Distinct Multisite Synergistic Interactions Determine Substrate Specificities of Human Chymase and Rat Chymase-1 for Angiotensin II Formation and Degradation*

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Human chymase and rat chymase-1 are mast cell serine proteinases involved in angiotensin II (Ang II) formation and degradation, respectively. Previous studies indicate that both these enzymes have similar P1 and P2 preferences, which are the major determinants of specificity. Surprisingly, despite the occurrence of optimal P2 and P1 residues at the Phe81 and Tyr41 bonds (where 1 indicates the scissile bond in peptide substrates) in Ang I (DRVYIHPFHL), human chymase cleaves the Phe81 bond with an ~750-fold higher catalytic efficiency (kcat/Km) than the Tyr41 bond in Ang II (DRVYIHPFPFH). Differences in the acyl groups IHPF and DRVYIHPFPFH of human chymase-1 cleaves the Tyr41 bond with an ~20-fold higher catalytic efficiency than the Phe81 bond. Differences in the acyl groups IHPF and DRVYIHPFPFH at the Phe81 and Tyr41 bonds, respectively, are chiefly responsible for the preference of human chymase for the Phe81 bond. We show that the IHPF sequence forms an optimal acyl group, primarily through synergistic interactions between neighboring acyl group residues. In contrast to human chymase, rat chymase-1 shows a preference for the Tyr41 bond, mainly because of a catalytically productive interaction between the enzyme and the P1, Ile83. The overall effect of this P1, Ile interaction on catalytic efficiency, however, is influenced by the structure of the acyl group and that of the other leaving group residues. For human chymase, the P1, Ile interaction is not productive. Thus, specificity for Ang II formation versus Ang II degradation by these chymases is produced through synergistic interactions between acyl or leaving group residues as well as between the acyl and leaving groups. These observations indicate that nonadditive interactions between the extended substrate binding site of human chymase or rat chymase-1 and the substrate are best explained if the entire binding site is taken as an entity rather than as a collection of distinct subsites.

Chymases1 are a family of mast cell serine proteinases involved in various functions including inflammation (1), parasite expulsion (2), and peptide hormone processing (3–5). These serine proteinases are synthesized as inactive precursors but are stored in secretory granules as active enzymes (6). Recent phylogenetic evidence indicates that mammalian chymases occur as two distinct isoenzyme groups, α and β (7). α-Chymases include human chymase, dog chymase, mouse chymase-5, rat chymase-3, and gerbil chymase-2 (7, 8). β-Chymases include rat chymase-1 and -2, mouse chymase-1, -2, -4, and -L, and gerbil chymase-1 (7, 8). Kinetic studies indicate that α- and β-chymases differ in their substrate specificity. For example, human chymase efficiently converts the decapeptide Ang I2 to the octapeptide hormone Ang II by splitting the Phe8-His9 bond in Ang I (5). The generated Ang II is not further degraded because the Tyr4-Ile6 bond in Ang II is resistant to cleavage by human chymase. Histochemical studies suggest a major role for primate chymases in regulating tissue Ang II levels. Rat chymase-1, in contrast, is an angiotensinase because it readily splits the Tyr4-Ile6 and the Phe8-His9 bonds in angiotensins (3, 7).

Early comparative studies on chymase specificities by Poitier et al. (11) using peptide 4-nitroanilide substrates indicated that the S1 to S4 substrates2 of human chymase and rat chymase are similar. In both human chymase and rat chymase-1, the key features for optimal acyl group interactions are a P1 hydrophobic aromatic residue, a P2 hydrophobic residue or Pro, and a P3 hydrophobic residue. S4 substrate interactions are less restrictive. Thus, these studies could not explain why human chymase is an Ang II-forming enzyme and rat chymase-1 is an angiotensinase (3, 5) and suggested to us that enzyme-substrate interactions other than those occurring at the S1 to S4 subsites of these enzymes could be important for determining specificity. In a previous paper we explored the S1 substrate as well as the S1 to S4 subsites of human chymase using decapetide Ang I analogs. We showed that a P1 hydrophobic aromatic residue was necessary but that several non-

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‡Mast cell chymotrypsin-like proteinases have in the past been variously referred to as chymases, mast cell chymases, and mast cell proteinases. The term mast cell protease has also been used to designate mouse mast cell tryptases and carboxypeptidases; thus, not all mouse enzymes referred to as mast cell proteinases are chymases; the number designation associated with this nomenclature is usually 1, 2, etc., but

L has also been used. The number assignment has usually been given in order of discovery. Because homologs in different species have been discovered in differing orders, numbers do not necessarily correspond to homologs. In this paper, we have used the term chymase to describe a distinct group of leukocyte serine proteinases (7) but have retained the original number designation given at the time of discovery.

