The Effects of Reactive Site Location on the Inhibitory Properties of the Serpin α1-Antichymotrypsin*

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The large size of the serpin reactive site loop (RSL) suggests that the role of the RSL in protease inhibition is more complex than that of presenting the reactive site (P1 residue) to the protease. This study examines the effect on inhibition of relocating the reactive site (Leu-358) of the serpin α1-antichymotrypsin either one residue closer (P2) or further (P1′) from the base of the RSL (Glu-342). α1-Antichymotrypsin variants were produced by mutation within the P4-P2′ region; the sequence ITLSSA was changed to ITLSSA to relocate the reactive site to P2 (Leu-357) and to ITILTS to relocate it to P1′ (Leu-359). Inhibition of the chymotrypsin-like proteases human chymase and chymotrypsin and the non-target protease human neutrophil elastase (HNE) were analyzed. The P2 variant inhibited chymase and chymotrypsin but not HNE. Relative to P1, interaction at P2 was characterized by greater complexity, lower inhibition rate constants, and increased stoichiometry of inhibition values. In contrast, the P1′ variant inhibited HNE (stoichiometry of inhibition = 4) but not chymase or chymotrypsin. However, inhibition of HNE was by interaction with Ile-357, the P2 residue. The P1′ site was recognized by all proteases as a cleavage site. Covalent complexes resistant to SDS-PAGE were observed in all inhibitory reactions, consistent with the trapping of the protease as a serpin-acyl protease complex. The complete loss in inhibitory activity associated with lengthening the Glu-342-reactive site distance by a single residue and the enhanced stability of complexes associated with shortening this distance by a single residue are compatible with the distorted-protease model of inhibition requiring full insertion of the RSL into the body of the serpin and translocation of the linked protease to the pole opposite from that of encounter.

Proteins have evolved a number of different mechanisms to inhibit serine proteases (1, 2). A feature common to all mechanisms is recognition of the inhibitor by the protease in a manner resembling that of a substrate. That is, S1-P1 interactions, according to standard nomenclature (3), determine the site of encounter and the specificity of the interaction. The P1 residue of a protease inhibitor is termed the reactive site. The reactive site is located within a specialized region of the inhibitor termed the reactive site loop (RSL) or bait region in the case of α2-macroglobulin.

Serpins possess a relatively large reactive site loop of ∼22–26 residues (4). Despite the size, the reactive site appears limited to a position 16 or 17 residues from the N-terminal base of the RSL, marked by the highly conserved residue Glu-342 (4, 5). The recent crystal structure of a trypsin-α1-proteinase inhibitor (α1-PI) complex suggests an explanation for this restriction. The crystal structure showed the protease ∼70 Å from the presumed point of encounter and residues P17-P3 of the RSL inserted into the face of the inhibitor as the 4th strand of a 6-strand A-β-sheet structure (6). At the interface region between the two proteins, the P1 residue of the serpin was covalently tethered to the protease through an ester bond with Ser-195. Active site structure, especially around the P1 residue of the serpin, was highly distorted, giving rise to the impression that the protease was trapped in a deformed-uncatalytic state by compression against the serpin. These observations imply that a length of about 17 residues was required to translocate the covalently tethered protease to the opposite end of the inhibitor where it was denatured by being forced against the rigid body of the serpin.

Although a number of spectroscopic studies using site-specific fluorescent labels support the location of the inhibited protease at the pole opposite from that of encounter (7, 8), other studies employing fluorescent labels (9), monoclonal antibodies (10, 11), and synthetic peptides resembling the RSL (12) place the enzyme closer to, if not at, the site of encounter. The latter studies suggest that only partial insertion of the RSL is required for inhibition. Among this group is a spectroscopic study similar to those that find the protease at the pole opposite of encounter (9). As suggested by O’Malley and Copperman (9), controversy over protease location and the extent of RSL insertion raises the possibility for multiple sites on the serpin capable of mediating inhibition.

Partial insertion models cannot easily explain the need for a large RSL and the restricted location of the reactive site within the RSL. Also not as evident from partial insertion models is why interaction at sites in the vicinity of P1 (typically P7-P3) produce cleavage and irreversible inactivation of the serpin instead of inhibition (13–17). To define the inhibition/substrate.
by the addition of increasing amounts of inhibitor to a fixed amount of protease. Protease-inhibitor reactions were in a total volume of 25–50 μl of solution containing 0.5–1.0 μM protease. Aliquots of 1–5 μl were typically removed for assay in 250–500 μl of substrate solution. The amount of protease in each aliquot was sufficient to produce a ΔA254/h of 0.15 in the absence of inhibitor. Reactions of inhibitors with chymase, chymotrypsin, and HNE were performed at 25 °C in 1.0 mM NaCl, 0.1 mM Tris-HCl (pH 8.0), 0.01% dodecyl maltoside, 0.5 mM NaCl, 0.1 mM Tris-HCl (pH 8.0), 0.01% dodecyl maltoside, and 0.5 mM NaCl, 0.1 mM HEPES (pH 7.5) or 0.1 mM Tris-HCl (pH 8.0), 0.01% dodecyl maltoside, respectively.

