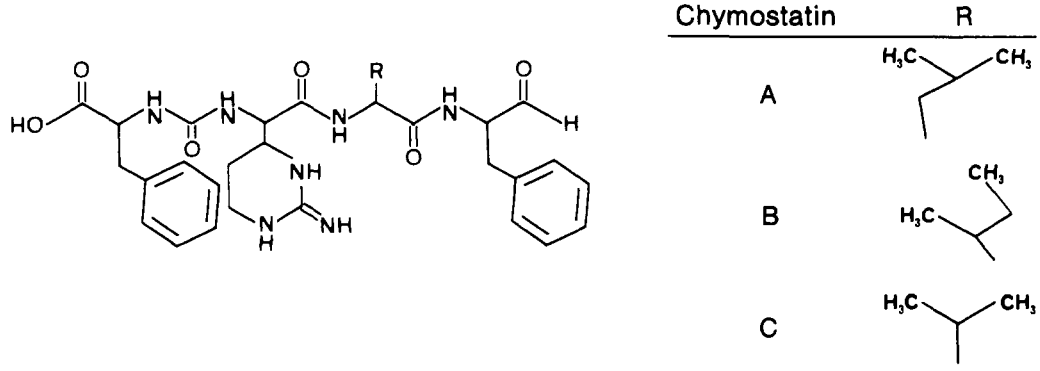
Chart I



mechanism of Scheme I, involving initial formation of an encounter complex of enzyme and aldehyde, E·I, followed by attack of the active-site serine on the carbonyl carbon of the inhibitor to form a hemiacetal, E–I (Delbaere & Brayer, 1985; Kennedy & Schultz, 1979; Thompson & Bauer, 1979). Using stopped-flow, rapid kinetic techniques, Kennedy and Schultz (1979) were able to determine the mechanistic constants K_i, k_i, and k_{-i} for the inhibition of chymotrypsin by Bz-Phe-H.[1] Conventional kinetic techniques are generally too slow for the determination of these parameters and limit the kinetic characterization of the inhibition of proteases by aldehydes to only the determination of K_i, the overall dissociation constant for E–I (see eq 1).

$$K_i = K_i' k_{-i}/k_i \quad (1)$$

In this paper, we report that, for the inhibition of chymotrypsin and cathepsin G by chymostatin, rates for the formation and decomposition of E–I are slow enough to readily quantitate by standard, spectrophotometric assay methods. This sort of inhibition has become known as "slow-binding" inhibition (Morrison, 1982) and has previously not been reported for reactions of serine proteases and aldehydes. We also report that previous studies greatly underestimate the potency of chymostatin for chymotrypsin. The original IC_{50} value of 0.3 μM (Umezawa et al., 1970) and a more recent K_i value of 0.03 μM (Feinstein et al., 1976) are very different from the K_i of 4×10^{-10} M that we find.

Materials and Methods

Materials. Chymostatin was purchased from Sigma Chemical Co. (lots 104F-0888 and 55F-0493) and Bachem Fine Chemicals (lot 6372). α-Chymotrypsin, the chromogenic substrates Ala-Ala-Phe-pNA and Suc-Ala-Ala-Pro-Phe-pNA,[1] and D_2O (98%) were also purchased from Sigma Chemical Co. Cathepsin G was a generous gift of Dr. James Travis. Sephadex G-25M was purchased as prepackaged, disposable PD-10 "desalting" columns from Pharmacia Fine Chemicals. Buffer salts and Me_2SO were analytical grade from several sources. Unless otherwise noted, pH 7.6 phosphate buffer containing 10 mM sodium phosphate and 500 mM NaCl was used. Buffer solutions in H_2O and D_2O were prepared as previously described (Stein, 1983).

Kinetic Procedures. Reaction progress was measured spectrophotometrically by monitoring the release of *p*-nitroaniline at 410 nm. In a typical experiment, a cuvette containing 2.89 mL of phosphate buffer and 50 μL each of Me_2SO solutions of chymostatin and Suc-Ala-Ala-Pro-Phe-pNA 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-2/RD bath. Injection of 10 μL of stock enzyme solution initiated the reaction. Absorbances were continuously measured, and the measurements were digitized and stored in a Digital Equipment Corp. PDP 11/73 minicomputer. The interface to the Cary spectrophotometer used its digital interface port. Progress curves were composed of from 500 to 1000 {absorbance, time} pairs.