The abbreviations used are: Ang I, angiotensin I; Ang II, angiotensin II; Scissile bond in peptide substrates; HPLC, high pressure liquid chromatography; ES complex, enzyme-substrate complex; ES1 complex, transition state complex; ΔG‡, difference between two substrates in the free energy required for transition state stabilization (i.e. free energy required to reach ES1 complex from E + S);

3 The nomenclature used for the individual amino acids (P1, P2, etc.) of a substrate and the substrates (S1, S2, etc.) of the enzyme is that of Schechter and Berger (21). Amino acid residues of substrates numbered P1, P2, etc. are toward the N-terminal direction, and P1, P2, etc. are toward the C-terminal direction from the scissile bond.
conservative changes in P'1 and P'2 positioned residues produced small effects (i.e. a ~3-fold change in the specificity constant $k_{cat}/K_m$) on the cleavage of the Phe8-His9 bond (12). To determine if the Tyr-Ile P'1-P'2 combination forms a poor cleavage site for human chymase or if the context of the bond within the substrate is important, we synthesized an peptide analog of Ang I that contained two Tyrs-Iles, one that naturally occurs at the 4–5 position and one introduced at the 8–9 position. The Tyr4-Ile5 bond was resistant to cleavage by human chymase, but the Tyr4-Ile9 bond was readily cleaved; the difference in $k_{cat}/K_m$ for the cleavage of the Tyr4-Ile9 bond versus the Tyr4-Ile5 bond was >500:1 (12). We proposed, therefore, that for human chymase, the structural context of the scissile bond within the polypeptide substrate is likely to be an important determinant of specificity. What is the structural context that makes the Phe8-His9 bond in Ang I, relative to the Tyr4-Ile5 bond in Ang II, highly susceptible to cleavage by human chymase? We show that in human chymase this structural context is generated through synergetic interactions between neighboring P4 to P1 residues of the substrate acyl group. Rat chymase-1, on the other hand, has a distinct P1 preference that distinguishes its specificity from human chymase.

**EXPERIMENTAL PROCEDURES**

**Peptides—**Peptides used in this study were synthesized by The Protein Core Facility of The Cleveland Clinic Foundation. Peptides were purified (purity >99%) on a C18 reverse phase HPLC column and characterized by amino acid analysis and by analytical C18 reverse phase HPLC. Peptide concentrations were standardized by amino acid analysis.

**Enzymes and Enzyme Kinetics—**Human chymase was purified to homogeneity from human left ventricular tissue (5). Rat chymase-1 was purified from rat peritoneal mast cells as described previously by Le Trong et al. (3). Identity of these enzymes was established by N-termini- nal sequence analysis. To determine $K_m$ and $V_{max}$ values for the human chymase and rat chymase-1 reactions, initial velocities ($v$) were determined as described by us previously (12). Fifteen concentrations of substrate ranging between 0.8 and 1,000 μM with human chymase or rat chymase-1 were incubated at 37 °C in 20 μl Tris-Cl buffer, pH 8.0, containing 0.5 M KCl, and 0.01% Triton X-100 (final volume, 50 μl) for 20 min (in the cleavage of Ang II by human chymase a 120-min incubation period was used). For each peptide substrate, enzyme concentration was adjusted to between 0.02 and 4 nM to ensure that $[S]/(V_{max})$ of each enzyme was >5. Correlation coefficients were routinely >0.97. The concentration of human chymase and rat chymase-1 was 0.97 PM. The concentration of human chymase and rat chymase-1 was 0.0046 μM−1s−1 occurs with a ~780-fold lower catalytic efficiency than Ang II formation. In Ang II degradation, IHPPF forms the leaving group. Cleavage of DRVYIHPPF (peptide hc7), an Ang II analog in which the leaving group side chains and the nonessential part of the peptide backbone are deleted, occurs with an ~11-fold higher catalytic efficiency than the cleavage of Ang II (Table I). Effect of individual leaving group side chain interactions on Ang II catalysis by human chymase are summarized in Table I. Deletion of P'1 (peptide hc8), P'2 (peptide hc9), P'3 (peptide hc10), or P'4 (peptide hc11) side chains in Ang II leads to a 2–14-fold increase in $K_{cat}$, a 7–170-fold increase in $k_{cat}/K_m$, and a 2–15-fold increase in $k_{cat}/M$. One way to interpret these data is that the binding energy im...
parted by these P′ interactions leads to a more stable ES complex, but the stability of the ES‡ complex is decreased. The activation energy of the $k_{cat}$ therefore increases substantially, so the reaction rate and catalytic efficiency both decrease. Thus, the IHPF leaving group at the Ang II Tyr$^{1}$ bond is detrimental for catalysis, and all leaving group side chains contribute to this effect.

