Serpins, serine proteinase inhibitors, form enzymatically inactive, 1:1 complexes (denoted E*I*) with their target proteinases, that only slowly release I*, in which the P1-P1’ linkage is cleaved. Recently we presented evidence that the serpin antichymotrypsin (ACT, I) reacts with the serine proteinase chymotrypsin (Chtr, E) to form an E*I* complex via a three-step mechanism, E + I → E · I → EI* → E*I* in which EI*, which retains the P1-P1’ linkage, is formed in a partly or largely rate-determining step, depending on temperature (O’Malley, K. H, Nair, S. A., Rubin, H., and Cooperman, B. S. (1997) J. Biol. Chem. 272, 5354–5359). Here we extend these studies through the introduction of a new assay for the formation of the postcomplex fragment, corresponding to ACT residues 359 (the P1’ residue) to 398 (the C terminus), coupled with rapid quench flow kinetic analysis. We show that the E-I encounter complex of wild type-rACT and Chtr forms both E*I* and postcomplex fragment with the same rate constant, so that both species arise from EI* conversion to E*I*. These results support our earlier conclusion that the P1-P1’ linkage is preserved in EI* and imply that E*I* corresponds to a covalent adduct of E and I, either acyl enzyme or the tetrahedral intermediate formed by water attack on acyl enzyme. Furthermore, we show that the A347R (P12) variant of rACT, which is a substrate rather than an inhibitor of Chtr, has a rate constant for postcomplex formation from the E-I complex very similar to that observed for WT-rACT and Chtr, implying that EI* is the common intermediate from which partitioning to inhibitor and substrate pathways occurs. These results are used to elaborate a proposed scheme for ACT interaction with Chtr that is considered in the light of relevant results from studies of other serpin-serine proteinase pairs.

Antichymotrypsin (ACT, I) is a human serine proteinase inhibitor (serpin), 398 amino acids long, that, as is typical of serpins (1, 2), forms an enzymatically inactive, 1:1 complex (denoted E*I*) with its target proteinases, releasing free enzyme (E) and cleaved ACT (I*) only very slowly (3, 4). The involvement of ACT in Alzheimer’s disease (5, 6) and in the regulation of the inflammatory response (7) as well as of prostate-specific antigen activity (8), makes it a particularly interesting protein for study. In the interaction of ACT with chymotrypsin (Chtr) to form an E*I* complex, both proteins undergo extensive conformational change (4, 9); the nonlability of E*I* may be due either to distortion of the enzyme active site within the complex (4, 9) or to inaccessibility of the covalent E-I linkage toward attacking nucleophilic water, or both.

Cleavage of I to form released I* occurs between residues 358 and 359 of ACT within the so-called “reactive center loop,” which in intact I extends out from the rest of the molecule, contains a segment of modified α-helix (10) and is the primary interaction site between the inhibitor and the target proteinase. Following standard nomenclature (11), these residues are designated P1 and P1’. In released I* residues P1-P14 are inserted into β-sheet A, the dominant structural element in ACT, as strand 4A (s4A). The β-sheet C is also reinforced, with the result that the P1 and P1’ residues are separated by 70 Å (12). Recent fluorescence energy transfer studies suggest substantial, if not complete, s4A insertion within E*I* for the serpin:serine proteinase pairs α1-proteinase inhibitor:trypsin (13) and plasminogen activator inhibitor-1:u-plasminogen activator (14).

E*I*’s formed from a variety of serpin-serine proteinase pairs are stable to both boiling water and SDS treatment, implying covalent bond formation between enzyme and serpin. Parallel studies by both Lawrence et al. (15) and Wilcynska et al. (16) have clearly shown that the P1-P1’ bond is cleaved within the complexes formed by several such pairs (including plasminogen activator inhibitor-1:plasminogen activator, plasminogen activator inhibitor-1:u-plasminogen activator, α1-proteinase inhibitor:human neutrophil elastase, α1-proteinase inhibitor:porcine pancreatic elastase and α1-antiplasmin:plasmin), providing strong evidence that E*I* corresponds either to an acyl enzyme, or, less likely given the stability of E*I*, to the tetrahedral intermediate formed by water attack to the acyl enzyme (17).