Progress Curve Analysis (Williams & Morrison, 1979; Morrison, 1982). Progress curves for inhibition were fit by nonlinear least-squares analysis to the integrated expression

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

where P is the product 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 first-order rate constant for the approach to steady state, and d is the displacment of P from zero at $t = 0$. Equation 2 predicts identical values of v_s and k_{obsd} regardless of whether the reaction is initiated by enzyme or substrate.

Reversal of Inhibition and Determination of k_{off}. The first-order rate constant for the dissociation of the stable complex of chymotrypsin and inhibitor was determined as follows: 2 mL of a solution of 0.05 μM chymotrypsin and 5.0 μM chymostatin 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-25M column (1.5 × 5.1 cm) that had been equilibrated with phosphate buffer at 4 °C. The column was eluted at 4 °C with phosphate buffer, and the two 1-mL fractions containing the isolated chymotrypsin–chymostatin complex were pooled and put on ice. Fifteen microliters of this solution was then diluted into 2.85 mL of a 0.5 mM solution of Suc-Ala-Ala-Pro-Phe-pNA ([S] = $10K_m$) at 25 °C, and the release of *p*-nitroaniline was 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 (Williams & Morrison, 1979; Morrison, 1982)

$$v = v_f[1 - \exp(-k_{off}t)] + v_i \quad (3)$$

where v is observed velocity at time t, v_f is the final velocity attained when the enzyme–inhibitor complex is completely dissociated, and v_i is the initial, near-zero velocity.

Results

Kinetics of Substrate Hydrolysis. By standard steady-state techniques (Stein, 1985a), kinetic parameters for substrate hydrolyses were estimated as follows: for Ala-Ala-Phe-pNA and chymotrypsin, $k_c = 7 \pm 1$ s^{-1} and $K_m = 800 \pm 100$ μM;

[1] Abbreviations: Bz, benzoyl; Suc, succinyl; pNA, *p*-nitroanilide; SIE, solvent deuterium isotope effect; Me_2SO, dimethyl sulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

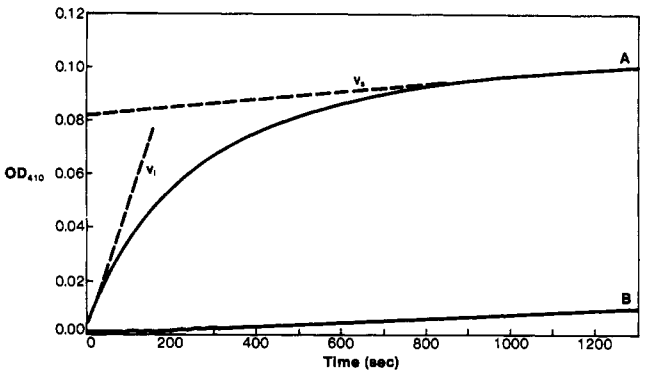


FIGURE 1: Progress curves for inhibition of chymotrypsin by chymostatin. Reaction conditions: [chymotrypsin] = 3.3 nM, [Suc-Ala-Ala-Pro-Phe-pNA] = 155 μM, [chymostatin] = 0.2 μM, phosphate buffer (10 mM sodium phosphate, 500 mM NaCl, pH 7.6), 3.3% Me_2SO, and 25 °C. Curve A, reaction initiated by enzyme; curve B, reaction initiated by substrate, after a 15-min incubation of enzyme and inhibitor.