Rate constants were measured under pseudo-first order conditions, where $[I]_0 = [E]_0 \cdot SI \cdot 10 \times 10$. Measurements were made using the progress curve method (25) or by monitoring the time-dependent loss of activity. Incubation conditions were the same as those of titrations except for the presence of substrate (and final 9% MeSO) in progress curve measurements. Chymase was measured using 0.5 mM Suc-AAPF-NA ($K_m = 0.8$ μM) or 0.05 mM Suc-AEPP-7-amido-4-methyl-coumarin ($K_m = 0.5$ μM). Chymotrypsin was measured using 0.1 mM Suc-AAPF-NA ($K_m = 0.05$ μM). Elastase was measured in 1 mM MeO-Suc-AVPF-NA ($K_m = 0.15$ μM). In the second method, used only for chymotrypsin, aliquots were removed, and residual activity was measured by dilution into media containing a saturating concentration of substrate.

Rate constants for complex breakdown were determined by following the return of enzyme activity at 25 °C. Complexes were formed at high protease and serpin concentrations with the serpin in excess. Thus complex formation was rapid, and virtually all free enzyme was inhibited. These conditions limit proteolysis of the serpin-complexed protease; proteases inhibited by serpins may become highly sensitive to proteolysis by either exogenous or endogenous protease (26, 27). Return of enzyme activity was monitored after a 250–500-fold dilution of the complex into a substrate-containing media. Breakdown of chymotrypsin-serpin complexes were monitored in a solution of 0.5 mM NaCl, 0.1 mM Tris-HCl (pH 8.0), 0.01% dodecyl maltoside, 9% MeSO, 2 mM Suc-AAPF-NA (>20 $K_m$), and breakdown of chymase-serpin complexes were in 1.0 mM NaCl, 0.1 mM Tris-HCl (pH 8.0), 0.01% dodecyl maltoside, 9% MeSO, 1.0 mM Suc-AVPF-NA (>7 $K_m$). Conversion of substrate to product was followed continuously for ~18 h in a thermostatted spectrophotometer.

**SDS-PAGE of Protease-Inhibitor Reactions**—Reactions were typically performed in a total volume of 25 μl, and proteins were resolved by discontinuous gel electrophoresis using a Bio-Rad mini gel system. 12% running gels were prepared according to Laemmli (28). Reactions were performed under the buffer conditions for titrations. Incubation times corresponded to at least five half-lives. Stoichiometrically, assuming second order conditions, reactions were stopped by the addition of inhibitors PMSF or MeO-Suc-AAPF-chloromethyl ketone, and samples were denatured in an SDS-DTT solution with or without heating at 90 °C for 10 min. DTT was not used in the denaturation step of the reactions between serpins and serine proteases, which was used above to prevent potential formation of complexes as well as to reduce residual content of protease in the complexes. Protease-serpin complexes were stopped instantaneously by the addition of trichloroacetic acid plus carrier RNA (10% final trichloroacetic acid concentration). Samples were allowed to precipitate in the cold for 1–2 h and then centrifuged to collect the precipitate. Precipitates were washed twice with ice-cold EtOH and then resolubilized in the SDS-DTT sample buffer and denatured by heating.

**MALDI-MS**—Material analyzed was obtained from reactions containing excess inhibitor. In reactions that did not form stable complexes, PMSF or MeO-Suc-AAPF-chloromethyl ketone was added before complete consumption of the inhibitor to limit secondary cleavages. Residual intact inhibitor was confirmed by SDS-PAGE analysis of aliquots removed from stopped reactions. Stoichiometries ([I]/[E]) for the reactions were as follows: chymase with rACT-RSP2 was 20 (chymase $= K_m$); chymase with rACT-RSP1 was 29 (chymase $= 0.8$ μM); chymase with rACT and rACT-L361V was 5.5 (chymase $= 2.8$ μM); HNE with rACT-RSP1 was 8 (HNE $= 2.0$ μM). N-terminal Sequence Analysis of HNE-rACT-RSP1 Complexes—Reactions contained an 8-fold excess of inhibitor in a solution of 0.1 mM HEPES, 0.4 mM NaCl, and 0.01% dodecyl maltoside. After completion, protease-serpin complexes were denatured by SDS-DTT at 75 °C without heating and resolved on 12% SDS gels. Bands were detected by dinitrofluorobenzene-dinitrophenylated or dinitrofluorobenzene-dinitrophenylated or dinitrofluorobenzene-dinitrophenylated chromogenic reagent and visualized by Coomassie Brilliant Blue. The gel band was cut from the paper and visualized with fluorescent gel imaging. The above procedure for identifying the reactive site was based upon the stoichiometric determination of the N termini of each protein and RSL cleavage sites.