In Ang II formation, the sequence DRVYIHPF (Ang II) forms the acyl group. Table I shows that catalytic efficiency does not change when this Ang I acyl group is reduced in length from DRVYIHPF to IHPF (peptide hc12). IHPF$^{1}$ HL (peptide hc12) and IHPF$^{2}$ GG (peptide hc13) are cleaved with similar catalytic efficiencies (Table I), again indicating that side chain interactions of the His-Leu leaving group are not consequential in the cleavage of the Phe$^{3}$-His$^{9}$ bond. Therefore, of the acyl group interactions, those provided by the IHPF residues are sufficient for the high catalytic efficiency with which human chymase cleaves the Phe$^{3}$-His$^{9}$ bond in Ang I. Because the acyl group involved in Ang II degradation, i.e. DRVY, is also four residues long, in the next series of experiments we directly compared the influence of the IHPF and DRVY acyl groups on catalytic efficiency.

The catalytic efficiency for human chymase-dependent cleavage of IHPF$^{2}$ GG (peptide hc13) was ~45-fold higher than for DRVY$^{1}$ GG (peptide hc7) cleavage (Table I). This difference in catalytic efficiency was almost entirely due to an ~44-fold difference in $k_{cat}$, indicating that the IHPF acyl group, relative to the DRVY acyl group, does not affect the stability of the ES, but instead its binding energy is realized only in the ES‡, i.e. the structure of the acyl group binding site is much more complementary to the transition state structure of the IHPF acyl group than to that of the DRVY acyl group.

The difference in DRVY and IHPF acyl groups reactivities was surprising because in both these acyl groups the P$_1$ and P$_2$ residues were previously predicted to be optimal (11). This observation prompted us to examine which residue(s) was the chief determinant of the reactivity difference observed between these acyl groups. Table II shows that replacement of the P$_2$ Asp with Ile (peptide hc15), P$_2$ Arg with His (peptide hc16), or P$_2$ Val with Pro (peptide hc17) in the DRVY acyl group of Ang II produced decreases in the free energy required to reach the ES‡ during hydrolysis ($\Delta G^\ddagger_{cat}$) $=$ $-3.67$ kJ mol$^{-1}$ for P$_2$ Asp $\rightarrow$ Ile, $-2.01$ kJ mol$^{-1}$ for P$_2$ Arg $\rightarrow$ His, and $-0.67$ kJ mol$^{-1}$ for P$_2$ Val $\rightarrow$ Pro. Replacement of the P$_1$ Tyr with Phe (peptide hc18) in the DRVY acyl group of Ang II produced a small increase (0.71 kJ mol$^{-1}$) in $\Delta G^\ddagger$.