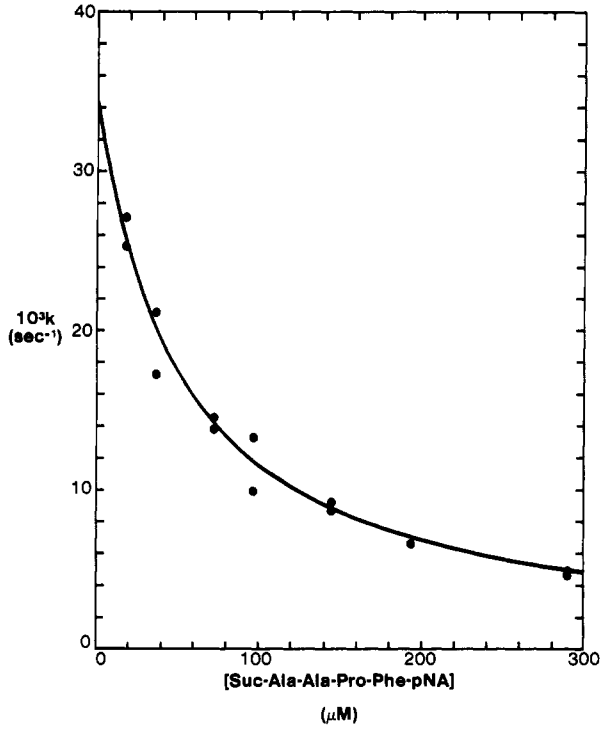


FIGURE 2: Dependence of the rate constant for association of chymotrypsin with chymostatin on substrate concentration. Solid line drawn according to eq 5 with $k_{on} = 3.5 \times 10^5$ M^{-1} s^{-1}, [chymostatin] = 0.1 μM, K_m = 50 μM, and $k_{off} = 0.0003$ s^{-1}.

for Suc-Ala-Ala-Pro-Phe-pNA and chymotrypsin, $k_c = 52 \pm 3$ s^{-1} and $K_m = 55 \pm 4$ μM; for Suc-Ala-Ala-Pro-Phe-pNA and cathepsin G, $k_c = 7.6 \pm 0.6$ and $K_m = 6.0 \pm 0.6$ mM.

Inhibition of Chymotrypsin. Progress curves for the inhibition of chymotrypsin by chymostatin were found to be biphasic, as illustrated in Figure 1, and could be successfully fit to eq 2 by nonlinear least-squares analysis. Using this method, we calculated values of v_s and k_{obsd} for a series of progress curves determined at various concentrations of inhibitor and substrate. Figure 2 is a plot of k_{obsd} vs. [S] determined at a single chymostatin concentration of 0.1 μM. The general shape of this plot together with the linearity of plots of k_{obsd} vs. [I] (see Figure 3) suggests the simple, competitive mechanism of Scheme II (Williams & Morrison, 1979; Morrison, 1982; Cha, 1976). The inhibition parameters of Scheme II are related to those of Scheme I in that $k_{on} = k_i/K_i'$ and $k_{off} = k_{-i}$.

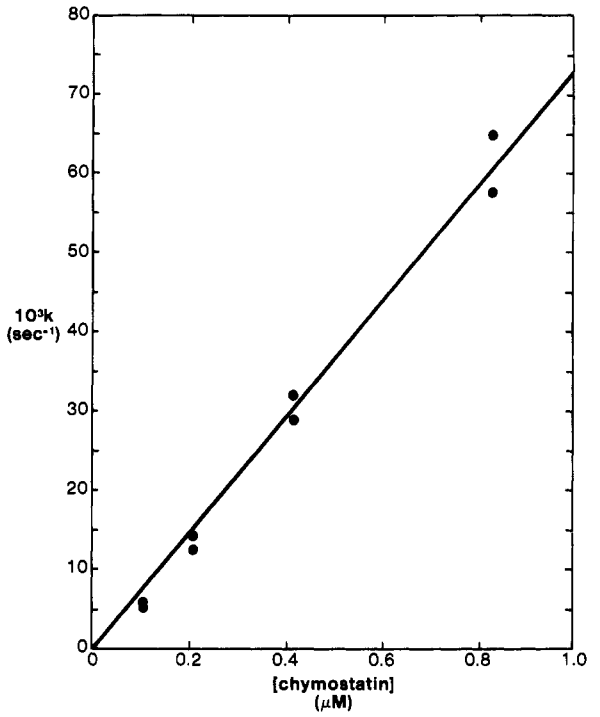


FIGURE 3: Dependence of rate constants for association of chymotrypsin with chymostatin on chymostatin concentration. Solid line drawn according to eq 5 with $k_{on} = 2.9 \times 10^5$ M^{-1} s^{-1}, [Suc-Ala-Ala-Pro-Phe-pNA] = 155 μM, K_m = 50 μM, and $k_{off} = 0.0003$ s^{-1}.