**Characterizations of Serpin-Protease Interactions**—SI values were determined by titration of protease activity. Titrations were performed...
an empirical observation in this laboratory. We have found that protease-ACT complexes treated with SDS-DTT without heating retain a significant fraction of the serpin C-terminal fragment produced during complex formation. We suspect that this resistance to complete denaturation reflects the enhanced stability of serpins when the reactive site is cleaved.

Data Analysis—Data was analyzed using the fitting routines of Igor Pro from Wavemetrics.

RESULTS

Design of Inhibitors—The RSL structure of ACT variants with reactive sites at the P2 position (16 residues from Glu-342) and the P1’ position (18 residues from Gly-342) are shown in Table I. The P2 reactive site variant, rACT-RSP2, was produced by mutating Leu-358 to Ser. This mutation makes Leu-357–Ser-358 the default reactive site. The P1’ reactive site variant rACT-RSP1 was produced by replacing the sequence P2-P2’ of rACT by Ile-Thr-Leu-Ser. This relocates Leu to P1’ and maintains the sequence of the surrounding residues. As a result of these mutations, position 358 is either Ser or Thr and, therefore, should not be recognized.

Characterization of the Interaction of Proteases with Reactive Loop Variants—The parameters, SI, k_{inh} and complex stability, describing the inhibition of chymase, chymotrypsin, and HNE with variants having the reactive site relocated to P2 or P1’ are reported in Table II. Also shown for comparison is the previously published parameters for the inhibition of the same proteases by rACT. The results show that each reactive site variant is functional, inhibiting at least one of the proteases being analyzed. Chymotrypsin and chymase were inhibited by rACT-RSP2, and HNE was inhibited by rACT-RSP1.

Inhibition of Chymotrypsin-like Proteases by rACT and rACT-RSP2—Titration of chymotrypsin and chymase hydrolytic activity by rACT-RSP2 and chymotrypsin hydrolytic activity by rACT are shown in Fig. 1A. All titrations demonstrated a linear loss of hydrolytic activity; only the chymotrypsin-rACT titration extrapolated to an end point consistent with an SI of 1. Endpoints for the inhibition of chymotrypsin and chymase by rACT-RSP2 were at \([I/E]_0\) values of 3 and 12, respectively. Both SI values are significantly higher than 1 and 4, observed for the inhibition of each protease by rACT (Table II). SI > 1 are due to the degradation of the inhibitor by a reaction in competition with the inhibition reaction.

SDS-PAGE analysis of the reaction of rACT-RSP2 with chymase (panels A and B) and chymotrypsin (panel C) are shown in Fig. 2. Lane 1 in each gel is the control, demonstrating the migration of active inhibitor (I) and PMSF-inhibited protease. Lanes 2–4 show the banding patterns of reactions produced at \([I/E]_0\) that range from below to above the SI established by titration. Bands consistent with the formation of covalent (1:1) protease-serpin complex (CM) are evident in reactions where \([I/E]_0\) ≥ SI. When \([I/E]_0\) is below the SI (Fig. 2, A–C, lanes 2), degradation of the protease-serpin complex is observed to variable degrees. Degradation is due to proteolysis of the complex, protease, and/or serpin components by excess protease. Such proteolysis does not result in the release of active protease (21, 26, 29). The patterns of protease disappearance (chymase or chymotrypsin) and that of intact inhibitor appearance over the \([I/E]_0\) range reflect the high SI values observed for each protease in titrations. Cleaved serpin (CL) is a consequence of the cleavage pathway producing SI > 1. The relative staining intensities of the complex and cleaved inhibitor bands also are consistent with the high SI values.

The close migration of intact and cleaved inhibitor in gels A and C indicate a minimal difference in mass. The small difference is consistent with hydrolysis within the RSL located in all serpins ~40–50 residues from the C terminus. Gels A and B represent the same chymase-rACT-RSP2 reaction, only differing in the temperature used for SDS denaturation. Gel B, in which SDS denaturation is performed at 25–37 °C instead of 90–100 °C, shows enhanced resolution of cleaved and intact inhibitor bands. The better separation confirms the extraordinarily high SI for the chymase-rACT-RSP2 reaction. The enhanced resolution is likely related to incomplete denaturation of cleaved inhibitor due to the greater structural stability of this serpin form (30, 31).

