Formation of the Covalent Chymotrypsin-Antichymotrypsin Complex Involves No Large-scale Movement of the Enzyme*

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α1-Antichymotrypsin is a member of the serine proteinase inhibitor, or serpin, family that typically forms very long-lived, enzymatically inactive 1:1 complexes (denoted E*I*) with its target proteinases. Serpins share a conserved tertiary structure, in which an exposed region of amino acid residues (called the reactive center loop or RCL) acts as bait for a target proteinase. Within E*I*, the two proteins are linked covalently as a result of loop or RCL) acts as bait for a target proteinase. Within E*I*, the two proteins are linked covalently as a result of nucleophilic attack by Ser195 of the serine proteinase on the P1 residue within the RCL of the serpin. This species is formally similar to the acyl enzyme species normally seen as an intermediate in serpin proteinase catalysis. However, its subsequent hydrolysis is extremely slow as a result of structural changes within the enzyme leading to distortion of the active site. There is at present an ongoing debate concerning the structure of the E*I* complex; in particular, as to whether the enzyme, bound to P1, maintains its original position at the top of the serpin molecule or instead translocates across the entire length of the serpin, with concomitant insertion of RCL residues P1-P14 within β-sheet A and a large separation of the enzyme and RCL residue P1'. We report time-resolved fluorescence energy transfer and rapid mixing/quench studies that support the former model. Our results indicate that the distance between residue P1' in α1-antichymotrypsin and the amino terminus of chymotrypsin actually decreases on conversion of the encounter complex E1 to E*I*. These results led us to formulate a comprehensive mechanism that accounted for both our results and for those of others supporting the two different E*I* structures. In this mechanism, partial insertion of the RCL, with no large perturbation of the P1' enzyme distance, is followed by covalent acyl enzyme formation. Full insertion can subsequently take place, in a reversible fashion, with the position of equilibrium between the partially and fully inserted complexes depending on the particular serpin-proteinase pair under consideration.

α1-Antichymotrypsin (ACT) is a member of the serine proteinase inhibitor, or serpin, family that typically forms very long-lived, enzymatically inactive 1:1 complexes (denoted E*I*) with its target proteinases. Serpins share a conserved tertiary structure, in which an exposed region of amino acid residues (called the reactive center loop or RCL) acts as bait for a target proteinase. For ACT, this loop extends from residues 342 to 367, denoted P17-P19', in which, following the nomenclature of Södhecurt and Berger (1), the scissile bond cleaved by the targeted proteinase chymotrypsin (Chtr) is between the P1 and P1' residues. The native serpin structure is unusual in that it is metastable and is considered to be in a stressed (S) conformation. The cleaved serpin released from the complex is much more stable than the intact serpin (2, 3) and is considered to be in a relaxed (R) conformation. Noteworthy among many structural differences between the S and R conformations (4) is the A β sheet, which is converted from a five-strand sheet into an antiparallel six-strand sheet by the insertion of RCL residues P1-P14 as strand e4A. As a result of this insertion, the P1 and P1' residues are separated by 70 Å.

There is now very good evidence that within E*I* the two proteins are linked covalently; an acyl enzyme has formed following attack by the nucleophilic Ser195 of the serine proteinase on the P1 residue of the serpin (5–7). This species is formed rapidly (5) and is formally similar to the acyl enzyme species normally seen as an intermediate in serpin proteinase catalysis. However, its subsequent hydrolysis is extremely slow, resulting in the observed inhibitory effect of serpins. This “trapped” acyl enzyme results from structural changes within the enzyme leading to distortion of the active site, which was inferred from proteolysis (8, 9) and NMR (10) studies in solution and recently confirmed by a crystal structure of the trypsin*antitrypsin* complex (11). Within this structure, the antitrypsin is in the R conformation, and the trypsin attached to the P1 residue has translocated from the top of the serpin molecule, defined as the position of the RCL in the intact, active serpin, across its entire length to the bottom of the molecule.

The trypsin*antitrypsin* structure provides strong support for one side in what has been an ongoing debate concerning the extent of RCL insertion within the E*I* complex, whether full insertion (R conformation), as first proposed by Wright and Scarsdale (12), or partial insertion, as suggested by Whisstock et al. (13). Earlier experimental results supporting each point of view are summarized in Stone et al. (14). More recently, studies employing fluorescence resonance energy transfer (FRET) (15) and donor-donor energy migration (16) have given results supporting the full insertion model, whereas monoclonal antibody binding studies to defined serpin epitopes (17,
18) favor the partial insertion model. These continuing disagreements raise questions as to whether the structure of the E2* complex may be different for different serpins and to what extent the time scale employed in measurement affects the structure observed, i.e. whether the partially inserted form may be an intermediate on the way to full insertion (19).