Scheme II

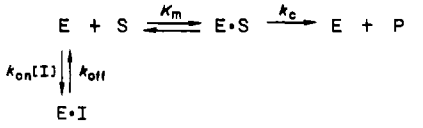


Table I: Inhibition of Chymotrypsin and Cathepsin G by Chymostatin

enzyme	chymostatin lot no.	10^9K_i (M)	$10^{-3}k_{on}$ (M^{-1} s^{-1})	$10^4k_{on}K_i$ (s^{-1})
chymotrypsin[a]	S1[b]	0.41	460	1.9
	S2	0.42	290	1.2
	B	0.36	340	1.2
		0.39 ± 0.04	360 ± 70	1.4 ± 0.5
cathepsin G[c]	S1	190	3.2	6.1
	S2	170	1.7	2.9
	B	80	1.2	1.0
		150 ± 60	2.0 ± 1.0	3.3 ± 2.6

[a] [Suc-Ala-Ala-Pro-Phe-pNA]$_0$ = 0.16 mM = 3K_m; [chymotrypsin]$_0$ = 3.3 nM; 10 mM sodium phosphate, 500 mM NaCl, pH 7.6, 3.3% Me_2SO, 25 ± 0.1 °C. [b] S1, Sigma 104F-0888; S2, Sigma 55F-0493; B, Bachem 6372. [c] [Suc-Ala-Ala-Pro-Phe-pNA]$_0$ = 0.50 mM = K_m/12; [cathepsin G]$_0$ = 120 nM; 10 mM sodium phosphate, 500 mM NaCl, pH 7.6, 3.3% Me_2SO, 25 ± 0.1 °C.

Given this mechanism, the following parameters can be defined (Williams & Morrison, 1979; Morrison, 1982; Cha, 1976):

$$v_s = k_c[E][S]/[K_m(1 + [I]/K_i) + [S]] \quad (4)$$

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

$$K_i = k_{off}/k_{on} \quad (6)$$

Values of K_i, calculated at several concentrations of inhibitor according to a rearranged form of eq 4, and k_{on}, calculated from the slope of plots of k_{obsd} vs. [I] (see eq (5), are summarized in Table I for three lots of chymostatin. The average of these data yields $K_i = (3.9 \pm 1.4) \times 10^{-10}$ M and $k_{on} = (3.6 \pm 0.7) \times 10^5$ M^{-1} s^{-1}.

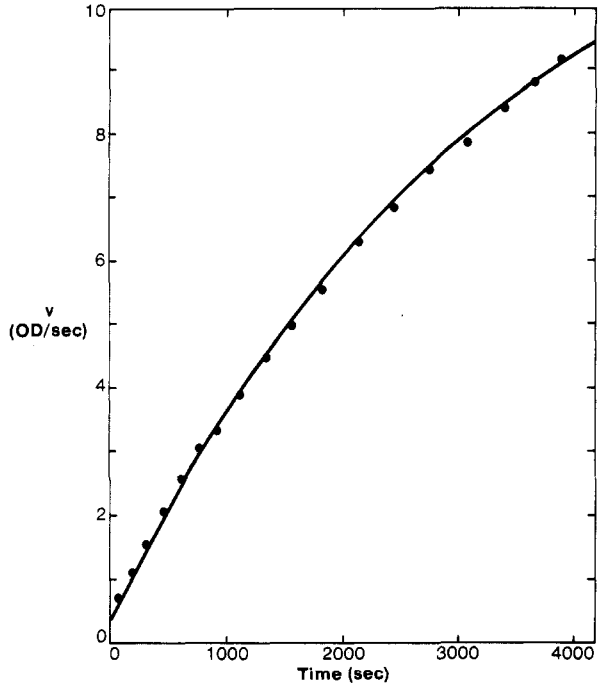


FIGURE 4: Reversal of inhibition of chymotrypsin by chymostatin. See text for details.