Sites of RSL cleavage were identified by MALDI-MS. This technique can be used to measure the mass of the 4–5-kDa C-terminal fragment generated in production of serpin-protease complex and cleaved serpin (29, 32). In Table III, the size of the C-terminal fragment(s) produced in the reactions of chymase with rACT, a P3’ variant of rACT, and rACT-RSP2 were reported. Experimentally determined masses are reported be-

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TABLE I

| Serpin | Reactive loop sequence |
|--------|------------------------|
| rACT   | 342P1367               |
| rACT-RSP2 | EEGTEASAATVKITLSSALVETRIT |
| rACT-RSP1’ | 5TLS                   |

| Summary of the inhibition characteristics for the reaction of rACT and rACT-reactive site variants with human chymase, chymotrypsin, and HNE |

| Enzyme | rACT-WT | rACT-RSP2 | rACT-RSP1’ |
|--------|---------|-----------|------------|
|        | SI | k_{inh} | t_{bkd}  | SI | k_{inh} | t_{bkd}  | SI | k_{inh} | t_{bkd}  |
| Chymase | 4 | 20,000 | ND* | 12 | 3,200b | ND | ND | >20,000c  | 0.05 |
| Chymotrypsin | 1 | 500,000d | 5–6 | 3 | 1,100f  | 100–200 | No inhibition | No inhibition |
| HNE     | No inhibition | No inhibition | 3.5 | 14,000f | >24 |

* ND, Not determined.

** Value is the average of 4 progress curve method measurements made over an [I] range of 0.5–3.5 μM, S.D. ± 750 μM s^{-1}.

** Due to the transient nature of the inhibition, k_{inh} is a crude estimate based on the observation that complete inhibition of chymase occurred within 30 s of the addition of 5.0 μM rACT-RSP1’ to 0.5 μM chymase.

** Determined by progress curve method, [I] = 1.2 μM.
low the closest mass calculated from the amino acid sequence. The cleavage products formed in the chymase-rACT and chymase-rACT-L361V reactions indicate two primary sites of en-
counter at the P1 and P2 residues of the RSL. A potential chymase cleavage site at P3'–P4' does not appear to be a primary site because its removal does not affect the SI (Table III). The only fragment detected for the chymase-rACT-RSP2 reaction corresponds to cleavage between Leu-357–Ser-358, defining this site as both the reactive site and the cleavage site producing the SI > 1. Given the similar substrate specificity of chymase and chymotrypsin, exclusive interaction with the P2 site also likely explains the SI increase for the chymotrypsin-rACT-RSP2 reaction.

The stability of complexes was determined by monitoring the return of enzyme activity after a large dilution of preformed complex into substrate (peptide-NA)-containing solution as described under “Experimental Procedures.” The release of protease was measured by following the progress of NA accumulation at 410 nm. The progress curve data were numerically differentiated to obtain the plots shown in Fig. 3. Only data for chymotrypsin-serpin complexes were determined by fitting the differentiated data to a single exponential function (solid line) with 100% release of the protease as the end point.

As shown in the Fig. 3, the chymotrypsin-rACT-RSP2 complex was ~20–40-fold more stable than the chymotrypsin-rACT complex. The half-life values estimated from the rate constants were 100–200 h and 5–6 h, respectively. The half-life for the chymotrypsin-rACT complex is comparable with the half-life of 7 h previously reported using a discontinuous method of assay (19) to ensure that the difference in breakdown rates was not influenced by the difference in inhibitor concentrations used to form each complex, a study similar to

FIG. 1. Titration of chymotrypsin, chymase (A), and HNE (B) hydrolytic activity by rACT variants with the reactive site relocated to P2 or P1' of the RSL. The structure of the reactive site variants is described in Table I. Enzyme activity is reported as fractional activity. To calculate this value, data points were divided by the y-intercept obtained from regression analysis of the original titration. Two sets of data (circles and squares) shown for HNE-rACT-RSP1' titration were obtained after 1 and 24 h of incubation. Panel A also shows titration of chymotrypsin with rACT for comparison.

