Converting Trypsin to Chymotrypsin: Ground-State Binding Does Not Determine Substrate Specificity†

Lizbeth Hedstrom,*,‡ Shauna Farr-Jones,§ Charles A. Kettner,∥ and William J. Rutter⊥

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254, Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143-0446, The DuPont Merck Pharmaceutical Company, Experimental Station, Wilmington, Delaware 19880, and Hormone Research Institute and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143-0534

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ABSTRACT: Rat trypsin II has been converted to a protease with chymotrypsin-like substrate specificity [Hedstrom, L., et al. (1994) *Biochemistry* (preceding paper in this issue)]. The key alteration in this conversion is the exchange of two surface loops for the analogous loops of chymotrypsin. k_{inact}/K_i for the inactivation of chymotrypsin, trypsin, a trypsin mutant with poor activity (D189S), and the chymotrypsin-like mutants Tr→Ch[S1+L1+L2] and Tr→Ch[S1+L1+L2+Y172W] by Suc-Ala-Ala-Pro-Phe-chloromethylketone correlates with k_{cat}/K_m for hydrolysis of Suc-Ala-Ala-Pro-Phe-AMC. k_{inact}'s for the inactivation of Tr→Ch[S1+L1+L2] and Tr→Ch[S1+L1+L2+Y172W] are comparable to that of chymotrypsin, while K_i's were much higher. K_i for the inhibition of these enzymes by the transition-state analog MeOSuc-Ala-Ala-Pro-boro-Phe also correlates with k_{cat}/K_m for hydrolysis of Suc-Ala-Ala-Pro-Phe-AMC. These results suggest that the surface loops stabilize the transition state for hydrolysis of chymotrypsin substrates by improving the orientation of bound substrates relative to the catalytic residues. Lastly, trypsin and chymotrypsin have comparable affinities for proflavin, while the K_d for the Tr→Ch[S1+L1+L2+Y172W]–proflavin complex is 10-fold higher. No proflavin binding could be observed for either D189S or Tr→Ch-[S1+L1+L2], which suggests that the S1 binding pockets of these two mutant enzymes are deformed. This work confirms that enzyme specificity is expressed in the chemical steps of the reaction rather than in substrate binding.

Among the challenges of protein engineering is the quest to design enzymes with novel substrate specificities. In general, approaches to this problem have focused on the design of substrate binding sites, with the belief that reactivity could be acquired with the addition of appropriate catalytic groups. These approaches have met with only modest success, in part because the structural requirements for specific substrate binding sites are not understood. A second approach is to study the structural determinants of substrate specificity in homologous enzymes, in the hope of delineating the strategies that nature uses to alter specificity. In some instances these strategies are remarkably straightforward—alteration of a small subset of enzyme residues that contact the substrate is sufficient to change specificity (Scrutton et al., 1990; Corbier et al., 1990; Wilks et al., 1988; Bradley et al., 1991; Wells et al., 1987; Bone et al., 1989). However, in other cases alteration of the residues that contact the substrate is not sufficient to completely exchange substrate specificity between homologous enzymes. Such results suggest that substrate specificity determinants exist which do not contact the substrate.

The best characterized example of substrate specificity determinants which do not contact the substrate is found in the trypsin family of serine proteases. Trypsin hydrolyzes peptides with Arg or Lys in the P1 position, chymotrypsin hydrolyzes peptides containing large hydrophobic residues in the P1 position, and elastase prefers small aliphatic P1 residues [nomenclature from Schechter and Berger (1967)]. Alterations of the S1 binding pocket of trypsin to the analogous residues of chymotrypsin have deleterious effects on enzyme activity, in general producing poor, nonspecific proteases (Hedstrom et al., 1992; Graf et al., 1988; Craik et al., 1985). We have recently shown that two surface loops, residues 185–188 (loop 1) and 221–225 (loop 2), which connect the walls of the S1 binding pocket but do not contact the substrate, are important substrate specificity determinants (Hedstrom et al., 1992). Trypsin is converted into a chymotrypsin-like protease when loops 1 and 2 of trypsin are exchanged for the analogous loops of chymotrypsin, in conjunction with the alterations of the S1 binding site. Subsequent work identified residue 172, Tyr in trypsin and Trp in chymotrypsin, as an additional specificity determinant (Hedstrom et al., 1994). Residue 172 interacts with one wall of the S1 binding pocket, but does not interact directly with the substrate. Structural analysis of these mutant enzymes suggests that loops 1 and 2 and residue 172 cause small changes in the S1 binding pocket of trypsin and chymotrypsin, which may account for the different specificities of these two enzymes (J. J. Perona, L. Hedstrom, W. J. Rutter, and R. Fletterick, manuscript in preparation).

Subsequent characterization of these mutant enzymes, Tr→Ch[S1+L1+L2][1] and Tr→Ch[S1+L1+L2+Y172W], revealed some rather surprising properties (Hedstrom et al., 1992, 1994). Although these enzymes possess chymotrypsin-like specificity for hydrophobic substrates, they do not bind hydrophobic substrates well. However, once bound, substrates react almost as efficiently as when bound to chymotrypsin. In contrast, although trypsin and the inactive mutant D189S

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‡ Graduate Department of Biochemistry, Brandeis University.

§ Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco.

∥ The DuPont Merck Pharmaceutical Company.

⊥ Hormone Research Institute and Department of Biochemistry and Biophysics, University of California, San Francisco.

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bind hydrophobic substrates at least as well as Tr→Ch-[S1+L1+L2] and Tr→Ch[S1+L1+L2+Y172W], trypsin and D189S do not possess chymotrypsin-like activity. These results demonstrate that the catalytic steps of an enzymatic reaction provide more substrate discrimination than the substrate binding steps.

