Inhibition of human chymase by α1-antichymotrypsin produces 3.5 mol of degraded inhibitor for every mol of chymase inhibited, resulting in a stoichiometry of inhibition (SI) of 4.5. In the present study, the substrate versus inhibitor properties of this reaction were examined further using wild type and mutant recombinant antichymotrypsins (rACT). Titration of chymase hydrolytic activity with rACT-L358W, L358M, and L358F indicated that the SI was sensitive to P1 residue replacements. SI values increased in the order of Trp < Met < Leu < Phe where SI values were 1.5, 2, 4, and 7, respectively. Chymase inhibitor complex and cleaved inhibitor were demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for all variants; the relative intensities of each band were consistent with SI values established by titration. NH2-terminal sequence analyses of the products formed in the reaction of chymase with rACT-L358F indicated that the P1-P1′ bond was the primary site of cleavage resulting in the hydrolysis and inactivation of this variant. The apparent second-order rate constant for chymase inhibition (k'/[I]) by rACT also was affected by P1 substitution. k'/[I] values increased in an order opposite that obtained for SI values (Phe < Leu < Met < Trp). The reactive loop mutant (rACT-P339P′) produced by replacing the reactive site region of ACT (Thr396-Val398) with that of α1-proteinase inhibitor (Ile396-Pro398) revealed a different reaction pattern. Although its SI was near 1, the value for k'/[I] was the lowest among variants. rACT-L358R, another P1 variant, did not inhibit chymase. These results are evaluated with respect to the substrate preferences of human chymase and with respect to partitioning schemes proposed to explain SI values greater than 1.

Human chymase from skin is a chymotrypsin-like proteinase that is stored within the secretory granules of mast cells (1–3). We have shown previously that this proteinase is inhibited by two human plasma inhibitors, ACT and α1-proteinase inhibitor (4). Both inhibitors are members of the serpin (serine proteinase inhibitor) protein family (5, 6). Inhibition of chymase by both inhibitors appeared irreversible, showing the typical tight binding SDS-stable complexes characteristic of serpin-target proteinase interactions (5, 6). Apparent second-order rate constants obtained under pseudo-first-order conditions were 21,000 M−1 s−1 for ACT and 7,500 M−1 s−1 for α1-proteinase inhibitor (4). The magnitude of these values is on the low side for serpin-proteinase interactions, approximately 1,000 × lower than values reported for the inhibition of neutrophil cathepsin G by ACT and neutrophil elastase by α1-proteinase inhibitor (7). The reaction of chymase with ACT and α1-proteinase inhibitor also displayed an unusually high SI, a value empirically defined as mol of inhibitor required to inhibit 1 mol of chymase measured by titration. SI values of 4.5 and 5.0 were obtained for inhibition of chymase by ACT and α1-proteinase inhibitor, respectively.

Further analysis of the interaction of chymase with each inhibitor revealed that the high SI for inhibition was caused by a competing reaction producing a hydrolyzed inactive inhibitor. SDS-PAGE and NH2-terminal sequence analyses indicated that the hydrolyzed inhibitor was generated by cleavage within the reactive loop (4); the cleavage site for ACT was provisionally located at the reactive site of the inhibitor (Leu288-Ser289), whereas the cleavage site for α1-proteinase inhibitor was located at a bond (Phe282-Leu283) several residues away from the reactive site (Met281). SI values obtained from titrations of chymase with either ACT or α1-proteinase inhibitor did not vary as a function of the initial enzyme concentration, indicating that the fraction of total inhibitor converted to hydrolyzed inhibitor was an invariant property of each reaction (4).

In this work, we provide additional evidence that the location of the chymase cleavage site producing hydrolyzed ACT
is the reactive site of the inhibitor which is referred to as the P1-P1′ site following the nomenclature of Schechter and Berger (6). We also investigated further the substrate versus inhibitor properties of the reaction of chymase with ACT using recombinant ACT (rACT) variants. These studies provide additional support for the inclusion of a rapid partitioning scheme in the general equation for serpin-proteinase interactions to explain high SI values, as proposed by Cooperman et al. (9) in the preceding article.

**EXPERIMENTAL PROCEDURES**

**Materials**—Peptide-pNA substrates were purchased from Sigma or Bachem. Bovine chymotrypsin and trypsin were from Calbiochem or Sigma. TSK-heparin-SPW and Mono Q HPLC columns were from Superdex and Pharmacia LKB Biotechnology, respectively. Imobilin-P membranes (PVP) from Millipore. Heparin-Sepharose was from Pharmacia. The detergents dodecyl maltoside and Triton X-100 were obtained from Anatrace and Sigma, respectively.