FIG. 2. SDS-PAGE analysis of the reaction products formed in the reaction of human chymase (A and B) and chymotrypsin (C) with rACT-RSP2. [I]/[E], ratios in reactions were obtained by varying [E], [I], was fixed at 3.0 μM. HC, Chymo, I, CM, and CL signify migration of human chymase (25 kDa), chymotrypsin (25 kDa), intact serpin (45 kDa), protease-serpin complex (roughly mass of protease + inhibitor), and inhibitor cleaved in the RSL, respectively. Controls of reactants, marked as C at the bottom of the gel contained protease inhibited by PMSF before incubation with inhibitor. The amount of protease in the control is the same as that in the lane with the [I]/[E], ratio nearest the SI. Gels A and B are identical except that samples in Gel B were denatured with SDS at room temperature instead of 100 °C. The faster migration of CL is assumed to be related to the increased structural stability obtained by serpins after cleavage within the RSL.
Effects of Reactive Site Location on Serpin Inhibition

TABLE III

MALDI-MS analyses identifying the C-terminal fragments produced by the reaction of chymase and HNE with rACT and variants

The RSL of ACT is located approximately 40–50 residues from the C terminus. Therefore, cleavage within the RSL produces polypeptide fragments of approximately 5000 Da. Reactions with SI > 1 may produce single or multiple fragments depending on whether the substrate site is same as or different from the reactive site. Shown below are the P6–P4′ sequences of rACT and rACT variants, the calculated mass for a peptide fragment extending from the indicated residue to the C terminus, and the experimentally measured masses of fragments produced upon reaction of chymase or HNE with each serpin. The experimental masses are shown below the closest matching calculated value. SI values were determined by titration as described under “Experimental Procedures.”

| rACT/Variant vs protease | Sequence position (mass, Da) |
|--------------------------|-----------------------------|
| rACT– | P6 | P5 | P4 | P3 | P2 | P1 | P1′ | P2′ | P3′ | P4′ |
| Calculated | 5293 | 5194 | 5066 | 4953 | 4852 | 4739 | 4625 | 4538 | 4467 | 4354 |
| Chymase (SI = 4) | 4746 | 4634 |
| rACT-L361V | Val | Lys | Ile | Thr | Leu | Ser | Ala | Leu | Val | |
| Calculated | 5279 | 5180 | 5052 | 4939 | 4838 | 4725 | 4611 | 4524 | 4453 | |
| Chymase (SI = 4) | 4727 | 4615 |
| rACT-RSP2 | Val | Lys | Ile | Thr | Leu | Ser | Ala | Val | Val | |
| Calculated | 5267 | 5168 | 5039 | 4926 | 4826 | 4713 | 4625 | 4538 | 4467 | |
| Chymase (SI = 12) | 4713 |
| rACT-RSP1′ | Val | Lys | Ile | Thr | Ile | Thr | Leu | Ser | Leu | Val |
| Calculated | 5323 | 5224 | 5096 | 4983 | 4882 | 4769 | 4668 | 4554 | 4467 | 4354 |
| Chymase (cleavage) | 4557a |
| HNE (SI = 3.5) | 5225 | 4986 | 4769a | 4555 |

* Major product based on peak heights over the mass range of 1000–8000 Da.

![Fig. 3. Breakdown of chymotrypsin-rACT and chymotrypsin-rACT-RSP2. Complexes were formed by incubation of chymotrypsin with rACT or rACT-RSP2 at [I]/[E] ratios of 2.8 and 13.5, respectively. [E]0 was 650 nM, and incubation time was 10–30 min at 25 °C. To monitor breakdown, aliquots of 1 and 2 μl were removed from the reactions, and each was diluted with 0.5 ml of a solution containing 1.0 M NaCl, 0.1 M Tris-HCl (pH 8.0) and saturating substrate (2 mM Suc-AAPF-NA, 20 × Km). Product formation for each dilution was monitored overnight or until an A410 nm of 2.00 was reached. The data were then numerically differentiated to obtain enzyme activities, reported as A410 nm/min. The rate of breakdown was compared for each inhibitor and inhibitor combination and the calculated half-life for breakdowns reported in the figure.](image)

![Fig. 4. Complex formation and stability in the reaction of chymase (A) and chymotrypsin (B) with rACT-RSP1′. Each protease (500 nM) was incubated with inhibitor at an [I]/[E]0 of 10 in solution containing 1.0 M NaCl, 0.1 M Tris-HCl (pH 8.0). Immediately after the addition of the inhibitor to the solution with protease, the reactions were diluted 250-fold into the same buffer containing 1 mM Suc-AVPF-NA (20 × Km), and product formation was measured by A410 nm as reported.](image)