In order to substantiate these results, we have evaluated the interaction of chymotrypsin, trypsin, D189S, Tr→Ch-[S1+L1+L2], and Tr→Ch[S1+L1+L2+Y172W] with inactivators, transition-state analog inhibitors, and proflavin. The results of these new studies confirm that Tr→Ch-[S1+L1+L2] and Tr→Ch[S1+L1+L2+Y172W] are defective in ground-state substrate/inhibitor binding. Moreover, this work suggests that the S1 binding pockets of D189S, Tr→Ch[S1+ L1+L2], and Tr→Ch[S1+L1+L2+Y172W] are deformed relative to chymotrypsin and trypsin. These results suggest that the current focus of protein engineering on the design of specific binding sites may be misplaced; high-affinity binding of a substrate is not required for catalytic efficiency.

MATERIALS AND METHODS

Materials. Suc-Ala-Ala-Pro-Phe-CMK was purchased from Enzyme Systems Products (Livermore, CA). Chymotrypsin (TLCK treated), proflavin, Suc-Ala-Ala-Pro-Phe-SBzl, Suc-Ala-Ala-Pro-Phe-pNA, Cbz-Lys-SBzl, Bz-Arg-pNA, and 4,4′-dithiodipyridine were purchased from Sigma Chemical Co. MeOSuc-Ala-Ala-Pro-boro-Phe was prepared as described previously (Kettner & Shenvi, 1984). Trypsin and trypsin mutants were isolated as described previously (Hedstrom et al., 1994).

Enzyme Assays. Assay mix contained 50 mM Hepes, pH 8.0, 10 mM $CaCl_2$, and 100 mM NaCl. Enzyme activity was determined by hydrolysis of Suc-Ala-Ala-Pro-Phe-SBzl and/or Suc-Ala-Ala-Pro-Phe-pNA for chymotrypsin, D189S, Tr→Ch[S1+L1+L2], and Tr→Ch-[S1+L1+L2+Y172W] and hydrolysis of Cbz-Lys-SBzl and/or Bz-Arg-pNA for trypsin as previously described (Hedstrom et al., 1994). Absorbance measurements were made on a Beckman DU650, Perkin-Elmer 552, or Hitachi U2000 UV–visible spectrophotometer.

Inactivation by Suc-Ala-Ala-Pro-Phe-CMK. Two methods were used to monitor Suc-Ala-Ala-Pro-Phe-CMK inactivation of chymotrypsin, trypsin, and trypsin mutants. For slowly reacting enzymes (trypsin, D189S, Tr→Ch-[S1+L1+L2], and Tr→Ch[S1+L1+L2+Y172W]), enzyme was incubated with Suc-Ala-Ala-Pro-Phe-CMK (50–250 μM) in assay buffer at 25 °C. Aliquots were removed at appropriate time intervals and diluted into excess substrate to measure activity and to determine the rate constant, k_{obs}, for pseudo-first-order inactivation. K_i, k_{inact}, and k_{inact}/K_i were determined by fitting the data to the equation $k_{obs} = k_{inact}[I]/(K_i + [I])$ using KinetAsyst software (Powers, 1977). Due to experimental limitations, only k_{inact} could be determined for Tr→Ch[S1+L1+L2+Y172W] in this experiment. For the rapidly reacting enzymes chymotrypsin and Tr→Ch-[S1+L1+L2+Y172W], enzyme was incubated with Suc-Ala-Ala-Pro-Phe-CMK in the presence of excess substrate. k_{obs} was determined from the reaction progress curves, and K_i, k_{inact}/K_i, and k_{inact} were determined from the equation $k_{obs} = k_{inact}[I]/\{K_i(1 + [S]/K_m) + [I]\}$. Because of experimental limitations, only k_{inact}/K_i could be determined for Tr→Ch-[S1+L1+L2+Y172W] in this experiment. All reported values are the average of at least two experiments.

Inhibition by MeOSuc-Ala-Ala-Pro-boro-Phe. The initial and final K_i's for the inhibition of chymotrypsin by MeOSuc-Ala-Ala-Pro-boro-Phe were determined from the initial and steady-state velocities as described previously (Kettner & Shenvi, 1984). Inhibition of trypsin and trypsin mutants by MeOSuc-Ala-Ala-Pro-boro-Phe was not time dependent. All K_i's were determined by fitting the data to the equation for competitive inhibition, $v = V_m[S]/\{K_m(1 + [I]/K_i) + [S]\}$ using KinetAsyst software. All reported values are the average of at least two experiments.

NMR Methods. Samples contained 800 μM enzyme, 0.5 M NaCl, and 10–15% D_2O. Spectra were acquired at 500 MHz with a GE500 Omega spectrometer equipped with a 16-bit digitizer and a 5-mm ^1H probe. Temperature ranged from 5 to 15 °C. Water suppression was achieved with the solvent-suppression sequence of Davis and Wimperis (1989) to avoid saturation of exchangeable protons. Sweep width was 14 000 Hz; 2000–8000 scans were acquired with a recycle time of 0.3–0.4 s. Chemical shift was referenced against DSS at 0 ppm. A convolution of the time domain was carried out before apodization with a Gaussian broadening function, and the baseline was corrected with a sixth-order polynomial.