Asp with Ile (peptide hc15), P$_3$ Arg with His (peptide hc16), or P$_4$ Val with Pro (peptide hc17) in the DRVY acyl group, and the P$_4$ Asp $\rightarrow$ Ile change has the most favorable effect on reactivity. Additivity analysis indicates a good agreement between the observed ($\Delta G^\ddagger_{cat}$ (multiple)) and calculated ($\sum \Delta G^\ddagger_{cat}$ (components)) decreases in transition state stabilization energy for two- or three-component transitions in the Ang II acyl group, e.g. DRVY $\rightarrow$ IIVY, DRVY $\rightarrow$ DRPY, or DRVY $\rightarrow$ IHVF (peptides hc19–21) (Table II). Remarkably, however, when we compared the observed decrease in transition state stabilization energy associated with the DRVY $\rightarrow$ IIVY change in the Ang II acyl group ($\Delta G^\ddagger_{cat}$ (multiple) $= -14.3$ kJ mol$^{-1}$) with that calculated on the basis of individual P$_1$ to P$_4$ changes, it is apparent that a significant component (>40%) of the high reactivity of the IHPF acyl group for human chymase is due to synergistic behavior between all four these acyl group residues. Additional additivity analyses based on calculations of $\Delta G^\ddagger_{cat}$ (multiple) from $K_m$ values in Table I and $\Delta G^\ddagger_{cat}$ (from $k_{cat}$ values in Table I) indicates that for the DRVY$^{1}$ IHPF $\rightarrow$ IHPF$^{2}$ IHPF transition there is good agreement between observed and calculated measurements of $\Delta G^\ddagger_{cat}$ (multiple) and $\Delta G^\ddagger_{cat}$ (components) $= 0.95$ kJ mol$^{-1}$, but deviations from simple additivity are clearly evident in $\Delta G^\ddagger_{cat}$ measurements ($\Delta G^\ddagger_{cat}$ (multiple) $> 8.7$ kJ mol$^{-1}$). Thus, synergistic behavior of the IHPF acyl group

| Peptide name/ reference number | Substrate | $K_m$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}$/K$_m$ (µM$^{-1}$ s$^{-1}$) |
|-------------------------------|-----------|------------|----------------|------------------|
| Ang I                         | DRVYIHPF$^1$ HL | 40.2 ± 2   | 146 ± 3.2     | 3.6             |
| Ang II                        | DRVY IHPF  | 8.9 ± 2    | 0.041 ± 0.003 | 0.0046          |
| h1                            | DRVYIHPF$^1$ GG | 49 ± 0.25 | 330 ± 0.4     | 6.7             |
| h2                            | DRVYIHPF$^1$ GG | 240 ± 2.5 | 29 ± 0.1      | 1.2             |
| h3                            | DRVYIHPF$^1$ G-NH$_2$ | 59 ± 3.3  | 100 ± 1.4     | 1.0             |
| h4                            | DRVYIHPF$^1$ GG | 48 ± 0.5  | 440 ± 5.0     | 9.0             |
| h5                            | DRVYIHPF$^1$ GG | 62 ± 0.5  | 350 ± 6.4     | 5.6             |
| h6                            | DRVYIHPF$^1$ GGGG | 54 ± 2.0 | 420 ± 6.6     | 7.7             |
| h7                            | DRVYIHPF$^1$ GGGG | 125 ± 7.6 | 6.4 ± 0.06    | 0.051           |
| h8                            | DRVYIHPF$^1$ GGGG | 31 ± 1.4  | 0.029 ± 0.015 | 0.0033          |
| h9                            | DRVY IGF    | 56 ± 0.45  | 2.5 ± 0.15    | 0.045           |
| h10                           | DRVY IGF    | 20 ± 0.46  | 0.56 ± 0.04   | 0.028           |
| h11                           | DRVY IHGF   | 102 ± 11   | 7.0 ± 0.5     | 0.068           |
| h12                           | IHPF$^1$ HL | 78 ± 2.3   | 274 ± 5       | 3.5             |
| h13                           | IHPF$^1$ G   | 123 ± 12   | 280 ± 6.7     | 2.3             |
| h14                           | IHPF$^1$ HH  | 29 ± 4.8   | 35 ± 6.6      | 1.2             |
| h15                           | IRYV IHPF   | 5.9 ± 0.1  | 0.32 ± 0.001  | 0.054           |
| h16                           | DRYV IHPF   | 12 ± 2.5   | 0.12 ± 0.001  | 0.01            |
| h17                           | DRPY IHPF   | 25 ± 2.13  | 0.21 ± 0.004  | 0.006           |
| h18                           | DRVF IHPF   | 8.0 ± 0.4  | 0.25 ± 0.001  | 0.0055          |
| h19                           | IIVY IHPF   | 13.6 ± 1.2 | 1.04 ± 0.03   | 0.071           |
| h20                           | DRPY IHGF   | 20 ± 0.9   | 0.09 ± 0.001  | 0.0045          |
| h21                           | DRPY IHGF   | 9.0 ± 2.6  | 0.25 ± 0.001  | 0.077           |
| h22                           | DRPY IHPF   | 19 ± 1.4   | 1.0 ± 1.7     | 9.2             |
| h23                           | DRPY IHPF, ethyl ester | 19 ± 1.0 | 1.744 ± 5.0   | 92              |

a For positional nomenclature of residues, see Ref. 16. Arrows indicate the peptide bond hydrolyzed by human chymase under assay conditions.
residues in producing a highly reactive human chymase substrate is seen in the energetics to reach ES complex but is not observed in the initial energetics of substrate binding that leads to the formation of the ES complex.