**rACT Variants**—Expression of rACTs, production of reactive site, reactive loop (rACT-P3P3′), and cassette mutants of ACT, and purification of rACTs, were accomplished as described by Rubin et al. (8). Phytoalbumin-Sepharose, rACT-P3P3′, and rACT-P3P3′cas refer to variants produced using a construction (11). Insertion of the cassette into ACT cDNA required the creation of two restriction sites which resulted in three amino acid changes in the inhibitor. In one, Ala384-Ala385 (P10-P9) was changed to Gly-Thr, and in the other, Val384 (P10') was changed to Thr384 (P10). Sites P9-P10 are located near reactive loop position P14, a site critical for serpin inhibitory function (12-15). Mutations at site P10 of antithrombin III (Ala384 to Ser or Pro) and C1 inhibitor (Ala384 to Thr) have been shown to reduce the inhibitory activities of these serpins (13). The P10 Ala to Gly mutation in our cassette variant of ACT was a more conservative substitution than those affecting the activity of antithrombin III. In addition, the P10 site of α1-proteinase inhibitor is Gly, suggesting that this amino acid residue may be acceptable in the P10 position of ACT. These changes alone did not alter the inhibition properties of ACT toward chymotrypsin (11). rACTs also contained a short NH2-terminal extension of 5 (10) or 4 (11) amino acids. Inhibition of chymase by rACT-Leu100 variants containing both types of NH2-terminal constructions was identical with respect to SI and inhibition rate constants.

**Purification of Human Chymase**—Most cell proteinases were extracted from skin and fractionated on a 500-ml heparin-Sepharose column. Heparin-Sepharose was eluted from the column with a linear gradient of 0.25-1.0 mM Suc-A-A-P-F-pNA, 2.5-3.0 nM chymase, and inhibitor. Absorbance was monitored continually for 15 min, and instantaneous velocities were determined over every 1-min interval by linear regression analysis of the data. Plots of the instantaneous velocity versus time (t) were fit by nonlinear methods to the expression \( V_0 = V_{0e} - k_{cat}[I] \), where \( V_0 \) corresponds to initial activity. The apparent second-order rate constant determined after correcting for the presence of substrate is equal to \( k' = k_{cat}([S]/K_m) + 1 \), where \( K_m \) is the inhibitor concentration, \([S]\) is the substrate concentration, and \( K_m \) is the Michaelis constant for chymase hydrolysis of the substrate under the experimental conditions. Reactions were monitored for at least three half-lives, except for rACT-P3P3′, where data covered one half-life. \( K_m \) for hydrolysis of substrate by chymase was 0.80 mM in 1.0 mM NaCl and 0.49 mM in 2.0 mM NaCl (4).

**SDS-Polyacrylamide Gel Electrophoresis**—Reactions of chymase with various inhibitors under standard assay conditions were fractionated on a 500-ml heparin-Sepharose column eluted with 0.25-1.0 mM Suc-A-A-P-F-pNA, 2.5-3.0 nM chymase, and inhibitor. Absorbance was monitored continually for 15 min, and instantaneous velocities were determined over every 1-min interval by linear regression analysis of the data. Plots of the instantaneous velocity versus time (t) were fit by nonlinear methods to the expression \( V_0 = V_{0e} - k_{cat}[I] \), where \( V_0 \) corresponds to initial activity. The apparent second-order rate constant determined after correcting for the presence of substrate is equal to \( k' = k_{cat}([S]/K_m) + 1 \), where \( K_m \) is the inhibitor concentration, \([S]\) is the substrate concentration, and \( K_m \) is the Michaelis constant for chymase hydrolysis of the substrate under the experimental conditions. Reactions were monitored for at least three half-lives, except for rACT-P3P3′, where data covered one half-life. \( K_m \) for hydrolysis of substrate by chymase was 0.80 mM in 1.0 mM NaCl and 0.49 mM in 2.0 mM NaCl (4).

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mixtures at the conclusion of the incubation period. After a further 20-min incubation, samples were concentrated, desalted as described above, dried under nitrogen, and finally resuspended in trifluoroacetic acid. A portion of the trifluoroacetic acid-solubilized sample was analyzed by automated Edman degradation, and another portion was subjected to quantitative amino acid analysis to confirm the protein concentration. rACT-L358F was reacted with chymase in the latter analyses were subjected to fractionation on a Mono Q column as an additional purification step.