Proflavin Binding. The binding of proflavin to chymotrypsin, trypsin, and trypsin mutants was determined as described by Bernhard (Bernhard et al., 1966). Enzyme was exchanged into assay mix and concentrated to approximately 500 μM using a Centricon (Amicon). Proflavin was added to 250 μL of enzyme solution to a final concentration of 1 μM, and the absorbance spectrum was recorded versus a solution of proflavin alone. The enzyme–proflavin solution was then diluted with assay mix containing 1 μM proflavin. Spectra were recorded at 5 or 6 different enzyme concentrations. A peak was observed at 465 nm for chymotrypsin, trypsin, and Tr→Ch[S1+L1+L2+Y172W]. The difference absorption coefficient of free and bound proflavin, $\Delta\epsilon_{465}$, and K_d were determined as previously described (Bernhard et al., 1966). No absorbance at 465 nm was observed for enzyme solutions in the absence of proflavin.

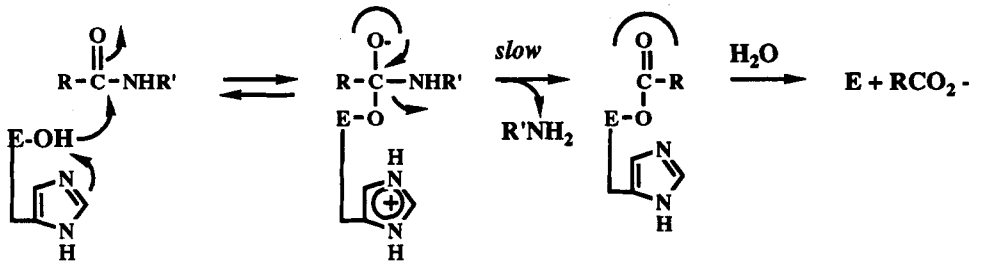
RESULTS AND DISCUSSION

Inactivation of Trypsin Mutants by Suc-Ala-Ala-Pro-Phe-CMK. Peptidyl chloromethyl ketones inactivate serine proteases by alkylating the active site histidine (His57 in the trypsin family) (Powers & Harper, 1986). Although the exact mechanism is in doubt, it is generally agreed that inactivation involves rapid formation of a hemiketal with the active site serine followed by slower alkylation of His57 (Figure 1). The oxygen of the hemiketal occupies the oxyanion hole of the enzyme, so that the final adduct resembles the tetrahedral intermediate of substrate hydrolysis. The inactivation process can be described by the following equation:

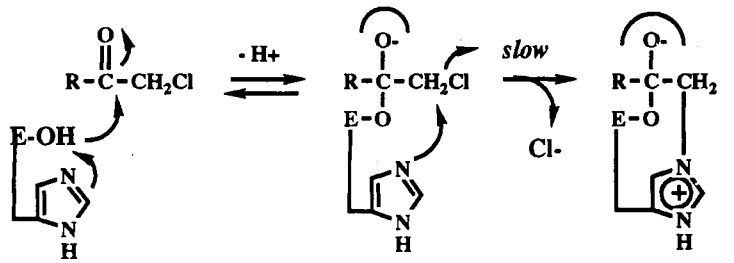
$$E + I \overset{K_i}{\rightleftarrows} E{\cdot}I \overset{k_{inact}}{\rightarrow} E\text{–}I$$

[1] Abbreviations: AMC, 7-amino-4-methylcoumarin; Suc-Ala-Ala-Pro-Phe-AMC, succinylalanylalanylprolylphenylalanyl-AMC; Suc-Ala-Ala-Pro-Phe-pNA, succinylalanylalanylprolylphenylalanyl-*p*-nitroanilide; Suc-Ala-Ala-Pro-Phe-CMK, succinylalanylalanylprolylphenylalanine chloromethyl ketone; TLCK, *N*-*p*-tosyl-L-lysine chloromethyl ketone; Suc-Ala-Ala-Pro-Phe-SBzl, succinylalanylalanylprolylphenylalanine thiobenzyl ester; Cbz-Lys-SBzl, *N*-(carbobenzoxy)lysine thiobenzyl ester; Bz-Arg-pNA, *N*-(benzoylarginyl)-*p*-nitroanilide; MeOSuc-Ala-Ala-Pro-boro-Phe, (methoxysuccinyl)-alanylalanylprolylphenylalanylboronic acid; DSS, 2,2-dimethyl-2-silapentanesulfonate; BPTI, bovine pancreatic trypsin inhibitor; D189S, rat trypsin II with Asp189 changed to Ser; Tr→Ch[S1+L1+L2], rat trypsin II containing the following alterations: Ile138 to Thr, Phe185 deleted, Leu186 to Ala, Glu187 to Ser, Lys188 deleted, Asp189 to Ser, Gln192 to Met, Tyr217 to Ser, insert Thr219, Ala221 to Ser, Leu222 deleted, Pro223 to Thr, Asp224 to Ser, and Asn225 to Thr. Tr→Ch[S1+L1+L2+Y172W] has the same changes as Tr→Ch-[S1+L1+L2] with the additional change of Tyr172 to Trp.

1. Mechanism of substrate hydrolysis.



2. Mechanism of chloromethylketone inactivation.



3. Mechanism of boronic acid inhibition.

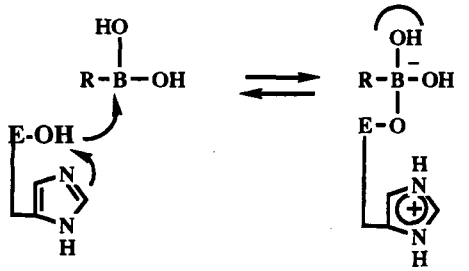


FIGURE 1: Mechanisms of serine protease catalysis and inhibition.