Although full inhibition of Chtr requires only 1 eq of WT-ACT (i.e. the interaction is characterized by a stoichiometry of inhibition, SI, of 1), ACT inhibition of another target proteinase, chymase, has an SI of 4 at pH 8 (18) and even with Chtr, some mutations within the reactive center loop yield inhibitors with SI > 1 (e.g. SI is 2.4 for the L358R variant) (3). In the extreme, some variants, such as A347R (P12) and T345R (P14), in which small, neutral side chains within the “hinge” region of rACT are replaced by the charged, bulky arginine side chain, show no inhibition of Chtr (SIs > 100) and no E*I* formation, but are cleaved by Chtr in the reactive center loop with complete (A347R) or almost complete (T345R) insertion of s4A (19, 20). These results, which parallel results obtained with other serpins (21, 22), have been interpreted as indicating that on
interaction of serpins and serine proteinases, there is a partitioning between substrate and inhibitor pathways, raising the following questions. First, what is the sequence of events leading to $E'$ formation? Second, where within the sequence does partitioning occur?

Recently we presented evidence for a minimal scheme (Scheme 1) for the interaction of rACT and Chtr leading to the formation of $E'$ (23). The main features of this scheme were that: (a) there was at least one intermediate between the encounter complex, $E'_{II}$, and $E'$; (b) conversion of $E'_{I}$ to $E'$ involved at least partial reactive center loop insertion into $\beta$-sheet A of rACT, as shown by a blue-shifted increase in fluorescence intensity when a fluorescent derivative of rACT (at position P7 within the reactive center loop) was reacted with Chtr; (c) the P1-P1’ linkage was preserved in $E'$, as inferred from the fact intact inhibitor was released on rapid acid denaturation of $E'$; and (d) $k_3 \gg k_2$ at 25 °C, but is similar in magnitude at 40 °C. We also speculated that $E'$, rather than $E'$, was the common intermediate from which partitioning between the inhibitor and substrate pathways occurred.

Here we extend these studies by introducing a new assay for quantitatively measuring the appearance of the so-called postcomplex fragment (16), formed as a result of P1-P1’ cleavage and corresponding to ACT C-terminal residues 359–398, and use this assay to measure the first-order rate constant for postcomplex fragment formation, at saturating enzyme concentration, both for WT-rACT and for the A347R variant of WT-rACT.

EXPERIMENTAL PROCEDURES

Materials—rACT and A347R-rACT were obtained as described earlier (20, 24). Bovine (N-pro-tosyl-L-lysine chloromethyl ketone-treated) Chtr was from Calbiochem. rACT and Chtr concentrations were determined as described earlier (23, 24). The concentration of A347R-rACT was determined assuming an $A_{280}$ of 8.0 cm$^{-1}$ (19). The chromophoric proteinase substrate SucAAPF-pNA, phenylmethylsulfonyl fluoride and bovine serum albumin were obtained from Sigma. Standard proteins for SDS-PAGE gel calibration were from Bio-Rad. HPLC-grade acetonitrile was from Fisher, and HPLC-grade trifluoroacetic acid was from Pierce (sequanal grade).

Rapid Kinetic Studies—Rapid quenched flow kinetic studies were carried out using a KinTek Chemical-Quench flow model RQP-3 machine as described earlier (23). Quenching was achieved with 0.1 M HCl.