Nonlinear least-squares fit of the data of Figure 2 to the expression for competitive inhibition of eq 5 gave a value for k_{on} of $(3.5 \pm 0.2) \times 10^5$ M^{-1} s^{-1}. The agreement of this value with that described above and the fact that the data of Figure 2 could not be fit to mechanistic models of mixed inhibition indicate that chymostatin is a competitive inhibitor of chymotrypsin.

k_{off} was determined to be $(3.2 \pm 0.3) \times 10^{-4}$ s^{-1} in triplicate experiments (see Figure 4) and is statistically different from the value for $k_{on}K_i$ of $(1.4 \pm 0.5) \times 10^{-4}$ s^{-1} ($p < 0.01$). $k_{on}K_i$ should equal k_{off} if the mechanism of Scheme I holds (see eq 16). That k_{off} and $k_{on}K_i$ are not more similar may signal that chymostatin does not interact with chymotrypsin according to the simple mechanism of Scheme I. In any event, it is clear that the stable complex of chymotrypsin and chymostatin dissociates slowly with a rate constant between 1×10^{-4} and 3×10^{-4} s^{-1}.

Inhibition of Cathepsin G. Progress curves for the inhibition of cathepsin G by chymostatin are also characteristic of slow-binding inhibition (data not shown) and were analyzed according to methods described for chymotrypsin. Values of K_i and k_{on} for the inhibition of cathepsin G by chymostatin are summarized in Table I.

The results summarized in Table I reveal that chymostatin inhibits chymotrypsin 380 times more strongly than it inhibits cathepsin G. The striking feature of these data is that this extreme difference in potency originates entirely in k_{on}: k_{off} values (assumed equal to $k_{on}K_i$) for the two inhibition reactions are identical within experimental error.

Solvent Deuterium Isotope Effects for k_{on}. To probe the catalytic mechanisms operative during the association of chymotrypsin with chymostatin, solvent deuterium isotope effects were determined for k_{on}. These studies were conducted with the substrate Ala-Ala-Phe-pNA at a concentration of 0.2 mM ($=K_m/4$). Under these conditions, eq 5 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 and analyzed as outlined under Materials and Methods.

Table II: Inhibition of Chymotrypsin by Bz-Phe-H[a] and Chymostatin

inhibitor	10^9K_i (M)	$10^{-6}k_{on}$ (M^{-1} s^{-1})	k_{off} (s^{-1})
Bz-Phe-H	28000	1.5	42
chymostatin	0.4	0.4	0.0003

[a] Data of Kennedy and Schultz (1979), pH 8.0.

The solvent isotope effect on k_{on} for the association of chymotrypsin with chymostatin was found to be of 1.6 ± 0.1. For comparison, solvent isotope effects on k_c/K_m for the chymotrypsin-catalyzed hydrolyses of Suc-Ala-Ala-Pro-Phe-pNA and Ala-Ala-Phe-pNA were determined and found to be 1.9 ± 0.1 and 2.8 ± 0.2, respectively.

Discussion

An important problem in contemporary enzymology is determining the mechanistic origins of slow-binding inhibition. Slow-binding inhibitors are distinguished from their classical counterparts by diminished values of k_{on} and k_{off} (Scheme II), which are typically several orders of magnitude smaller than those of classical inhibitors. To understand slow-binding inhibition, we therefore need to understand the mechanisms that determine the magnitudes of k_{on} and k_{off}.

In the present case, our goal is to understand why chymostatin is a slow-binding inhibitor of the serine proteases chymotrypsin and cathepsin G. To solve this problem, we will not only require information about association transition states, to account for k_{on}, but we will also need information about stabilizing features that operate in enzyme–inhibitor complexes, to account for k_{off}.