The specificity of a peptidyl chloromethyl ketone for a given enzyme is described by the parameter k_{inact}/K_i, which represents the apparent second-order rate constant for the reaction of free enzyme and inactivator. K_i is probably a combination of rate constants representing inactivator binding and reversible formation of the hemiketal intermediate. k_{inact} most likely represents the rate constant for alkylation of His57. Specificity of peptidyl chloromethyl ketone inactivation generally correlates with the specificity of substrate hydrolysis for a serine protease (Powers & Harper, 1986).

Figure 2 shows that k_{inact}/K_i for inactivation of chymotrypsin, trypsin, D189S, Tr→Ch[S1+L1+L2], and Tr→Ch-[S1+L1+L2+Y172W] by Suc-Ala-Ala-Pro-Phe-CMK correlates with their respective k_{cat}/K_m's for the hydrolysis of Suc-Ala-Ala-Pro-Phe-AMC. This correlation spans 5 orders of magnitude, with a slope of 1.00 and $r^2 = 0.969$. A similar correlation exists between k_{inact}/K_i and k_{cat}/K_m for the hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA by the five enzymes. Suc-Ala-Ala-Pro-Phe-CMK inactivation of trypsin and D189S is characterized by high K_i and low k_{inact} values relative to those of chymotrypsin (Table 1). The high K_i's suggest that inactivator binding and hemiketal formation are not favored for either trypsin or D189S, while the low k_{inact} suggests that the hemiketal intermediate is not oriented optimally for reaction with His57 in these two enzymes. The K_i for Suc-Ala-Ala-Pro-Phe-CMK inactivation of Tr→Ch[S1+L1+L2] is 40-fold greater than that for inactivation of chymotrypsin, and only 4–7-fold better than that for D189S or trypsin. This observation suggests that Tr→Ch[S1+L1+L2] is defective in hemiketal formation relative to chymotrypsin. In contrast,

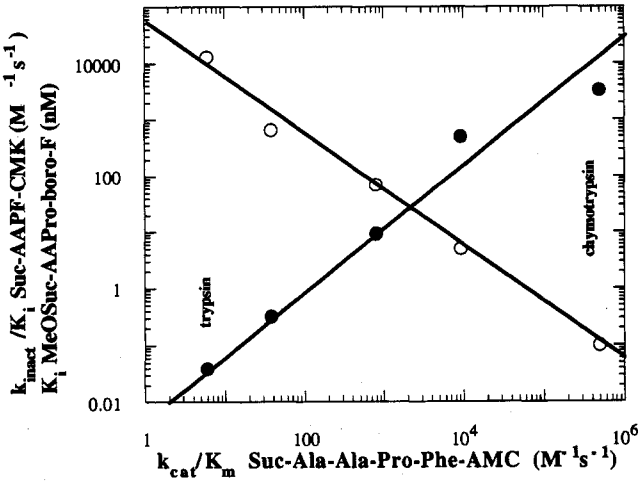


FIGURE 2: Correlation of substrate hydrolysis with Suc-Ala-Ala-Pro-Phe chloromethyl ketone inactivation and Suc-Ala-Ala-Pro-boro-Phe inhibition for chymotrypsin, trypsin, and trypsin mutants. Conditions were as described in Materials and Methods. (○) K_i, Suc-Ala-Ala-Pro-boroPhe; (●) k_{inact}/K_i, Suc-Ala-Ala-Pro-Phe chloromethyl ketone.

k_{inact} for Suc-Ala-Ala-Pro-Phe-CMK inactivation of Tr→Ch-[S1+L1+L2] is equivalent to k_{inact} for chymotrypsin. This observation suggests that the hemiketal intermediates formed in the inactivation of chymotrypsin and Tr→Ch[S1+L1+L2] have the same orientation relative to His57. These results suggest that the chymotrypsin-like specificity of Tr→Ch-[S1+L1+L2] results from an improvement in the orientation of bound substrate relative to D189S and trypsin.

Table 1: Inactivation of Chymotrypsin, Trypsin, and Trypsin Mutants with Suc-Ala-Ala-Pro-Phe-CMK[a]

enzyme	K_i (μM)	k_{inact} (s^{-1})	k_{inact}/K_i (M^{-1} s^{-1})
chymotrypsin	27 ± 15	0.1 ± 0.04	(3.3 ± 0.2) × 10^3
trypsin	(7 ± 3) × 10^3	(3 ± 1) × 10^{-4}	0.039 ± 0.001
D189S	(3.7 ± 0.8) × 10^3	(1.2 ± 0.2) × 10^{-3}	0.32 ± 0.01
Tr→Ch[S1+L1+L2]	1000 ± 400	0.1 ± 0.03	9.2 ± 0.5
Tr→Ch[S1+L1+L2+Y172W]	120 ± 40	0.06 ± 0.01	500 ± 100

[a] Conditions were as described in Materials and Methods

The addition of the Tyr172 to Trp mutation to Tr→Ch-[S1+L1+L2] increases k_{inact}/K_i for Suc-Ala-Ala-Pro-Phe-CMK inactivation by lowering K_i. This result suggests that hemiketal formation is improved in Tr→Ch-[S1+L1+L2+Y172W] relative to Tr→Ch[S1+L1+L2]. This improvement very likely reflects an improvement in inactivator binding; the reactivity of the chloromethyl ketone and Ser195 will drive hemiketal formation if the two functional groups are proximal.