In preparing samples for HPLC analysis and quantification of rACT C-terminal peptide formation, the quenched rACT:Chtr sample (85 μl) was treated with 12 M urea (118 mM) and 300 mM phenylmethylsulfonyl fluoride (2 μl) and incubated for 1 h at 25 °C. Following centrifugation at 12,000 rpm in an Eppendorf centrifuge (no. 5414) for 2 min, 200 μl of supernatant was injected into a Perkin-Elmer Series 4 system, equipped with an LC-95 (Perkin-Elmer, 4.5 μl flow cell) variable wavelength detector, using a Rainin C-18 column (Microsorb-MV, 50 × 4.6 mm, 300 A, 5 μm, 0.7 ml/min). HPLC data were analyzed using Turbochrom Navigator software from Perkin-Elmer. A$_{215}$ values were used to quantitatively estimate the eluted peptide (25).

**Other Methods**—Quenched rACT:Chtr samples were analyzed on SDS-PAGE, and stained protein bands were quantified, as described earlier (23). Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) was performed on a VG Tofspec linear time-of-flight mass spectrometer (Fisons Instruments, Danver, MA) at the Protein Chemistry Laboratory in the Medical School of the University of Pennsylvania.

RESULTS

Purification and Quantification of Postcomplex Fragment—Treatment of rACT with Chtr leads to postcomplex fragment formation. Following denaturation of the sample with dilute HCl and concentrated urea, to ensure dissociation of the postcomplex fragment from the remainder of the rACT molecule, the peptide was purified from other components of the reaction mixture by RP-HPLC, as shown in Fig. 1A. Its retention time was close to the value predicted for amino acids 359–398 based on the use of Rekker’s constants for the constituent amino acids (26). Proof that the indicated postcomplex fragment peak contains the C-terminal peptide was provided by MALDI-MS analysis, which gave a mass of 4624 (for Ala$_4$Arg$_3$Asn$_3$Asp$_2$Glu$_2$Leu$_2$Lys$_2$Met$_2$Phe$_2$Pro$_2$Ser$_2$Thr$_2$Val$_4$), the theoretical M$^+$ is 4623.5. In simple preparative experiments, the yield of recovered postcomplex fragment, calculated from HPLC peak areas as described previously (25), was found to be 85 ± 5%. The difference from 100% is attributable to losses on the RP-HPLC column.

Rate of Postcomplex Fragment Release from the rACT-Chtr Complex—Rate constants for postcomplex fragment release following mixing of rACT and excess Chtr were measured using a quenched stopped flow apparatus, and determining the amount of released fragment (Fig. 1B) as a function of incubation time.
prior to the quench. The yield of recovered peptide was 75 ± 9%. This value, slightly lower than that above, reflects losses in the rapid quench apparatus in addition to those on the RP-HPLC column. Rates were measured under strictly first-order conditions, as demonstrated in Fig. 2 (upper panel). Earlier we showed that quantitative scanning of stained SDS-PAGE analyses of quenched reaction mixtures, in which E°I° complexes relied on assay of the newly formed α-NH₂ terminus at P1’ (15, 16), an approach only applicable in the absence of lysines in the postcomplex fragment. Coupling these new assays to a rapid quench flow kinetic analysis, we provide the first demonstration that the overall first-order rate constants for both postcomplex fragment and E°I° formation from the E-I encounter complex are indistinguishable from one another over a range of experimental conditions, including at 40 °C when k₂ is similar in magnitude to k₉. Thus, within the context of Scheme 1, postcomplex fragment formation must accompany EI conversion to E°I°, supporting our earlier conclusion that the P1-P1’ linkage is preserved in EI.

The second major result of this work is the demonstration that postcomplex fragment formation from Chtr complexes of both the inhibitor WT-rACT and the substrate A347R-rACT proceed with very similar first-order rate constants. This result straightforwardly supports the hypothesis that partitioning between the inhibitor and substrate pathways, leading to formation of the inhibited complex E°I° or the cleaved inhibitor I*, respectively, proceeds from a common intermediate formed in a step that is largely rate-determining, i.e. EI. By contrast, partitioning from E-I rather than from EI’ would require that two quite different transformations, conformational change of E-I to yield EI’ and hydrolysis of I within EI’ to yield E + I’, coincidently proceed with very similar rate constants, which we consider unlikely.