We speculate that the extended substrate binding site of human chymase, particularly the region that binds the P$_2$ to P$_3$ acyl group residues, has specialized to allow the Phe$_8$-His$_9$ bond in Ang I to bind in a highly productive mode. This optimal acyl group is generated through synergistic interactions between neighboring acyl group residues; these interactions, we believe, form the basis of the "structural context" that has allowed human chymase to become an efficient Ang II-forming enzyme. The critical nature of these synergistic interactions in determining specificity is illustrated by the fact that the Ang II Tyr$_4$-Ile$_5$ bond and the Ang I Phe$_8$-His$_9$ bond, which seemingly determining specificity is illustrated by the fact that the Ang II cleavage ($k_{cat}/K_m = 0.085$ $\mu$M$^{-1}$s$^{-1}$) is compared to DRVY$^1$GG cleavage (peptide rc6; $k_{cat}/K_m = 0.0039$ $\mu$M$^{-1}$s$^{-1}$). These findings suggest that interaction of the Ang II leaving group with the rat chymase-1 S' subsite is dependent on secondary structure of the substrate leaving group or perhaps that of the entire substrate and that the P$_1$ interaction is dominant over other P' interactions. These speculations led us 1) to consider whether the effect of the Ang II leaving group would be different if this leaving group was attached to a different acyl group and 2) to examine the effect of a single P$_1$ Ile side chain on catalysis.

Direct comparisons were made between the Ang II acyl group DRVY and the Ang I acyl group IHPF. Specificity constants for the cleavage of IHPF$^3$GG (peptide rc2) and DRVY$^1$GG (peptide rc6) were identical (Table III), indicating that within the context the Gly-Gly leaving group rat chymase-1 does not differentiate between these acyl groups. To determine if interactions between the acyl and the leaving group can influence transition state stabilization, we compared the effects of IHPF and Gly-Gly leaving groups on DRVY and IHPF acyl groups (peptides rc2, rc6, rc11, and Ang I). Table IV shows that the IHPF leaving group, relative to the Gly-Gly leaving group, causes a $7.94$ kJ/mol$^{-1}$ decrease in $\Delta G_T^{-1}$ when DRVY is the acyl group and a $13.8$ kJ/mol$^{-1}$ decrease in $\Delta G_T^{-1}$ when IHPF is the acyl group. Thus, acyl-leaving group interactions can greatly influence the overall effect of the leaving group on transition state stabilization.

To show if the favorable effect of the IHPF leaving group in IHPF$^1$GG catalysis can be mimicked by the introduction of an Ile side chain at the P$_1$ position, we examined DRVY$^1$GG (peptide rc11), IHPF$^1$GGG (peptide rc4), IHPF$^1$IOGG (peptide rc12), and IHPF$^1$GGGG (peptide rc5) catalysis by rat chymase-1. IHPF$^1$GG was cleaved by rat chymase-1 with a 138-fold higher catalytic efficiency than IHPF$^1$GGG. IHPF$^1$IOGG was cleaved with a 86-fold higher catalytic efficiency than IHPF$^1$GGGG; peptides in which Ile side chains were introduced at additional leaving group sites, e.g. IHPF$^1$BGG and IHPF$^1$BGG (peptides rc13 and rc14), were catalyzed with an efficiency similar to that of IHPF$^1$IGGG (Table III). These findings are consistent with the view that the P$_1$ Ile side chain of the IHPF leaving group provides the dominant favorable effect. This Ile effect on catalytic efficiency is produced by a 12.5-fold decrease in $K_m$ and a 5.3-fold increase in $k_{cat}$ (peptides rc5 and rc12) and is position-dependent.