Here, we report time-resolved stopped-flow FRET and rapid mixing/quench studies of E2* formation between α-ACT, derivatized with a fluorescence donor at the P1 position, and δ-Chtr, derivatized with a fluorescence acceptor at the amino terminus. Our results demonstrate multistep formation of the E2* complex in which the distance separating the two fluorophores is inconsistent with formation of the fully inserted R conformation. In consequence, we propose a mechanism for E2* formation that rationalizes the apparent disagreements mentioned above. The chief features of this mechanism are that: (a) partial insertion of the RCL occurs with the P1-P1’ peptide linkage intact; (b) acyl enzyme formation occurs following partial insertion and is not concomitant with full insertion; and (c) the R conformation forms reversibly, but not necessarily rapidly, from the partially inserted conformation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine N-p-tosyl-1-leucine chloromethyl ketone-treated δ-chymotrypsin, α-chymotrypsinogen, all chromophoric proteinase substrates, N-α-tosyl-l-lysine chloromethyl ketone (TLCK), diithreitol, quinine sulfate, hydroxylamine hydrochloride, and phenylmethylsulfonyl fluoride were obtained from Sigma. Bovine N-p-tosyl-l-phenylalaninechloromethyl ketone-treated trypsin was obtained from Worthington. The concentrations of these enzymes and of rACT were determined as described earlier (20). All HPLC solvents, H2O, and formic acid were obtained from Fisher Scientific. 4-Bromomethyl-7-methoxycoumarin (BMMC) and 7-diethylaminocoumarin-3-carboxylic acid (DEACA)-sucinimidyl ester were acquired from Molecular Probes, Inc. (Eugene, OR). SDS-PAGE analysis was performed according to Laemmli (21).

**Preparation of Derivatized S359C-rACT**—The preparation of derivatized S359C-rACT was conducted using sequence overlap expression polymerase chain reaction and the ACT expression vector described previously (20, 22). The internal primers coding for the Ser195 mutation are as follows: 5′-GGGATTTTGAC-3′ and 5′-GATTTTGAC-3′ (the mutation sites are in boldface). The polymerase chain reaction product, representing the entire coding region, was cut with BstXI, gel-purified, and inserted in the correct reading orientation in pZMS. Full gene sequencing confirmed a single codon change. S359C-rACT and rACT were purified to homogeneity as described earlier (22).

**Preparation of Derivatized δ-Chymotrypsin—Zymogen** was derivatized prior to activation, permitting specific labeling of the unique amino terminus present in the proenzyme. Chymotrypsinogen (19 μM in 20 mM sodium phosphate, pH 7) was reacted with a 6-fold excess of 7-diethylaminocoumarin-3-carboxylic acid (DEACA)-sucinimidyl ester from Molecular Probes, Inc. (Eugene, OR). SDS-PAGE analysis was performed according to Laemmli (21).

**Characterization of Derivatized S359C-rACT**—Second-order rate constants for inhibition kS and stoichiometry of inhibition (SI) were determined for all serpin-proteinase pairs. Inhibition rate constants were determined by incubating equimolar concentrations of enzyme and inhibitor under second-order conditions and removing aliquots for residual enzyme activity determination as described earlier (20). SI values were determined by densitometric analysis of SDS-PAGE gels by comparing the intensity of cleaved serpin following complex formation with proteinase with the band intensity of uncleaved serpin (23).

**Mass Spectral Analysis**—Electrospray ionization was performed on a Micromass Platform LC Electrospray mass spectrometer (Micromass® UK, division of Waters Corp., Milford, MA) at the Mass Spectrometry Facility of the Department of Chemistry at the University of Pennsylvania. MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) was performed on a VG Tofspec (Fisons Instruments, Danvers, MA) at the Protein Chemistry Laboratory in the Medical School of the University of Pennsylvania.