Kinetic Constants for Peptide pNA Substrates—$k_{\text{cat}}$ and $k_{\text{cat}}/[I]$ values for the hydrolysis of Suc-A-A-P-F-pNA by chymase in solutions containing 1 and 2 M NaCl were determined according to the Michaelis-Menten rate equation by nonlinear regression analysis of initial velocity versus substrate concentration data. Six to eight different substrate concentrations ranging from 0.25 to 3 mM were used in each experiment. Fits were all statistically significant with standard errors of $K_m$ and $k_{\text{cat}}$ values of < 18 and 10%, respectively.

RESULTS

The Large SI for the Reaction of Human Chymase with ACT Is Not the Result of a Contaminating Proteinase or Poorly Folded Inhibitor—To confirm the purity of chymase preparations routinely used in reactions with rACTs, an enzyme preparation was subjected to additional fractionation on a TSK-heparin HPLC column. Only one peak of chymotryptic-like activity was observed eluting at the column at 0.75 M NaCl, even when fractions were assayed for long time periods to allow for minor contaminating activities to be detected. The SI value for rACT and rACT-L358F of highly purified chymase was the same as that obtained with routinely purified chymase, indicating that our routine preparations were not contaminated with another proteinase capable of hydrolyzing rACTs.

In a second test of enzyme purity, chymase purified by routine procedures was reacted with rACT at [rACT]/[chymase]$_o$ > SI, and then reactions were analyzed by SDS-PAGE to show that excess inhibitor was not subject to further hydrolysis by a possible contaminating proteinase. The presence of intact inhibitor in addition to degraded inhibitor at [I]$_o/[E]_o$ ratios > SI is shown in Fig. 1A. Even after long incubation times intact inhibitor was observed on gels (Fig. 1B). Residual intact inhibitor was shown to be active in a parallel experiment in which chymotrypsin was added to the reaction after inhibition of chymase. SDS-PAGE analysis of this reaction mixture (not presented) showed the disappearance of the intact rACT band and the appearance of an additional high $M_r$ band corresponding to the chymotrypsin-rACT complex. These results also argue against the presence of a proteinase contaminant for which rACT is uniquely a substrate.

High SI values also could result from the presence of improperly folded inhibitor in our ACT preparations. Previous studies by us have shown that serum-ACT, as well as rACT and variant ACTs purified from bacterial extracts, have SI values near 1 in reactions with bovine pancreatic chymotrypsin or trypsin and that all ACTs behave as single species in experiments that determine their rate constants for inhibition of chymotrypsin or trypsin (10, 11). This biochemical characterization indicates that serum ACT used previously (4) and the rACTs used in the present study were properly folded. The stoichiometries of rACTs used in the present work were determined by titration of each inhibitor with chymotrypsin or trypsin, and only the concentration of active inhibitor established from these titrations was used to calculate SI values for chymase inhibition.

The studies and arguments just described for ruling out artifactual possibilities strongly indicate that the large SI values observed for the inhibition of chymase by serum ACT and by rACTs are an intrinsic property of the interaction between these proteins.

Reaction of Chymase with Variant rACTs—Titrations for five reactive site (P1 site) variants denoted as rACTs L358 (wild type), L358M, L358F, L358W, and L358R, and a more extensively substituted variant, denoted as rACT-P3P3', are shown in Fig. 2. In the latter variant, the P3-P3' sequence of ACT was replaced by the corresponding sequence of α-chymotrypsin inhibitor. The difference in the titrations demonstrate that the SI value was sensitive to mutation of the P1 site as well as to mutation of the P3-P3' sequence. The variant rACT-L358R did not inhibit chymase even at an [I]$_o/[E]_o$ ratio of 20 (not shown in Fig. 2). SI values for each variant are reported in Table I along with SI values for three additional rACT variants that were used using a cassette construction (denoted as cas) as described under "Experimental Procedures." Examples of time courses for reactions show that the inhibition obtained at different [I]$_o/[E]_o$ ratios was stable, even at the high ionic strength (1 M NaCl) conditions employed (Fig. 3). Residual activities in these studies were achieved within minutes and remained constant for at least 24 h. Consistent with the stability of residual activities, the SDS gel band representing the chymase-rACT complex remained intact for at least 24 h of incubation (Fig. 1B).

Apparent second-order rate constants ($k'/[I]$) for the inhibition of chymase by rACT variants are reported in Table I. They were measured at [rACT]/[chymase]$_o$ ratios at least 10 times the SI determined for each variant. At these high [I]$_o/[E]_o$ ratios, we have shown previously that the reaction of chymase with serum ACT follows pseudo-first-order kinetics (4). Chymase activity loss in the presence of all rACT variants was first-order, proceeding to a complete loss of catalytic activity. Inhibitor concentration for these measurements did not exceed 300 nM. This concentration is not likely saturating for the reaction based on our previous study with serum ACT, which showed a linear relationship between $k'$ and [I] up to an inhibitor concentration of 1 μM.