**Inhibition of Chymotrypsin-like Proteases by rACT-RSP1′**

In contrast to the inhibitory activity observed for the interaction at P2, moving the reactive site to the P1′ position, 18 residues from Glu-342, virtually abolished inhibitory activity of rACT toward chymotrypsin and chymase. The reactive site variant, rACT-RSP1′ did not form a stable complex with either protease even at high [I]/[E]0. Despite the absence of titratable inhibition, a transient inhibitory effect was observed for the reaction of chymase with rACT-RSP1′. This transient effect is characterized in Figs. 4A and 4B. In Fig. 4A, the progress curve of free chymase and an equivalent amount of chymase pretreated with a 10-fold excess of rACT-RSP1′ is shown. Monitoring was
initiated immediately after a 100-fold dilution of free and inhibitor-treated chymase into reaction buffer containing a saturating concentration of substrate. The progress curve for inhibitor-treated chymase demonstrates a lag before steady state hydrolysis is attained. The lag indicates that chymase had reacted with the inhibitor but in an unstable manner.

SDS-PAGE monitoring of a chymase-rACT-RSP1 reaction performed at an [I]_0/[E]_0 of 1 demonstrates the transient formation of a covalent serpin-protease complex immediately after mixing (Fig. 5A) and the rapid turnover of this complex to free enzyme and cleaved inhibitor. The slow, but catalytic turnover of inhibitor is demonstrated in the time courses where only inhibitor is present. In panel C, aliquots from a single reaction were snap-frozen using liquid N\(_2\) and stored at \(-70^\circ\)C until all the time points were obtained. Because of the high concentration of inhibitor, only half the material was loaded on the gel. Lanes marked C contain inhibitor and inactivated protease, except in panel B, where only inhibitor is present. In panel C, lane E, the migration of HNE denatured in SDS without heating is shown; the protease migrates with an usually high mass of 100 kDa compared with 30 kDa for the free enzyme.

Fig. 5. SDS-PAGE analysis of reactions of human chymase (A and B) and HNE (C) with rACT-RSP1'. [I]_0/[E]_0 of the reactants are reported under each gel, and the length of the incubation period is reported above lanes. Bands corresponding to migration of reagents and products are labeled to the right of each gel as in Fig. 2. Reactions in panel A were stopped by trichloroacetic acid precipitation and in panel B by the addition of PMSF (2 mM final concentration). Samples in panels B and C were denatured without boiling. In panel C, HNE and rACT-RSP1' were preincubated together for 1 h at 25°C to form complexes. After the incubation, MeO-Suc-AAPV-chloromethyl ketone (200 mM final concentration) was added to ensure that any HNE released by complex breakdown could not react with the excess inhibitor. To obtain the time points in panel C, aliquots from a single reaction were snap-frozen using liquid N\(_2\), and stored at \(-70^\circ\)C until all the time points were obtained.

DISCUSSION

The current study evaluates the effect of reactive site relocation on the interaction of ACT with chymotrypsin, chymase, and HNE. This study parallels a recent report using the interaction of r-rPI Pittsburgh with factors Xa and thrombin as a model system (18). In the study by Zhou et al. (18), the reactive site was relocated nearer or further from the base of the RSL, Glu-342, by inserting Ala residues (Add-1, Add-2 variants) or removing RSL residues (delete 1–3 variants) at sites 2–3 residues preceding the reactive site. In our study, the method of reactive site relocation to the P2 or P1' position of ACT did not affect the overall length of the RSL. Relocation was accomplished by mutation of reactive site residues rather than by

identify the reactive site for the HNE-rACT-RSP1' reaction. Reaction products analyzed by MALDI-MS revealed cleavage products consistent with hydrolysis of the RSL at several sites (Table III). Cleavage at P6-P5 and P4-P3 have been reported previously for the reaction of HNE with rACT (17) and variants of rACT (29). Because HNE is not inhibited by rACT, these sites likely do not mediate inhibition. Two other fragments not previously observed revealed masses consistent with cleavage at P2-P1 and P1'-P2'. Peak heights of analyses suggest that the fragment corresponding to cleavage at P2-P1 was the most abundant reaction product.

N-terminal sequence analysis of HNE-rACT-RSP1' complexes resolved on SDS-PAGE demonstrated four sequences. Two sequences were the N termini of each protein, and two corresponded to cleavage within the RSL. One RSL-derived sequence (H-TLXLVETRTIVRFN-OH) was produced by cleavage of the P2-P1 peptide bond, whereas the other was produced by cleavage of the P4'-P5' peptide bond. Cleavage at P4'-P5' has been observed by MALDI-MS in HNE reactions with rACT variants capable of inhibiting HNE by interaction at the P1 site (17, 29). Cleavage at this site was variable and is believed to occur after complex formation by exposure of the site to enzyme not yet inhibited (29).