**Oxidative Liberation of the Amino-terminal Fragment**—The amineterminal fragment (α-chain) from the remainder of the molecule was obtained from the Nα-acetylated (27, 28) δ-Chtr or DEACA-δ-CT was suspended in formic acid (88% in H2O), and a 2-fold volume of performic acid (prepared by adding 1/20 volume 30% H2O2 to formic acid followed by room temperature incubation for 1 h (29, 30)) was added, bringing the final concentration of H2O2 to 100%. The reaction mixture was kept on ice for 10 min, the reaction mixture was washed and concentrated, and the concentrations of these enzymes and of rACT were determined using Amicon, Centriprep-10, and Centricon-10 concentrators (Amicon, Inc.).

**Mass Spectral Analysis**—Electrospray ionization was performed on a Micromass Platform LC Electrospray mass spectrometer (Micromass® UK, division of Waters Corp., Milford, MA) at the Mass Spectrometry Facility of the Department of Chemistry at the University of Pennsylvania. MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) was performed on a VG Tofspec (Fisons Instruments, Danvers, MA) at the Protein Chemistry Laboratory in the Medical School of the University of Pennsylvania.
previously (22). SDS-PAGE analysis was performed on aliquots of the acid-quenched reaction mixture using gels containing 12% polyacrylamide. Prior to analysis, all samples were precipitated with freshly prepared 20% trichloroacetic acid, incubated on ice 30 min, centrifuged and redissolved in 10 μl of 20 mM sodium Pφ, pH 7, to which 2 μl of 1 M Tris (pH 7.4) was added to neutralize the excess trichloroacetic acid present in the quenched samples. Phenylmethylsulfonyl fluoride at a final concentration of 2 mM was also added to rapidly inactivate any reanimated chymotrypsin. Gels were stained overnight in GelCode® Blue Stain Reagent (Pierce), destained overnight in water, then dried prior to densitometric analysis. Data were fit to a three-step kinetic scheme using Hopkinsim, version 1.7 (32).

**Measurement of FRET Efficiency**—Energy transfer was determined by the acceptor enhancement method as well as by the decrease in donor fluorescence (33). In calculating efficiencies, observed values of fluorescence were corrected for the inner filter effect (Equation 1) (33), in which \(\alpha_{D}\) is the absorption of all species at \(\lambda_{D}\), and \(\alpha_{E}\) is the absorption of all species at the emission wavelength (\(\lambda_{E}\)). Such corrections varied from 3 to 19% for both acceptor and donor fluorescence (see below).

\[
F = F_{\text{norm}} \times 10^{-\alpha_{D}/22.5} \left(\frac{\alpha_{E}}{22.5}\right) (\text{Eq. 1})
\]

Fluorescence acceptor efficiencies (34) were calculated by Equation 2, in which \(F_{DA}\) is the fluorescence of the donor/acceptor pair (MCM-S359C-rACT/DEACA-chymotrypsin) when irradiated at the excitation wavelength of the donor and detected at the emission maximum of the donor (\(\lambda_{D}\) of MCM-S359C-rACT/DEACA-chymotrypsin), \(F_{DE}\) is the fluorescence of the donor in the absence of acceptor, \(F_{DA}\) is the fluorescence of the donor/acceptor pair, \(\alpha_{D}\) and \(\alpha_{E}\) are the absorbances of the donor and acceptor, respectively, at an excitation wavelength (\(\lambda_{D}\)) of 330 nm; and \(f_{D}\) (equal to 0.75) is the fraction of labeled acceptor protein.

\[
E = \left(\frac{1}{f_{D}}\right) \left[\frac{F_{DA}}{F_{DA} - F_{DE}} - 1\right] (\text{Eq. 2})
\]

The decrease in donor efficiency (35) was calculated by Equation 3, in which \(F_{DA}\) is the fluorescence of the donor/acceptor pair, with irradiation at the excitation wavelength of the donor and detection at the emission maximum of the donor (\(\lambda_{D}\) of MCM-S359C-rACT/DEACA-chymotrypsin), \(F_{DE}\) is the fluorescence of the donor in the absence of acceptor, \(\alpha_{D}\) and \(\alpha_{E}\) are the absorbances of the donor and acceptor, respectively, at an excitation wavelength (\(\lambda_{D}\)) of 330 nm; and \(f_{D}\) is the fraction of labeled acceptor protein.

\[
E = \left(\frac{1}{f_{D}}\right) \left[\frac{F_{DA}}{F_{DA} - F_{DE}} - 1\right] (\text{Eq. 3})
\]

**Calculation of Distance**—The measured efficiency is a function of the distance between donor and acceptor, \(R_{O}\), equal to \(2\left|\omega_{1}\right|^{2} \times 10^{-6} \text{cm}^{-1} \text{nm}^{2}\). The Förster distance at which the efficiency is 50%, is calculated by Equation 4.