Inhibition of Trypsin Mutants by MeOSuc-Ala-Ala-Pro-boro-Phe. Peptide boronic acids are potent inhibitors of serine proteases. These inhibitors form two types of covalent adducts with serine proteases (Bachovchin et al., 1988; Tsilikounas et al., 1992; Bone et al., 1989). Inhibitors that resemble specific substrates form transition-state-like complexes (type I adducts) where Ser195 is covalently bonded to the boron atom (Figure 1). In type I complexes, His57 is protonated and hydrogen bonded to both the boron adduct and Asp102. The K_i's for type I inhibitors correlate with the k_{cat}/K_m's for hydrolysis of the analogous substrates, as expected for transition-state analogs (Kettner et al., 1988; Bone et al., 1989). Type I adducts of porcine trypsin have characteristic low-field ^1H NMR spectra with two proton resonances, one at ~16.3 ppm from the $N^{\delta 1}$ proton and a second at ~17.0 ppm from the $N^{\epsilon 2}$ proton. Compounds that do not resemble good substrates (e.g., borate) often form covalent adducts with His57 (type II adducts). K_i's of type II inhibitors are lower than expected from the k_{cat}/K_m for hydrolysis of the analogous substrate (Kettner et al., 1988; Bone et al., 1989). The ^1H NMR spectra of type II adducts are characterized by a single low-field proton resonance at ~15.8 ppm from the $N^{\delta 1}$ proton.

The inhibition of chymotrypsin, trypsin, D189S, Tr→Ch-[S1+L1+L2], and Tr→Ch[S1+L1+ L2+Y172W] by MeO-Suc-Ala-Ala-Pro-boro-Phe was characterized as shown in Table 2A. MeOSuc-Ala-Ala-Pro-boro-Phe inhibition of chymotrypsin is time dependent as previously described, and the initial and final K_i's are in good agreement with those reported previously [lit. initial K_i = 3.4 nM; final K_i = 0.16 nM (Kettner & Shenvi, 1984)]. No time-dependent inhibition was observed for the inhibition of trypsin or trypsin mutants, which is not surprising because MeOSuc-Ala-Ala-Pro-boro-Phe is a much less potent inhibitor of these enzymes. The K_i's for MeOSuc-Ala-Ala-Pro-boro-Phe inhibition of chymotrypsin, trypsin, D189S, Tr→Ch[S1+L1+L2], and Tr→Ch-[S1+L1+L2+Y172W] correlate with k_{cat}/K_m for the hydrolysis of the analogous substrate Suc-Ala-Ala-Pro-Phe-AMC (Figure 2). This correlation spans 5 orders of magnitude, with a slope of −1.05 and r^2 = 0.992. A similar correlation exists between K_i and k_{cat}/K_m for the hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA. No correlation exists between K_i for MeOSuc-Ala-Ala-Pro-boro-Phe inhibition and K_m for substrate hydrolysis. These results suggest that MeOSuc-Ala-Ala-Pro-boro-Phe forms type I adducts with all five enzymes.

Low-field ^1H NMR also supports formation of type I adducts between MeOSuc-Ala-Ala-Pro-boro-Phe and D189S (Figure 3 and Table 2B). The low-field ^1H NMR spectrum of D189S complexed with MeOSuc-Ala-Ala-Pro-boro-Phe displayed pH-independent resonances at 17.5 and 16.5 ppm, while the spectrum of D189S complexed with borate displayed a single resonance at 15.9 ppm that was pH dependent (Figure 3). Previous work suggested that MeOSuc-Ala-Ala-Pro-boro-Phe forms type I adducts with chymotrypsin, as evidenced by low-field ^1H NMR resonances at 16.9 and 16.1 ppm (Farr-

Table 2: Inhibition of Chymotrypsin, Trypsin, and Trypsin Mutants by MeOSuc-Ala-Ala-Pro-boro-Phe

A. Kinetic Parameters[a]

enzyme	initial K_i (nM)	final K_i (nM)
chymotrypsin	1.5 ± 0.2	0.10 ± 0.02
trypsin	n.a.	13 000 ± 700
D189S	n.a.	660 ± 90
Tr→Ch[S1+L1+L2]	n.a.	70 ± 20
Tr→Ch[S1+L1+L2+Y172W][b]	n.a.	5 ± 3

B. Low-Field ^1H NMR Resonances for Complexes of Boric Acid andMeOSuc-Ala-Ala-Pro-boro-Phe with D189S[c]

sample	^1H chemical shifts at pH 4.0	8.6
no inhibitor	17.5	14.7
boric acid, 0.8 M	no complex	15.9
MeOSuc-Ala-Ala-Pro-boro-Phe, 4.7 mM	17.3, 16.3	17.5, 16.4

[a] Conditions were as described in Materials and Methods. [b] k_{inact} and k_{inact}/K_i were determined separately as described in Materials and Methods. K_i is calculated from k_{inact} and k_{inact}/K_i. [c] Conditions were as described in Materials and Methods (800 μM enzyme, 0.5 M NaCl, and 15% D_2O, 5–15 °C).