The results in Table I show that $k'/[I]$ values were also sensitive to P1 and reactive loop substitutions. $k'/[I]$ values for P1 variants decreased in the order of Trp > Met > Leu > Phe, which was opposite to that observed for SI values. This trend was not followed by the rACT-P3P3' variant which demonstrated a low SI and a low $k'/[I]$ value. Only minor changes in SI and $k'/[I]$ values were observed at the two different NaCl concentrations (1 and 2 M) employed. Although the reaction properties of cassette variants differed somewhat from those of the corresponding non-cassette variants, the change in reaction properties between cassette variants paralleled those of corresponding non-cassette variants. Presumably, the different SI and $k'/[I]$ values for both types of variants are related to the additional mutations at sites P9-P10 and/or P10' required for construction of the cassette variants.

SDS-PAGE Analysis of Products Generated in Reaction of Chymase with rACTs—Banding patterns of reaction products obtained for rACTs at indicated [I]$_o/[E]_o$ ratios are shown in Fig. 4. The patterns clearly show the presence of a complex band with an apparent molecular mass corresponding to the addition of chymase (30 kDa) and rACT (46 kDa), a band corresponding to rACT when the [I]$_o/[E]_o$ > SI, as well as a band or bands migrating slightly slower than rACT (37-40 kDa) presumably corresponding to rACT cleaved in the reactive loop. The relative intensities of these bands vary in qualitative agreement with the SI values obtained from titrations for each variant; i.e., rACTs with low SI values, like rACT-L358W and rACT-P3P3', have an intense complex...
Reaction of Human Chymase with Variant Antichymotrypsins

![Image: SDS-PAGE banding patterns showing Chymase vs rACT-L358](image)

**Fig. 1.** Analysis by SDS-PAGE of the products formed in the reaction of human chymase with rACT-L358 as a function of $[I]/[E]$ ratio (panel A) and time (panel B). Abbreviations on the sides of the panels signify migration of proteinase inhibitor complex (C), undegraded inhibitor (UI), and degraded inhibitor (DI). The $M_I$ of the complex band is roughly the sum of the $M_I$ of rACT (46,000) and chymase (30,000). The amount of inhibitor resolved in each analysis was equivalent to 3 pg of native inhibitor; various $[I]/[E]$ ratios were monitored in an $I/E$ ratio of 7 was monitored in each analysis. The amount of inhibitor resolved in each analysis was equivalent to 3 pg of native inhibitor; various $[I]/[E]$ ratios were not noticeable degradation of complex bands as well as two bands migrating faster than intact inhibitor. These extra bands are presumably degradation of complex bands as well as two bands migrating faster than intact inhibitor. These extra bands are presumably caused by the presence of uninhibited chymase. A single reaction at an $[I]/[E]$ ratio of 7 was monitored in panel A. At the indicated times, shown below the lanes, reactions were stopped by addition of PMSF (2 mM final concentration) and denatured in SDS by standard procedures. Denatured samples were then analyzed by SDS-PAGE at the end of experiment. Lanes 6 and 7 in panel B are rACT-L358 alone and chymase alone (1 µg of chymase in this lane does not correspond to that used in the time course). Chymase migrates as broad band because of denatured samples were then analyzed by SDS-PAGE at the end of experiment. Lanes 6 and 7 in panel B are rACT-L358 alone and chymase alone (1 µg of chymase in this lane does not correspond to that used in the time course). Chymase migrates as broad band because of glycosylation (17).

**Fig. 2.** Titration of human chymase with different rACT variants. Chymase concentrations were held constant in each titration, and the amount of inhibitor was varied to obtain the different $[I]/[E]$ ratios. Different symbols denote separate experiments. The enzyme concentration used for titration ranged between 150 and 400 nM. Among individual variants, SI values extrapolated from titrations did not change as a function of the initial chymase concentration.

| rACT variant | NaCl | $k'/[I]^a$ | SI |
|--------------|------|------------|----|
| L358W        | 1    | 151,000    | 1.5|
| L358M        | 1    | 46,500     | 2.0|
| L358         | 1    | 27,000     | 4.0|
| L358F        | 1    | 24,000     | 7.0|
| P3P3'        | 1    | 10,300     | 1.3|
| L358W        | 2    | 280,000    | 1.2|
| L358M        | 2    | 64,000     | 2.0|
| L358         | 2    | 42,500     | 3.5|
| L358F        | 2    | 21,500     | 7.0|
| L358cas      | 1    | 58,000     | 2.0|
| L358Fc     | 1    | 12,000     | 8.0|
| P3P3'cas    | 2    | 8,250      | 1.4^4|

* Experimental conditions for rate constant measurements were similar to those of titrations except for the presence of substrate and 9% Me2SO. Most $k'/[I]$ values are the average of two experiments, and deviation from the average was typically less than 15%. $k'/[I]$ values for rACT-L358 in 2 M NaCl and rACT-L358W in 1 M NaCl were based on four and five measurements, respectively; S.D. were 8 and 15% of the reported values, respectively.