\[
R_{O}^{2} = 8.8 \times 10^{8} \left(\frac{\text{cm}^{2}}{\text{nm}^{2}}\right)(\text{Eq. 4})
\]

In this equation, \(J\), the overlap integral that relates the degree of spectral overlap between the emission spectrum of the MCM group covalently bound to serpin and the absorption spectrum of the DEACA group attached to proteinase, was determined to be 4.52 \times 10^{-14} \text{cm}^{2} \text{nm}^{-1}\text{m}^{-1}\text{cm}^{-1}\text{mm}^{-1}\) using the method of Conrad and Brand (36); \(\theta_{F}\), the quantum yield of the donor in the absence of acceptor, was determined to be 0.023 for labeled serpin complexed to unlabeled proteinase by comparison with quinine sulfate as standard (37); \(n\), the index of refraction of the solvent, is assumed to be 1.33 (38); and \(k_{D}\) is the orientation factor, describing the relative orientation in space of the transition dipoles of the donor and acceptor. \(k^{2}\) may be set equal to 0.667 for donors and acceptors that randomize by rotational diffusion prior to energy transfer or to 0.476 assuming that a range of static donor-acceptor orientations exists that does not change during the lifetime of the excited state (33). Using either of these values gives similar values for \(R_{O}\), of 23.5 Å (\(k^{2} = 0.23\)) or 21.0 Å (\(k^{2} = 0.476\)).

**Modeling**—Modeling of the MCM-S359C-rACT/DEACA-b-Cht complex was performed using the program Quanta version 98.1111 (Molecular Simulations, Inc., San Diego, CA). The coordinates for the ACT-Cht docked model proposed by Katz and Christianson (39) were used as a starting point. The fluorescent probes were constructed in the two-dimensional Sketcher application and then converted to molecular structure files. These were simultaneously imported into Quanta along with the published model, moved into proximity with their attachment sites, and covalently attached with the modeling palette. The Molecular Editor tool was used to add or remove hydrogens as necessary for proper valency. The initial orientation of the probes was varied for both probes by a rotation around the bond directly connected to the fluor in 90° increments, generating a total of 16 structures. The probe aromatic rings were constrained to remain planar during the minimization and subsequent energy calculations. Each structure was then minimized first by the method of Steepest Descent, followed by the method of Adopted-Basis Newton-Raphson. These structures were then used to measure an average center-to-center distance between the probes in the encounter complex.

**RESULTS**

**Site of DEACA Labeling of b-Chtr**—The site or sites of labeling within b-Chtr depended on the overall stoichiometry of chymotrypsinogen labeling. Preparations having ≥1.0 DEACA groups per Chtr molecule had measurable labeling of the Ile\(^{16}\)-Asn\(^{245}\) polypeptide chain, rising to ~0.22 mol/mol for the sample containing 1.2 DEACA groups per Chtr. This most likely reflects partial labeling of some of the 14 free Lys residues present. On the other hand, preparations containing ≤0.75 DEACA groups per chymotrypsinogen molecule showed no (<0.01 mol/mol) such labeling of Ile\(^{16}\)-Asn\(^{245}\). For these samples, all labeling was confined to the α-chain (residues 1–13) and, by inference, to the only amino group within this chain, the α-amino group of Cys\(^{1}\). Accordingly, preparations containing 0.75 DEACA per α-Chtr were used in all kinetics experiments.

Direct determination of the stoichiometry of α-chain labeling was provided by RP-HPLC analysis of the soluble fraction of a performic acid oxidized sample of DEACA-b-Chtr (Fig. 1) as described under "Experimental Procedures." An analysis of the 75% labeled DEACA-b-Chtr gave a labeled α-peptide/unlabeled α-peptide ratio of 3:1, measured by corrected peak area at 215 Å. The assignment of peak 2 as the DEACA-labeled α-chain was indicated by its absorbance at 430 nm and confirmed by mass spectral analysis (1589, M + 2 Na). The assignment of peak 1 as an undervanated α-chain was also confirmed by mass spectral analysis (1301, M + H).