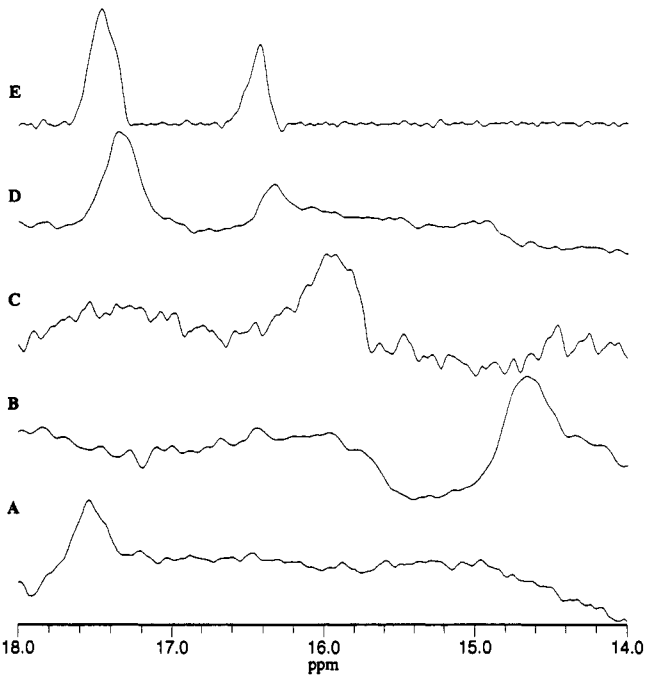


FIGURE 3: Low-field ^1H NMR spectra of D189S. Conditions were as described in Materials and Methods. (A) Resting enzyme, pH 4.0; (B) resting enzyme, pH 9.0; (C) complex with borate, pH 8.6; (D) complex with Suc-Ala-Ala-Pro-boro-Phe, pH 4.0; (E) complex with Suc-Ala-Ala-Pro-boro-Phe, pH 8.6.

Table 3: Proflavin Binding to Chymotrypsin, Trypsin, and Trypsin Mutants[a]

enzyme	K_d (μM)	$\Delta\epsilon_{465}$ (cm^{-1} mM^{-1})
chymotrypsin	70 ± 20	23
trypsin	140 ± 30	38
D189S[b]	≥2000	n.a.
Tr→Ch[S1+L1+L2]	≥4000	n.a.
Tr→Ch[S1+L1+L2+Y172W]	700 ± 90	65

[a] Conditions were as described in Materials and Methods. [b] Estimated by assuming $\Delta\epsilon_{465}$ = 23 cm^{-1} mM^{-1} from the minimum observable ΔA_{465} values from D189S (770 μM) and Tr→Ch[S1+L1+L2] (470 μM) of 0.05 and 0.002, respectively.

Jones, 1989). Unlike porcine trypsin, rat trypsin displayed no low-field ^{1}H NMR resonances in the absence or presence of MeOSuc-Ala-Ala-Pro-boro-Phe.

Binding of Proflavin to Trypsin Mutants. Proflavin binding is a direct probe of the integrity of the S1 binding pocket of both chymotrypsin and trypsin. The absorbance maximum of proflavin shifts from λ_{max} = 444 to 458 nm on binding to chymotrypsin or trypsin, with the maximum difference at 465 nm (Bernhard & Gutfreund, 1965; Bernhard et al., 1966). Proflavin is displaced by competitive inhibitors of trypsin and chymotrypsin, which indicates that it binds in the S1 binding site. Proflavin does not bind to chymotrypsinogen, where the S1 binding pocket is collapsed (Fersht, 1972). The abilities of chymotrypsin, trypsin, and the trypsin mutants to bind proflavin were compared as shown in Table 3. The K_d's and $\Delta\epsilon_{465}$ for chymotrypsin are comparable to previous reports [lit. K_d = 37 μM, $\Delta\epsilon_{465}$ = 18 cm^{-1} mM^{-1} (Bernhard et al., 1966); K_d = 45.4 μM, $\Delta\epsilon_{465}$ = 17.1 cm^{-1} mM^{-1} (Brady & Abeles, 1990)]. The K_d for proflavin binding to rat trypsin is similar to that reported for bovine trypsin [lit. K_d = 40 μM (Bernhard & Gutfreund, 1965)]. Unlike chymotrypsin and trypsin, neither D189S nor Tr→Ch[S1+L1+L2] cause a large shift in the absorbance spectrum of proflavin. These results indicate that the mutant enzymes cannot bind proflavin and demonstrate that the S1 binding pockets of D189S and Tr→Ch[S1+L1+L2] are deformed relative to those of trypsin and chymotrypsin. Tr→Ch[S1+L1+L2+Y172W] binds proflavin 5–10-fold less tightly than chymotrypsin and trypsin, which suggests that the binding pocket of this mutant is stabilized relative to those of D189S and Tr→Ch[S1+L1+L2], but still deformed relative to those of chymotrypsin and trypsin.

Loops 1 and 2 Improve the Orientation of Enzyme-Bound Substrates. The experiments reported here and previously demonstrate that the two mutants containing loops 1 and 2 can process bound substrates and inactivators with rates comparable to chymotrypsin. These observations suggest that the orientation of bound substrate/inactivator relative to the catalytic groups is the same for chymotrypsin, Tr→Ch-[S1+L1+L2], and Tr→Ch[S1+L1+L2+Y172W]. Amide hydrolysis and chloromethyl ketone inactivation are likely to be very dependent on the relative orientation of substrate/inactivator and His57. Amide hydrolysis is believed to involve rapid formation of the tetrahedral intermediate, followed by slow expulsion of the amine leaving group (Polgar, 1989). Protonation of the amine leaving group by His57 is believed to be critical for the catalytic efficiency of serine proteases. Similarly, chloromethyl ketone inactivation is believed to involve rapid addition of Ser195 to form the hemiketal intermediate, followed by slow alkylation of His57. Therefore the improvements in both amide hydrolysis and chloromethyl ketone inactivation observed in Tr→Ch[S1+L1+L2] versus D189S likely reflect an improvement in the orientation of bound substrate/inactivator relative to His57. This interpretation argues that the small differences in the structure of the S1 binding pockets of trypsin and chymotrypsin are crucial for substrate specificity. Significantly, the orientation of the substrate relative to the catalytic groups is more important than the affinity of binding in determining enzyme specificity.