Structural Features of the k_{on} Transition State. Two sets of experimental results relate to the structure of the transition state for k_{on}: (i) the similarity of k_{on} values for the inhibition of chymotrypsin by chymostatin and Bz-Phe-H (Table II) and (ii) the solvent isotope effects. The fact that chymostatin and Bz-Phe-H interact with chymotrypsin with such similar values of k_{on} suggests that the predominant rate-limiting step in both of these reactions is the same: the attack of the active-site serine on the aldehydic carbon of the inhibitor (Kennedy & Schultz, 1979). Thus, k_{on} transition states for both reactions resemble, at least to some degree, the tetrahedral hemiacetal intermediate.

We should note, however, that although the k_{on} values for these two reactions are similar, the k_{on} for the interaction of Bz-Phe-H with chymotrypsin is 4 times larger than k_{on} for the interaction of chymostatin with chymotrypsin. This is opposite the trend generally observed for reactions of serine protease with peptide-based substrates. For these reactions, k_c/K_m *increases* with substrate prolongation (Kraut, 1977). This suggests that k_{on} for the interaction of chymostatin with chymotrypsin is not entirely rate limited by hemiacetal formation and that some other reaction step must also contribute to the rate determination of k_{on}.

The existence of multiple rate-limiting steps for k_{on} is also supported by solvent isotope effect data. The solvent isotope effect on k_{on} for the association of chymostatin with chymotrypsin is 1.6 and should be compared to the isotope effect of 2.8 on k_c/K_m for the hydrolysis of Ala-Ala-Phe-pNA by chymotrypsin. Since Ala-Ala-Phe-pNA is a relatively poor substrate for chymotrypsin, k_c/K_m should be entirely rate limited by the chemical events of acylation. The isotope effect of 2.8 must therefore reflect the general-acid/general-base catalysis that operates in this step. The simplest explanation for the small isotope effect on k_{on} involves an association

Scheme III

$$E + I \overset{K_i'}{\rightleftharpoons} E{\cdot}I \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} E\text{-}I \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} (E\text{-}I)'$$

mechanism in which hemiacetal formation is only partially rate limiting. The large isotope effect that accompanies hemiacetal formation is "diluted" to the observed value of 1.6 by the near-unity isotope effect of the other partially rate-limiting step. A likely candidate for this step is a conformational change of one of the complexes of enzyme and inhibitor.

Stable Complex of Protease and Chymostatin. The interactions of chymostatin with both chymotrypsin and cathepsin G ultimately lead to enzyme–inhibitor complexes of significant stability. As seen in Table I, these complexes dissociate with k_{off} values around 0.0002 s^{-1}, corresponding to dissociation half-times of nearly 1 h. Data that are relevant to understanding how these enzymes are able to stabilize these complexes to such an extent are in Table II. Here we see that Bz-Phe-H is 5 orders of magnitude less potent than chymostatin as an inhibitor of chymotrypsin. Significantly, this difference in potency is reflected entirely in k_{off}.

This large difference in k_{off} suggests that, during the interaction of chymostatin with chymotrypsin, a unique enzyme–inhibitor complex is formed that is inaccessible to complexes formed during the interaction of Bz-Phe-H with chymotrypsin. Scheme I is expanded to incorporate this proposal (see Scheme III). According to Scheme III, chymostatin and chymotrypsin initially interact to form the encounter complex, E·I. Within this complex the active-site serine, assisted by histidine general-base catalysis, attacks the carbonyl carbon of the inhibitor to form a hemiacetal, E–I. In the final step of the reaction, E–I undergoes a conformational change that produces the greatly stabilized complex (E–I)′. (E–I)′ accumulates only during the interaction of chymostatin with chymotrypsin, and this indicates that remote subsite interactions play a critical role in stabilizing (E–I)′ relative to E–I. The mechanism of Scheme III may apply with equal validity to interactions of chymostatin with cathepsin G. This is supported by the observation of identical k_{off} values for the complexes of chymostatin with both chymotrypsin and cathepsin G.

Registry No. Ala-Ala-Phe-pNA, 61043-41-2; Suc-Ala-Ala-Pro-Phe-pNA, 107409-54-1; chymotrypsin, 9004-07-3; cathepsin G, 56645-49-9; chymostatin, 9076-44-2; deuterium, 7782-39-0.

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