**Functionality of Fluorescently Labeled Proteins**—As shown by the results in Table I, MCM-S359C-rACT retains full activity toward Chtr inhibition, as measured by both the second-

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**Fig. 1.** RP-HPLC separation of DEACA-modified N-terminal fragment (Cys\(^{1}\)-Leu\(^{13}\)) from unmodified fragment. Overlay (lower trace) of traces monitored at 215 Å (a) and 330 Å (b). Focusing on DEACA-labeled Cys\(^{1}\)-Leu\(^{13}\) from unmodified fragment. Overlay of (a) and (b) shows that the DEACA-labeled Cys\(^{1}\)-Leu\(^{13}\) fragment elutes at a lower molecular weight compared to the unmodified fragment, indicating that the DEACA label is covalently attached to the Cys\(^{1}\)-Leu\(^{13}\) fragment. The peak at 215 Å corresponds to the DEACA-labeled Cys\(^{1}\)-Leu\(^{13}\) fragment, while the peak at 330 Å corresponds to the unmodified fragment. The results are consistent with the conclusions drawn from the stoichiometry analysis and support the hypothesis that the DEACA label is covalently attached to the Cys\(^{1}\)-Leu\(^{13}\) fragment.
order rate constant for inhibition, $k_i$, and the SI of $\sim 1$. Similarly, DEACA-$\delta$-Chtr retains full activity toward rACT and toward hydrolysis of the standard substrate N-succinimidyl-
AAPF-$p$-nitroanilide (data not shown). Finally, the reaction of
N toward hydrolysis of the standard substrate

to prevent proteolysis of the $rACT^*$ complex formed at 40 °C. Incubations were performed in

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and the amino-terminal α-amino group in α-Chtr is 33.6 Å. In our modeling, Ser359 was first mutated to Cys359, an MCM group was attached to ACT-Cys359, and a DEACA group was attached to the amino-terminal α-amino group in Chtr. Energy minimization, performed using a large variety of starting orientations, gave an estimated MCM center-to-DEACA center distance of 35.8 ± 2.0 Å, in reasonable accord with the range of values estimated by FRET for the EzxEI, Eia,o, or Eib complexes (26.4–34.6 Å, Table III).

**DISCUSSION**

**Kinetic Scheme—** Recent pH-dependent studies of the reaction of a fluorescent derivative of a cysteine variant of rACT at the P13 position with Chtr enabled us to formulate a scheme for rACT interaction with Chtr (Scheme 2), similar to Scheme 1 but containing three intermediates between Ei and E*I* (23). The nature of the pH dependence of the fluorescent changes and the similarity of Scheme 2 to the interaction of substrates with Chtr (40, 41) led us to propose that Ei conversion to EIa principally involves rearrangement of the RCL to the required canonical conformation. This rearrangement is followed by conversion of EIa to E*I*, corresponding to covalent reaction between Chtr residue Ser195 and the P1 residue of ACT to give acyl enzyme.

\[
\begin{align*}
Ei & \rightarrow EIa \\
EIa & \rightarrow EIb \\
EIc & \rightarrow E*I*
\end{align*}
\]

**SCHEME 2**

Our current results are consistent with Scheme 1. In Luo et al. (23), the time dependence of fluorescent change and of E*I* formation, measured at pH 7 and 10 °C, could be adequately fit with three rate constants, and it was only in considering all of the rate data collected over the pH range of 5.0 to 8.0 that the need for four rate constants became clear. Thus, it is appropriate to compare rate constants \(k_1\) (27.5 s\(^{-1}\)), \(k_2\) (2.5 s\(^{-1}\)), and \(k_3\) (0.6 s\(^{-1}\)) measured in this work with rate constants \(k_{1,\text{app}}\) (75 s\(^{-1}\)), \(k_{2,\text{app}}\) (5.6 s\(^{-1}\)), and \(k_{3,\text{app}}\) (0.7 s\(^{-1}\)) respectively, measured in Luo et al., where \(k_{3,\text{app}}\) the overall rate constant for conversion of EIa to E*I*, is equal to \(k_2 k_3 / (k_2 + k_3)\). This comparison shows each of the constants measured at 5 °C to be lower within a factor of 2 ± 1 than the values measured by Luo et al. at 10 °C, a reasonable result for a conserved kinetic scheme.