**SI was measured under 1 M NaCl conditions.**

Identification of the Site of Cleavage of rACT-L358F by Human Chymase—SDS-PAGE banding patterns showing major degradation products slightly smaller than rACTs suggested that inhibitors were cleaved by chymase within the reactive loop region (the reactive center is 40 residues from the COOH terminus). Small peptide products (~4 kDa) formed in the reaction of chymase with rACT-L358Fcas were identified on highly cross-linked SDS gels as described previously (4). Following electroblotting of these gels onto PVDF membranes, the small peptides were subjected to NH$_2$-terminal sequence analysis. As shown in Table II, experiment 1,
produced by cleavage at the reactive site. The only peptide identified in the low M, band began with Ser<sup>369</sup>, the P<sub>1</sub>' position of ACT. Since rACT-L358Fcas has an SI of 8 (Table I), cleavage arising from its hydrolysis as a substrate should predominate over its hydrolytic product (P<sub>1</sub>'-COOH-terminal peptide) released upon SDS denaturation of proteinase-inhibitor complexes (23, 24). Thus, only finding peptide beginning with Ser<sup>369</sup> is a result which indicates that inhibitor inactivation (hydrolysis as substrate) was produced by cleavage at the reactive site.

A similar analysis for the reaction of chymase with serum ACT, which has an SI of 4.5, was described previously (4). Although two apparent sites of cleavage were observed (Table II, experiment 4), the peptide corresponding to cleavage at P<sub>1</sub>-P<sub>1'</sub> (Leu<sup>368</sup>-Ser<sup>369</sup>) was formed apparently in a higher yield than the peptide corresponding to cleavage at P<sub>3</sub>-P<sub>4</sub>' (Leu<sup>361</sup>-Val<sup>362</sup>). Since Leu<sup>361</sup> is located on the COOH-terminal side of Leu<sup>368</sup>, we suggest that P<sub>3</sub>'-P<sub>4</sub>' cleavage may have occurred following P<sub>1</sub>-P<sub>1'</sub> cleavage. This suggestion is supported by the results of Baumann et al. (25) who showed that the peptide Ser<sup>369</sup>Leu<sup>368</sup> was removed from ACT on prolonged incubation with chymotrypsin.

The identity of the cleavage site producing degraded rACT-L358Fcas was confirmed in an experiment where NH<sub>2</sub>-terminal sequence analysis was performed on the entire reaction mixture. The results obtained for two reactions performed at different [I]<sub>e</sub>/[E]<sub>e</sub> ratios (ratios = SI and 2SI) demonstrated only one NH<sub>2</sub>-terminal sequence starting at Ser<sup>369</sup> (Table II, experiments 2 and 3). Based on yields of phenylthiohydantoin derivatives, the estimated recoveries of peptide for the two reactions were roughly 90% and 50%, respectively. Reactions were estimated assuming that 1) the coupling efficiency of protein to the sequencing support was 50%, and 2) yields of phenylthiohydantoin-Ala and phenylthiohydantoin-Leu in the second and third Edman degradation cycles were representative of peptide recoveries. These high recoveries indicate that the majority of the Ser<sup>369</sup>-peptide identified in this analysis was a product generated by the hydrolysis of rACT-L358Fcas as a substrate. Amino acid sequences corresponding to the NH<sub>2</sub> termini of rACT and chymase were not detected. The absence of the latter sequence was because of its low concentration. The absence of the former sequence is unclear. Although rACT is a bacterial product, other rACT variants prepared for analysis differently have demonstrated a free NH<sub>2</sub> terminus.

For analyses of whole reaction mixtures, reactions of chymase with rACT-L358Fcas were performed at 4 °C to take advantage of the high SI (SI = 15–17) we observed at this lower temperature. The SI of rACT-L358F, the non-cassette analog of rACT-L358Fcas, also increased as the temperature was lowered (SI increased from 7 to 12), and a similar effect of temperature has been reported for the reaction of C1 inhibitor with kallikrein (26). SDS-PAGE analyses of low temperature reactions confirmed the high SI and demonstrated that altering the temperature had no effect on the size of the hydrolyzed inhibitor product. The latter observation suggests that the location of the cleavage site producing hydrolyzed inhibitor was not changed by temperature.