Loops 1 and 2 Stabilize the Transition State of Substrate Hydrolysis. The correlation of K_i with k_{cat}/K_m is important confirmation that an inhibitor is acting as a transition-state analog. Such a correlation has been demonstrated for a handful of enzyme/inhibitor systems, most notably phosphonate inhibitors of Zn^{2+} proteases and boronic acid and trifluoromethyl ketone inhibitors of serine proteases (Bartlett & Marlowe, 1983; Brady & Abeles, 1990; Kettner et al., 1988). This correlation is difficult to demonstrate in most enzyme/inhibitor systems because enzyme specificity precludes the use of multiple substrates. Recently, Bartlett has suggested that this problem can be circumvented by using a series of mutant enzymes rather than a series of different substrates (Phillips et al., 1992). In that study, K_i for a series of phosphonic acid inhibitors correlated with k_{cat}/K_m for substrate hydrolysis by a set of mutants of carboxypeptidase A. Previous work revealed a similar correlation between k_{cat}/K_m of substrate hydrolysis and K_i for boronic acid inhibition of mutant α-lytic proteases (Bone et al., 1991). Our data is yet another example of a correlation between K_i of a transition-state analog and k_{cat}/K_m for substrate hydrolysis by a series of mutant enzymes. Together these results suggest that the use of a series of mutant enzymes to confirm transition-state analogy of a given inhibitor may be widely applicable. More importantly, the correlation for mutant trypsins indicates that the transition states of substrate hydrolysis have geometric and electronic properties similar to those of the boronic acid inhibitor. Therefore, the transition state must be similar for all five enzymes, and substrate hydrolysis most likely proceeds via the same mechanism for all of the enzymes studied. This conclusion is notable given the extremely low activity of trypsin and D189S on Suc-Ala-Ala-Pro-Phe-AMC.

Ground-State Substrate/Inhibitor Binding Is Defective in TrfCh[S1+L1+L2] and TrfCh[S1+L1+L2+Y172W]. Previous work suggested that Tr→Ch[S1+L1+L2] and Tr→Ch-[S1+L1+L2+Y172W] are defective in ground-state substrate binding, as demonstrated by the high K_s's relative to chymotrypsin. Our present studies suggest that Tr→Ch-[S1+L1+L2] and Tr→Ch[S1+L1+L2+Y172W] are also defective in ground-state binding of the chloromethyl ketone inactivator, as evidenced by the correspondingly high K_i's relative to chymotrypsin. The most revealing experiment is the demonstration that Tr→Ch[S1+L1+L2] fails to bind proflavin, while Tr→ Ch[S1+L1+L2+Y172W] binds proflavin 10-fold less tightly than chymotrypsin. The proflavin binding experiments conclusively demonstrate that the S1 binding pocket of these two mutants is deformed relative to those of chymotrypsin and trypsin. This result is supported by X-ray structural analysis of the Suc-Ala-Ala-Pro-Phe-CMK adducts of Tr→Ch[S1+L1+L2] and Tr→Ch-[S1+L1+L2+Y172W], which reveals increased disorder in loops 1 and 2 of both enzymes relative to chymotrypsin and trypsin (J.J. Perona, L. Hedstrom, W. J. Rutter, and R. Fletterick, manuscript in preparation).

The deformed S1 binding pockets of these trypsin mutants may be analogous to the deformed binding pocket of trypsinogen. Four sections of trypsinogen, including the section from the N terminus to residue 19 and residues 142–152, 184–193 and 216–223, cannot be observed in the structure of trypsinogen, although these regions are well ordered in trypsin (Huber & Bode, 1978). Loop 1 (residues 185–188) and loop 2 (residues 221–225) are part of this disordered "activation

domain". This observation suggests that trypsinogen may be a useful model for the S1 binding pocket of these mutants.

The inability of D189S to bind proflavin suggests that the S1 binding pocket of this mutant is also deformed. However, this interpretation is seemingly in conflict with recent structural studies (Perona et al., 1994), which determined the structure of the complex between D189S, BPTI, and acetate. This complex is identical to the bovine trypsin–BPTI complex; the acetate is in a position similar to the position occupied by Asp189 in wild type, and no significant differences in side-chain position or thermal factors are observed. However, this structure does not provide any information about the unliganded enzyme structure. Indeed, Bode has shown that BPTI can induce trypsinogen, which has a disordered S1 binding pocket, to assume a trypsin-like conformation (Bode et al., 1978). The side-chain positions and thermal factors of the trypsinogen–BPTI–Ile-Val complex are virtually indistinguishable from those of the trypsin–BPTI complex. Therefore, it seems likely that BPTI and acetate induce D189S to form a conformation indistinguishable from that of trypsin. This example illustrates the dangers inherent in analyzing mutant enzyme structures with inhibitor complexes, for such complexes may mask large structural perturbations.

Implications for the Design of Novel Enzymes. Our studies suggest two mechanisms for the lack of chymotrypsin-like activity of D189S. First, D189S cannot orient the substrate correctly relative to the catalytic residues; k_{cat} for substrate hydrolysis and k_{inact} for chloromethyl ketone inactivation are low relative to those of chymotrypsin. This defect can be alleviated by the exchange of loops 1 and 2. This observation suggests that the small differences in the S1 binding sites of trypsin and chymotrypsin are crucial for specificity. Indeed, X-ray crystal structural studies reveal that the S1 binding pockets of both Tr→Ch[S1+ L1+L2] and Tr→Ch-[S1+L1+L2+Y172W] resemble chymotrypsin rather than trypsin (J. J. Perona, L. Hedstrom, W. J. Rutter, and R. Fletterick, manuscript in preparation). Second, the stability of the S1 binding pocket of trypsin appears to be finely tuned; mutation of Asp189 to Ser destabilizes the pocket. This observation suggests that alternate strategies for stabilizing the S1 binding pocket must have evolved with changes in enzyme specificity. Residue 172 is involved in stabilizing the S1 binding pocket; again, X-ray crystal studies confirm that the S1 binding pocket of Tr→Ch[S1+L1+L2+Y172W] is more structured than that of Tr→Ch[S1+L1+L2] (J. J. Perona, L. Hedstrom,W. J. Rutter, and R. Fletterick, manuscript in preparation). These two mechanisms are not necessarily independent. A deformed S1 binding pocket may bind substrate but may not constrain the substrate in the proper orientation relative to the catalytic groups.