If partitioning between the inhibitor and substrate pathways is from EI’, then the putative partial insertion accompanying EI’ formation must be insufficient to distort the catalytic machinery of the proteinase. As a result, further conformational change in the serpin-proteinase complex accompanies EI’ to E°I° conversion, and it is this further change that must be coupled to proteinase distortion and stabilization of E°I° toward hydrolysis.

Is Scheme 1 generally applicable for serpin-serine proteinase interactions? Scheme 1 is similar to those proposed by Olson et al. (27) and Stone and Le Bonniec (28) in positing that conversion of the E-I encounter complex to E°I° proceeds via an intermediate, E’I, and that the reactive center loop is at least

| Experiment | [rACT] | [Chtr] | Temp | pH | k_COMP | k_PCFC |
|------------|--------|-------|------|----|--------|--------|
| 1          | Wild-type | 15    | 3.3  | 25 | 7.5    | 6.8 ± 0.7 (2) |
| 2          | Wild-type | 6.6   | 7.5  | 6.9 ± 0.6 (3) | 8 ± 2 (1) |
| 3          | Wild-type | 6.6   | 40 | 7.5  | 17 ± 2 (3) |
| 4          | Wild-type | 6.6  | 25 | 9.0  | 6 ± 6 (2) |
| 5          | A347R   | 3     | 25 | 7.5  | 11 ± 4 (1) |
| 6          | A347R   | 6     | 25 | 7.5  | 3.9 ± 0.9 (2) |
| 7          | A347R   | 22    | 40 | 7.5  | 3.8 ± 0.2 (2) |

* From O’Malley et al. (23).
Antichymotrypsin Interaction with Chymotrypsin

partially inserted into β-sheet A in EI'. Also noteworthy is the similarity in values of the overall first-order rate constants for E-I conversion to E*1' in this work (7 s⁻¹ for rACT and Chtr at 25 °C and pH 7.5) and in reactions of the heparin complex of antithrombin and thrombin (5 s⁻¹) (29) and of t-plasminogen activator and two different reactive center loop fluorescent derivatives of plasminogen activator inhibitor-1, at P1' (8 s⁻¹) and P9 (4 s⁻¹) (15), under similar conditions (25 °C and pH 7.4).

However, the schemes proposed by Olson et al. (27) and Stone and Le Bonniec (28) differ from one another in two significant respects. The first concerns the structure of EI'. Olson et al. propose that EI' involves a covalent bond (either tetrahedral intermediate or acyl enzyme) between the active site Ser-195 and the serpin, based mainly on the lack of fluorescence change following anhydrotrypsin, whereas the schemes proposed by Olson et al. placing it at E*. In their scheme (as in ours) kₙ₂, although only WT thrombin can complete the reaction to form E*1'. In their scheme (as in ours) E-I' is largely rate-determining for E*1' formation. A possible reason for this difference is that dehydroalanine substitution for Ser-195 might be more disruptive than Ala-195 substitution, effectively blocking E-I' conversion to EI'. Alternatively, different serpin-serine protease pairs may simply react via different pathways. Our present results are compatible either with no interaction between E and I in EI' or with formation of the initial tetrahedral intermediate on Ser-195 attack on the P1 carbonyl, and thus shed no further light on this issue.

The second point of difference concerns the species from which partitioning into substrate and inhibitor pathways occurs, Olson et al. placing it at EI', as in Scheme 1 and Stone and Le Bonniec placing it at E1', although in neither case is the supporting evidence strong. As discussed above, our results support EI' as the species from which partitioning occurs.

We conclude that Scheme 1, in which (a) formation of EI' from E-I is at least partly rate-determining in overall E*1' formation from the E1 encounter complex, (b) the P1-P1' linkage is preserved in EI', (c) partitioning between substrate and inhibitor pathways occurs from EI', and (d) E*1' corresponds to a covalent adduct of E and I, most likely acyl enzyme, is likely to provide a general, minimal description of serpin-serine protease interaction, but that whether there is covalent bond formation between E and I in EI' might vary from system to system.

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