In contrast, in the case of human chymase, introduction of an Ile in the P$_1$ position of the pentaglycyl leaving group (pepti
Contributions of leaving group substrate residues-rat chymase-1 subsite interactions to the catalytic efficiency of peptide substrates
rc6, rc7, rc8, rc9, rc10, and rc11 listed in Table III

| Peptide name/ reference number | Substrate group (Pn, P1, P2, P3, P4, P5, P6, P7, P8, P9) | Km (μM) | kcat (s⁻¹) | kcat/Km (μM⁻¹s⁻¹) |
|--------------------------------|---------------------------------------------------------|---------|-------------|-------------------|
| Ang II                        | DRVY₄IHPF                                               | 55 ± 2.7| 4.7 ± 0.08  | 0.085             |
| rc1                           | IHFP₄HL                                                | 250 ± 7.8| 1.08 ± 0.011| 0.0043            |
| rc2                           | IHFP₄GG                                                | 420 ± 6.5| 1.7 ± 0.005 | 0.004             |
| rc3                           | IHFP₄GGGG                                              | 320 ± 5.0| 1.6 ± 0.03  | 0.005             |
| rc4                           | IHFP₄GGGGG                                             | 310 ± 14 | 2.0 ± 0.02  | 0.006             |
| rc5                           | IHFP₄GGGGG                                             | 200 ± 10 | 1.6 ± 0.03  | 0.008             |
| rc6                           | DRVV₄GG                                               | 180 ± 5.0| 0.7 ± 0.04  | 0.0039            |
| rc7                           | DRVV₄IHPF                                              | 54 ± 3.0 | 0.71 ± 0.004| 0.013             |
| rc8                           | DRVV₄IHPGF                                             | 35 ± 0.4 | 14 ± 0.08   | 0.4               |
| rc9                           | DRVV₄IHPGF                                             | 25 ± 2.1 | 8.0 ± 0.11  | 0.32              |
| rc10                          | DRVV₄IHPG                                              | 58 ± 2.0 | 33 ± 0.21   | 0.56              |
| rc11                          | IHFP₄IHPF                                              | 9.0 ± 1.0 | 7.5 ± 0.1   | 0.83              |
| rc12                          | IHFP₄IHPGF                                             | 16 ± 0.1 | 8.4 ± 0.1   | 0.53              |
| rc13                          | IHFP₄IHPGG                                             | 14 ± 0.86| 6.9 ± 0.38  | 0.49              |
| rc14                          | IHFP₄IHPGI                                             | 14 ± 2.2 | 7.8 ± 0.07  | 0.55              |

* For positional nomenclature of residues, see Ref. 16. Arrows indicate the peptide bond hydrolyzed by rat chymase-1 under assay conditions.

**ΔΔG‡(A → B) = −RT ln [kcat/Km(B)/kcat/Km(A)], i.e. difference in transition state stabilization energy of the two substrates A and B.**

**Table IV**
Contributions of leaving group substrate residues-rat chymase-1 subsite interactions to the catalytic efficiency of peptide substrates
rc6, rc7, rc8, rc9, rc10, and rc11 listed in Table III

| Substrate pair | ΔΔG‡(A → B) (kJ·mol⁻¹) |
|----------------|------------------------|
| DRVY₄IHPF → DRVY₄GHP | 4.84 |
| DRVY₄IHPF → DRVY₄IHPF | 4.0 |
| DRVY₄IHPF → DRVY₄IHPF | -3.42 |
| DRVY₄IHPF → DRVY₄IHPF | -4.86 |
| IHFP₄IHPF → IHFP₄IHPF | -7.84 |
| IHFP₄IHPF → IHFP₄IHPF | -13.8 |

**Table III**
Hydrolysis of Ang II and its analogues by rat chymase-1

The kinetic constants were determined in 20 mM Tris-HCl buffer, pH 8.0, containing 0.5 M KCl, and 0.01% Triton X-100 at 37 °C. Km and kcat values were determined by nonlinear regression; values are the means ± S.D. of three independent determinations for each peptide.
that interactions between the binding site of human chymase or rat chymase-1 and the substrate are best explained if the entire substrate binding site is taken as an entity rather than as a collection of distinct \( S_x \) and \( S'_x \) subsites. Thus, these studies suggest that the identification of highly reactive novel substrates for human chymase as well as the design of substrate-derived inhibitors cannot be predicted from simple subsite mapping; on the other hand, combinatorial approaches are likely to be effective. This speculation is strengthened by the recent studies of Bastos et al. (20) that show highly synergistic behavior between certain \( P_3-P_2 \) combinations in combinatorial human chymase inhibitor libraries that could not be predicted from simple subsite maps of human chymase (11). Combinatorial approaches could also prove be useful in defining the specificity of related leukocyte serine proteases such as cathepsin G where linear approaches have failed in identifying highly efficient natural substrates.

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