Changes in SI among Variants Compared to the Substrate Specificity of Human Chymase—Kinetic constants in Table III for Suc-V-P-X-pNA substrates were reported by Powers et al. (3), and those for angiotensin I analogs were reported by Kinoshita et al. (27). Human chymase cleaves angiotensin I at the Phe<sup>9</sup>-His<sup>10</sup> peptide bond, thus for this substrate position 8 corresponds to the P<sub>1</sub> position (28, 29). As shown in Table III, the substrate preference (k<sub>cat</sub> and k<sub>cat</sub>/K<sub>m</sub> values) of chymase for a series of P<sub>1</sub>-substituted peptide-pNA substrates followed an order (Phe > Leu > Met > Trp), which parallels the substrate preference of chymase for rACT's, as shown by the SI values in Table I. Kinetic constants for P<sub>1</sub>-substituted angiotensin I analogs did not follow this order, however. For this series of substrates, the catalytic preference of chymase was Trp > Phe > Leu.

**DISCUSSION**

Inhibition of proteinases by serpins may occur with SI values greater than 1 as reviewed by Cooperman et al. (9) in the preceding article. The high SI is the result of a concurrent reaction producing a hydrolyzed form of the inhibitor which is inactive. Results presented in this study and a previous study (4) demonstrate that the interaction of human chymase with ACT is another example of this type of inhibition. The dual outcomes of the chymase-ACT interaction were evidenced by titrations that demonstrated SI values greater than 1 and SDS-PAGE analyses of reactions that demonstrated the presence of both a hydrolyzed inhibitor and a stable chymase-ACT complex (Fig. 1).

NH<sub>2</sub>-terminal analysis of the reaction products from inhibitors with high SI values (serum-ACT and rACT-L358F) identified the cleavage site resulting in inhibitor inactivation as the P<sub>1</sub>-P<sub>1'</sub> bond (Table II). The absence of a minor sequence in these analyses indicating a second proteolytic site within the reactive loop suggests that the P<sub>1</sub> site also was
compared with rACT-L358F (compare major degraded inhibitor product in products in cas. About 3 pg equivalents of (2.5 bands.

tion studies with other proteinases (5, 6). This result was peptide product corresponding to cleavage at P6-P7, thereby certain reaction conditions exhibited an SI of 9.

Fig. 4. SDS-PAGE analysis of reactions of human chymase with P1- variants of ACT (panel A), rACT-L358F (panel B), rACT-P3P3' cas (panel C), and rACT-L358W (panel D). P1 variants analyzed in panel A are reported under each lane; the Phe variant was rACT-L358Fcas. About 3 µg equivalents of (2.5 µM in reactions) rACT were resolved in each lane. Symbols signifying various reaction products in panels B–D are C, proteinase inhibitor complex; UI, undegraded inhibitor; DI, degraded inhibitor. [I]/[E]$_0$ ratios reported under each lane were obtained by varying [chymase$_0$]. Lanes containing inhibitor alone or chymase alone (panel D, lane 1, contained 1 µg of chymase) are signified by a dash. Reactions (25 °C) were stopped by the addition of PMSF after 1 h (panel A) or 30 min (panels B–D) of incubation. For reasons that are not clear, degradation products for many variants have slightly different migration patterns. Most notable is the dimeric pattern observed in the case of rACT-L358M (panel D) and the slower mobility of the rACT-L358Fcas degradation product compared with rACT-L358F (compare major degraded inhibitor product in panel A, lanes 4 and 8, with the corresponding product in panel B). Since both Phe variants had approximately the same SI and showed a similar increase in SI when the reaction temperature was lowered, we do not believe that the difference in the mobility of their hydrolyzed products is indicative of a different hydrolytic site. At [I]/[E]$_0$ ratios < SI there is noticeable degradation of complex bands as well as the appearance of additional bands migrating below degraded inhibitor bands.

responsible for chymase inhibition, as expected from inhibition studies with other proteinases (5, 6). This result was different from that obtained for the interaction of chymase with another serpin, $\alpha_1$-proteinase inhibitor, which under certain reaction conditions exhibited an SI of 9 (4). In this case, NH$_2$-terminal analysis demonstrated a minor peptide product corresponding to cleavage at P1-P1' and a major peptide product corresponding to cleavage at P6-P7, thereby indicating that the hydrolytic site was different from the reactive site. A second observation supporting the P1 site as the chymase inhibitory site was that replacement of Leu$_{358}$ with Arg produced an rACT variant active toward trypsin and chymotrypsin but not chymase. Since human chymase does not readily hydrolyze ester or peptide substrates with Arg at P1, the lack of inhibition by rACT-L358R agrees with the expectation of reduced interaction at the P1 site. In contrast, chymase inhibition was observed with all other P1 variants of rACT having residues recognized by chymase in substrates. Other serpin-proteinase pairs that demonstrate reactive site-bond cleavage concurrent with proteinase inhibition are thrombin-antithrombin and kallikrein-C1 inhibitor (26, 31, 32).