The finding of P2-P1 cleavage and no other by both N-terminal sequencing of the complex and by MALDI-MS indicate that the P2-Ile residue of rACT-RSP1' is the site mediating inhibition. Cleavage at P6-P5, P-4-P3, and P1'-P2' are likely responsible for the SI > 1.
Effects of Reactive Site Location on Serpin Inhibition

Adding or deleting residues (Table I). Despite the difference in the design of variants, similar effects on the inhibition parameters of complex stability, $k_{on}$, and SI, were observed with changes in reactive site location. The agreement between these two studies suggests that $\alpha$1-PI and ACT follow a similar mechanism of inhibition.

**Complex Stability**—Reaction at the P2 site, located 16 residues from Glu-342, produced inhibitory complexes that were as stable or more stable than those produced at P1. Chymotrypsin-rACT-RSP2 complexes were $\sim$20–40-fold more stable than chymotrypsin-rACT complexes. The stability of the HNE-rACT-RSP1' complex formed by interaction of HNE with the P2-Ile residue was even more striking considering the relative instability of complexes produced by interaction with numerous ACT-reactive site variants maintaining the reactive site at P1 (17, 29).

The Glu-342-reactive site distance cannot be reduced to less than 15 residues without loss of inhibitor activity. We have shown previously that HNE cleaves ACT at P6-P5 and P4-P3 bonds in a substrate-like manner (17). Similarly HNE cleavage of peptide bonds 12–14 residues from Glu-342 have been shown to inactivate several other serpins including C1 inhibitor (15), antiplasmin (13), and antithrombin (16). Zhou et al. (18) produced three RSL-shortened mutants (Delete 1–3), placing the reactive site at P4, P3, and P2, 14–16 residues from Glu-342. Although interaction of thrombin at P2 (Delete-1) produced stable inhibition similar to that observed in our study, reaction at P3 and P4 produced only cleaved serpin. Factor Xa was somewhat less sensitive to loop shortening, demonstrating stable inhibition with Delete-1 and 2 but not Delete-3. Taken together these observations strongly indicate that the placement of a functional reactive site has a lower limit of 15 or 16 residues from Glu-342.

In contrast to the apparent increase in complex stability associated with the P2 site, relocating the reactive site to P1' led to complete loss or diminished inhibitory activity. Chymotrypsin and HNE appeared to recognize the P1' site only as a cleavage site. The interaction of chymase with the P1' site demonstrated transient inhibition ($t_{50} < 5$ min), defined by the rapid formation and then disappearance of an SDS-stable complex. Similar transient inhibition was observed for the interaction of Xa with the Add-1 (reactive site at P1') variant of $\alpha$1-PI Pittsburgh (18). Inhibition of thrombin by the Add-1 variant was more stable, demonstrating a half-life for enzyme return of 10 h compared with 14 weeks (virtually irreversible) with native $\alpha$1-P1-Pittsburgh. The Add-2 variant behaved as a transient inhibitor of thrombin, supporting a general trend toward rapid complex breakdown upon increased Glu-342-reactive site distance.

The abrupt changes in complex stability observed by us and by Zhou et al. (18) upon relocation of the reactive site suggests that serpin functionality is dependent on the reactive site being located 15–17 residues from Glu-342. This finding is consistent with the recent crystal structure of a protease-serpin complex (6) and studies proposing protease distortion as the mechanism of inactivation (18, 26, 33–35). The crystal structure showed a distorted protease located at the pole opposite that of encounter and insertion of the RSL from P17-P3 into $\beta$-sheet A. Full insertion of the RSL into $\beta$-sheet A appeared to underpin the distortion process. The embedding of the RSL forced the close apposition of both proteins, resulting in strain on Ser-195 and distortion of protease around the “rigid” body of the cleaved serpin. In this insertion-distortion model, inhibition would be dramatically reduced if the Glu-342-reactive site distance was too long to provide strain on the protease after full insertion (≥18 residues) or too short to complete the insertion process (≤15 residues). The improved stability observed for protease-serpin complexes upon shortening the Glu-342-reactive site distance to 16 instead of 17 residues would be consistent with greater strain and distortion of the protease.