Our studies suggest that it may be easier to design new enzymes by optimizing substrate binding after catalytic activity is achieved, rather than the converse. The substrate must be perfectly oriented relative to the catalytic groups for efficient catalysis. Clearly, it is very difficult to design ligand binding sites with the necessary precision. Moreover, this perfect positioning has a high entropic cost, which results in low observed binding affinity (Jencks, 1987). Thus a low-affinity binding site, which could escape a simple screen for binding affinity, may be the site which provides the correct orientation. Alternatively, a flexible binding site may access an active orientation relative to the catalytic groups and thus be active despite its poor substrate binding properties. Our studies show that catalytic activity and binding affinity do not correlate.

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REFERENCES

Anderson, D. E., Becktel, W. J., & Dahlquist, F. W. (1990) *Biochemistry 29*, 2403–2408.
Bachovchin, W. W., Wong, W. Y. L., Farr-Jones, S., Shenvi, A. B., & Kettner, C. A. (1988) *Biochemistry 27*, 7689–7697.
Bartlett, P. A., & Marlowe, C. K. (1983) *Biochemistry 22*, 4618–4624.
Bernhard, S. A., & Gutfreund, H. (1965) *Proc. Natl. Acad. Sci.. U.S.A. 53*, 1238–1243.
Bernhard, S. A., Lee, B. F., & Tashjian, Z. H. (1966) *J. Mol. Biol. 18*, 405–420.
Bode, W., Schwager, P., & Huber, R. (1978) *J. Mol. Biol. 118*, 99–112.
Bone, R., Frank, D., Kettner, C. A., & Agard, D. A. (1989) *Biochemistryy 28*, 7600–7609.
Bone, R., Fujishige, A., Kettner, C. A., & Agard, D. A. (1991) *Biochemistry 30*, 10388–10398.
Bradley, M., Bucheler, U. S., & Walsh, C. T. (1991) *Biochemistry 30*, 6124–6127.
Brady, K., & Abeles, R. H. (1990) *Biochemistry 29*, 7608–7617.
Corbier, C., Clermont, S., Billard, P., Skarzynski, T., Branlant, C., Wonacutt, A., & Branlant, G. (1990) *Biochemistry 29*, 7101–7106.
Craik, C. S., Largman, C., Fletcher, T., Roczniak, S., Barr, P. J., Fletterick, R., & Rutter, W. J. (1985) *Science 228*, 291–297.
Davis, A. L., & Wimperis, S. (1989) *J. Magn. Reson. 84*, 620–626.
Farr-Jones, S. (1989) Ph.D. Thesis, Sackler School of Graduate Biomedical Sciences, Tufts University.
Fersht, A. R. (1972) *J. Mol. Biol. 64*, 497–509.
Graf, L., Jancso, A., Szilagyi, L., Hegyi, G., Pinter, K., Naray-Szabo, G., Hepp, J., Medzihradsky, K., & Rutter, W. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4961–4965.
Hedstrom, L., Szilagyi, L., & Rutter, W. J. (1992) *Science 255*, 1249–1253.
Hedstrom, L., Perona, J., & Rutter, W. J. (1994) *Biochemistry* (preceding paper in this issue).
Huber, R., & Bode, W. (1978) *Acc. Chem. Res. 11*, 114–122.
Jencks, W. P. (1987) *Cold Spring Harbor Symp. Quant. Biol. 52*, 65–73.
Kettner, C. A., & Shenvi, A. B. (1984) *J. Biol. Chem. 259*, 15106–15114.
Kettner, C. A., Bone, R., Agard, D. A., & Bachovchin, W. W. (1988) *Biochemistry 27*, 7682–7688.
Perona, J. J., Hedstrom, L., Wagner, R. L., Rutter, W. J., Craik, C. S., & Fletterick, R. (1994) *Biochemistry 33*, 3252–3259.
Phillips, M. A., Kaplan, A. P., Rutter, W. J., & Bartlett, P. A. (1992) *Biochemistry 31*, 959–963.
Polgar, L. (1989) *Mechanisms of Protease Action*, CRC Press, Inc., Boca Raton, FL.
Powers, J. C. (1977) *Methods Enzymol. 46*, 197–209.
Powers, J. C., & Harper, J. W. (1986) In *Proteinase Inhibitors* (Barrett, A. J. & Salvasen, G., Eds.) pp 95–105, Elvesier, Amsterdam.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.
Scrutton, N. S., Berry, A., & Perham, R. N. (1990) *Nature 343*, 38–43.
Tsilikounas, E., Kettner, C. A., & Bachovchin, W. W. (1992) *Biochemistry 31*, 12839–12846.
Wells, J. A., Cunningham, B. C., Graycar, T. P., & Estell, D. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5167–5171.
Wilks, H. M., Hart, K. W., Feeney, R., Dunn, C. R., Muirhead, H., Chia, W. N., Barstow, D. A., Atkinson, T., Clarke, A. R., & Holbrook, J. J. (1988) *Science 242*, 1541–1544.