Analysis of the interaction of chymase with reactive site variants of ACT (Table I) and a more extensively substituted variant, rACT-P3P3', demonstrated that SI values and apparent second-order inhibition rate constants ($k'/[I]$) are altered by mutation. Variation of the P1 residue in rACT with residues recognized by chymase in the P1 site of substrates produced inhibitors with different SI values that followed the order of Trp < Met < Leu < Phe; SI values ranged from 1.5 to 7. $k'/[I]$ values for P1 variants, in contrast, increased following a trend opposite to that of SI values (Table I). Thus rACT-L358 had the highest inhibition rate constant and the lowest SI among variants. The more extensively altered variant, rACT-P3P3', which has a reactive site Met, had an SI value near 1 for reaction with chymase. However, its $k'/[I]$
TABLE II
Identification of chymase cleavage sites in rACT's by NH2-terminal sequence analysis of reaction products

Experiments 1 and 4 are of low M, peptide products (M, between 3,000 and 6,000) obtained after resolving reactions on SDS gels and electrophoretic proteins to PVDF membranes. Experiments 2 and 3 are of whole reaction mixtures. The native sequence of ACT from P1' to P15' is S-A-L-V-E-R-T-T-L-V-R-F-N-R-P. Values in parentheses are net yields reported in pmol.

| rACT | Cycle |
|------|-------|
| L358F | S A L V E T R T I T R F N R P |
| L358F | S A L V E T R T I T R F N R P |
| L358F | S A L V E T R T I T R F N R P |
| L358F | S A L V E T R T I T R F N R P |

The theoretical yield of product should have been 120 pmol based on the amount of reaction mixture used in the analysis and a resin coupling efficiency of 50%.

The theoretical yield of product should have been 315 pmol based on the same assumptions as described in Footnote b.

Two sequences, (a) and (b), were deduced from data. The results, but not the actual data, were reported previously (4).

No residue was identified in this cycle because of a high background. Based on the sequence following this cycle it is assumed that serine (a) and valine (b) are the true NH2-terminal residues of these peptides.

### TABLE III
Catalytic properties of human chymase for substrates differing in the identity of the P1 residue

Kinetic constants for peptide-pNA substrates were determined with human chymase isolated from skin tissue; those for angiotensin I and analogs were determined by Kinoshita et al. (37) with human chymase isolated from heart tissue. Based on the identity of the NH2-terminal amino acid sequences (35 residues) for both skin and heart enzymes, they are presumed to be identical proteins (30).

| Substrate (peptide-pNA or angiotensin I) | Km | kcat | kcat/Km |
|------------------------------------------|----|------|---------|
| Suc-A-A-P-F-N-NA | 0.8 | 65.0 | 8.1 x 10^6 |
| Suc-A-A-P-L-NA | 3.0 | 6.7 | 2.2 x 10^5 |
| Suc-A-A-P-M-NA | 2.6 | 2.5 | 1.0 x 10^6 |
| Suc-V-P-F-N-NA | 0.1 | 75.0 | 7.5 x 10^5 |
| Suc-V-P-L-NA | 0.9 | 21.0 | 2.4 x 10^4 |
| Suc-V-P-M-NA | 1.2 | 8.7 | 7.3 x 10^3 |
| Suc-V-P-W-NA | 1.6 | 8.4 | 5.3 x 10^3 |
| D-R-V-W-I-H-P-F-H-L' | 0.06 | 160 | 2.7 x 10^5 |
| D-R-V-W-I-H-O-L-H-L | 0.31 | 46 | 0.2 x 10^5 |
| D-R-V-W-I-H-P-W-H-L | 0.11 | 210 | 2.8 x 10^5 |

P1 residues are in boldface. Assay conditions were 0.2 M Tris-HCl (pH 8.0), 1.0 M NaCl, 9.0% Me2SO at 25 °C. Kinetic constants determined in 2 M NaCl (not shown) were similar, except that the Km values were approximately half those obtained in 1 M NaCl.

Assay conditions were 0.05 M sodium phosphate (pH 8.0), 1.5 M KCl, and 10% Me2SO at 25 °C (3).

Chymase cleaves angiotensin I at the Phe-His bond. Assay conditions were 0.02 M Tris-HCl (pH 8.0), 0.5 M KCl, 0.01% Triton X-100 (27).