The dependence of complex stability on reactive site location was not the same for all proteases. This variation may be related to active site structure influencing the extent of distortion (18, 34, 35). It was suggested by Zhou et al. (18) that the stability of thrombin with the Add-1 variant was due to its unusually deep active site cleft. The deep cleft is primarily produced by the large size of two loops termed 60 and 147. Based on the backbone structures of chymotrypsin, chymase, and HNE shown in Fig. 6 (24, 36, 37), chymase appears to have the deepest active site cleft, and HNE appears to have the most shallow. A three-residue insertion makes the 37 loop of chymase more prominent than the corresponding loops of the other proteases, and the 145 loop is not cleaved like that of chymotrypsin. These features may explain the virtual irreversible inhibition of chymase upon interaction with reactive sites located at P1 and P2 and the transient inhibition upon the interaction at P1'. Chymotrypsin did not form a stable complex at P1, did not form even a transient complex at P1', and only approached the stability of chymase at P2. HNE has the shallowest-appearing active site cleft and virtually no 170–180 loop. The shallowness of the active site cleft may explain why reaction at the P2 site of rACT-RSP1' produced a highly stable HNE-serpin complex, whereas numerous other rACT variants with the reactive site at P1 produced relatively unstable complexes (17, 29). An additional distinguishing feature of the chymase structure, possibly enhancing its susceptibility to distortion, is the absence of a disulfide bond between residues 191 and 220. Residues 191 and 220 join the two major sequence

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**Figure 6:** Structure of the active site clefts of chymase, HNE, and chymotrypsin. Ca-tracings of chymase (24), chymotrypsin (49), and HNE (50) were rendered using Rasmol. Accession numbers for the crystal structures are 1PJP, 1HNE, and 4CHA, respectively. The loops forming the walls of the active site cleft and the S1 pocket are presented in color (24, 37, 51). The S7 loop is in green, the 60, 70–80, 99, and 147 loops (autolysis loop in chymotrypsin) are in blue, and 174 loop is in orange. The loops producing the S1 pocket, 189–195 and 213–228, are in red. Specific residues are presented over some loops as well; all numbering is based on homology to chymotrypsin as used in the above crystal structures. Ser-195 is shown as a black stick model in each structure.
Segments, 189–195 and 213–228, that form the S1 pocket. In chymase, the cystine is replaced by Phe-191 and Ala-220.

As stated in the introduction, not all studies of serpin-protease complexes find the protease at a position consistent with full insertion. Studies implying partial insertion of the RSL do not provide a mechanism for protease inactivation. In the absence of an alternative mechanism for complex stability, it is difficult to understand the correlation provided here between complex stability and a length of RSL residues just appropriate for translocation of the protease to the pole opposite that of encounter. This correlation also argues against the suggestion that stable complexes can be produced at different locations.

**Stoichiometry of Inhibition**—The increases in SI values for chymase and chymotrypsin upon interaction with the P2 site (see Table II) reproduces a similar trend to that reported by Zhou et al. (18). The mechanism primarily assumed for generation of an SI > 1 involves a competition for the serpin acyl protease between deacylation on the hydrolytic pathway and the initiation of RSL insertion on the inhibition pathway (38–41). This mechanism is derived mainly by analogy with suicide substrates (38, 39) and from studies involving mutation of the hinge region near Glu-342 (42, 43). It does not straightforwardly explain the current observation pertaining to reactive site relocation. A reactive site located at P2 could pose difficulty for completion of the insertion process at the pole of the serpin, where distortion of the acyl enzyme is presumed to occur. An enzyme translocated on a shortened arm might not align properly with the pole, thereby requiring increased time for completion of the insertion process and subsequent distortion of the protease.

Another finding related to SI values is that the unusually high SI for the chymase-rACT reaction (SI = 4) is due at least in part to a partitioning of the reaction between the P1 and P2 sites (Table III). MALDI-MS analysis of chymase-rACT interactions suggested that both sites are recognized by chymase. The SI for the reaction of chymase with rACT-RSP2 was greater than 10, whereas the SI for the reaction with a variant having a P2 site not recognized by chymase, rACT-Val557Leu (32), was about 1.7. Interaction with each site at an approximately equal rate, as will be discussed subsequently, would produce an SI = 4.

**Inhibition Rate Constants**—Inhibition through reaction with the P2 site of ACT decreased $k_{inh}$ values. The decrease was more dramatic for chymotrypsin (500-fold) than chymase (6-fold). Because both reactions exhibited increased SI values (Table II), the recognition of the P2 site is somewhat greater than that estimated by $k_{inh}$. Considering the SI, recognition of the P2 site by chymotrypsin is still poor relative to the P1 site of rACT. On the other hand, the high SI (12) for the chymase-rACT-RSP2 reaction suggests that the P2 site is recognized by chymase at a rate comparable with that of the P1 site. As pointed out above, approximately equal recognition of P2 and P1 could explain the high SI of 4 for the chymase-rACT reaction. The most efficient inhibition reaction was that of HNE where distortion of the acyl enzyme is presumed to occur. An enzyme translocated on a shortened arm might not align properly with the pole, thereby requiring increased time for completion of the insertion process and subsequent distortion of the protease.

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Effects of Reactive Site Location on Serpin Inhibition

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