One involves a branching mechanism described by Cooperman et al. (9) in the preceding article. A compressed version of that scheme sufficient to account for the results presented in this paper is shown below as Scheme 1. Similar branching schemes to this Scheme 1 have been proposed by Bjork et al. (33) and Patston et al. (26). In this model, I binds reversibly to E to form a complex E-I, which is then partitioned along two pathways: one leading to the formation of EI*, a stable enzyme-inhibitor complex, and the other leading to cleavage of the inhibitor as a substrate producing free E and P, a reactive site-cleave inhibitor. EI* formation for serum-proteinase reactions have been proposed to involve a structural rearrangement within the inhibitor induced by binding to the enzyme (13, 33). If, for a fraction of its encounters with the inhibitor, a proteinase is capable of hydrolyzing the P1-P1' bond before the conformational change is complete, the SI for the reaction will be greater than 1 (33). The second scheme (Scheme 2) is based on the studies of Dierckx et al. (34) which suggest that a single serpin may have interconvertible inhibitor (Ia) and substrate (Ib) conformations. The value SI would then depend on the relative rates of chymase interaction with each conformer and the position of the conformer equilibrium.

\[
\begin{align*}
E + I &\rightleftharpoons E\cdot I \\
&\rightleftharpoons E\cdot I^* \\
&\rightleftharpoons E + P \\
\end{align*}
\]

**Scheme 1**

\[
\begin{align*}
E + I_a &\rightleftharpoons E\cdot I_a \\
E + I_b &\rightleftharpoons E\cdot I_b \\
\end{align*}
\]

**Scheme 2**

The order of SI values (Phe > Leu > Met > Trp) as a function of P1 substitution qualitatively paralleled the substrate preference of chymase (Phe > Leu > Met ~ W) toward...
peptide-pNA substrates as measured by $k_{cat}$ or $k_{cat}/K_m$ values (Table III). This correlation is consistent with the notion that the substrate pathway in either Scheme 1 or Scheme 2 is more sensitive to P1 substitution than is the inhibitor pathway and suggests a parallelism for the effects of P1 substitution on SI values and on the rate determining step(s) for peptide-pNA hydrolysis. Similar agreement, however, was not observed for chymase catalysis of P1-substituted angiotensin I analogs where $k_{cat}$ and $k_{cat}/K_m$ values were reported to vary in the order of Phe $\sim$ Trp $> \text{Leu}$ (27). The difference observed for the two simple sets of substrates might reflect differences in the nature of the rate-determining steps for chymase catalyzed hydrolysis of each substrate type (35).

Partitioning occurs as two first-order reactions in Scheme 1 and as two second-order reactions in Scheme 2. As a result, the naive expectation is that in Scheme 1, SI-1 values (rate of the substrate pathway reaction relative to the rate of the inhibitor pathway) may parallel $k_{cat}$ values, whereas in Scheme 2 they may parallel $k_{cat}/K_m$ values. Quantitatively, relative SI-1 values (Phe:Leu:Met:Trp) for P1 variants agree better with $k_{cat}$ than $k_{cat}/K_m$ values of peptide-pNA substrates; however, the results do not permit a clear choice between Schemes 1 and 2.

The result of P1 substitutions on $k'/[I]$ values showed that these values decreased as SI values for corresponding variants increased (Table I). Although this result for P1 variants is a possible consequence of the branch points in Scheme 1 (the partitioning of E-1 by reaction steps with rate constants $k_2$ and $k_0$) and Scheme 2 ([Ia]b equilibrium), the low $k'/[I]$ and low SI values obtained for the rACT-P3P3' variant demonstrate that these two parameters do not necessarily vary inversely. rACT-P3P3' is a more extensively substituted variant compared with P1 variants. The greater degree of change in its behavior.

The order of $k'/[I]$ values for inhibition of chymase by Met, Leu, and Phe variants was opposite to that observed for inhibition of chymotrypsin (Phe $> \text{Leu} > \text{Met}$) by the same variants (10, 11), despite both proteases having the same general substrate preferences (3, 36). In contrast to chymase, the SI value for the interaction of chymotrypsin with each of these variants is close to 1 (10, 11), suggesting that suppression of the substrate pathway may account for the observed difference in the order of inhibition rate constants.

In summary, the high SI observed for the interaction chymase with ACT and its wide variation in mutants allow for the analysis of serpin-proteinase interactions in a manner not available for other target proteinases. Our results, however, do not provide definitive evidence permitting a clear choice between the branched or conformer model to explain SI values greater than 1. Nevertheless, these studies demonstrate the importance of including a rapid partitioning scheme in the general equation for serpin-proteinase interactions and the need for further defining the mechanism producing high SI values.

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