**A General Mechanism for E*I* Formation**—The results col-
lected in Table III indicate a small decrease in distance between the fluorophores attached to the amino terminus of Chtr and P1′ on conversion of the E-I encounter complex to the SDS-stable E*I* complex. This is consistent with only small-scale movement of Chtr relative to ACT during this conversion, but it would not exclude larger movements that would leave the fluorophore-fluorophore distance little changed, such as those that could accompany partial insertion of the RCL in the A β-sheet. What is excludable is a large-scale movement of the enzyme across the length of the serpin molecule, as in the fully inserted (R conformation) model proposed by Wright and Scarsdale (12) and seen recently in the high resolution crystal structure of the trypsin*antitrypsin* complex (11). This conclusion is independent of the value of χ^2 in Equation 4. Above (see “Experimental Procedures”), we have provided the rationale for using χ^2 values of 0.667 or 0.476, showing that the resulting calculated distance in the E-I complex is consistent with that estimated from a docked model. However, even making the extremely unlikely assumption that χ^2 in the E*I* complex has its upper limit value of 4.0 (corresponding to the two fluorophores being held rigidly parallel to one another with aligned dipoles) leads to an upper limit fluorophore distance of only 42.6 Å, well below the value demanded by the fully inserted model.

Although our results appear incompatible with the crystal structure of the trypsin*antitrypsin* complex, both sets of results, as well as the apparently contradictory results of others (15, 16), can be rationalized within the general mechanism for serpin-proteinase complex formation that we propose in Fig. 6, which is based in part on the recent results of Gooptu et al. (19) (see below). The important features of this mechanism are that: (a) partial insertion of the RCL, involving substantial structural change, occurs with the P1-P1′ peptide linkage intact; (b) acyl enzyme formation occurs following partial insertion and is not concomitant with full insertion; and (c) the fully inserted form of the acyl enzyme, E*I*, is formed reversibly from the partially inserted form E*I*, and differences in serpin structure are capable of affecting both the equilibrium position between these two states and the activation energy barrier for state-to-state interconversion. Here it should be noted that there is long-standing evidence for the reversibility of E*I* formation from E-I (42–44), although the overall equilibrium generally favors the acyl enzyme form.

Feature a is supported by both time-resolved and structural studies. In our own work, both current and previous (5, 22, 23), on the ACT/Chtr interaction, we have shown that large fluorescent changes, from probes placed at the P7, P13, and P1′ positions in ACT precede E*I* formation. Similar conclusions from time-resolved studies have been reported for the interactions of antithrombin (AT) and thrombin (14) and antitrypsin and elastase (45). In addition, structural studies have indicated that the RCL of intact serpins can insert either partially (19, 46) or fully into the A β-sheet (13, 47), the latter yielding the latent serpin. In the proposed mechanism, complexation with enzyme would favor partial insertion of the intact RCL in forming EL.

Feature b is a consequence of the present work, which unequivocally shows that acyl enzyme forms in the reaction of ACT and Chtr without full insertion of the RCL. This result differs from that of Stratikos and Gettins (15), who, employing FRET measurements from tryptophans within trypsin-to-dansyl groups placed at different specific positions within antitrypsin, demonstrated that enzyme was proximal to the bottom of the serpin as in E*I*₂, in agreement with the crystal structure of the antitrypsin/trypsin complex (11). Moreover, earlier work by Stratikos and Gettins (48) showed that such insertion occurs reasonably rapidly. Similarly, Fa et al. (16), using donor-donor energy migration and several fluorescently labeled double Cys variants of PAI-1, showed that, within the PAI-1-u-PA complex, the P3 position (amino acid 344) was far removed from the P1′ position (amino acid 347) at the top of the serpin but was proximal to amino acid 313 at the bottom of the serpin; in these studies, however, there was no clear indication of how quickly P3 separates from P1′.

Feature c rationalizes the accumulation of E*I*₁ in the interaction of ACT and Chtr, as opposed to the accumulation of E*I*₂ in the antitrypsin/trypsin and PAI-1-u-PA interactions, if it is assumed that the E*I*₁ complex has a higher relative stability for the ACT-Chtr pair. A structural basis for this assumption is provided by the crystal structure of the so-called δ-conformation of the naturally occurring L55P variant of intact ACT, as recently determined by Gooptu et al. (19). In this structure, the space between the s3A and s5A strands is filled by partial insertion of residues from the RCL, which bends out of the A sheet at P12 and turns to join s1C, and by residues...
75% labeling of DEACA-δ-Chtr calculated from stopped-flow traces corrected for internal filtering and the addition of MCM-S359C-rACT to DEACA-δ-Chtr structure. We used the following conditions: 

- 5 μM were obtained at 5 °C, pH 7.0, on the reaction of 10 μM enzyme.

Excitation at 330 nm, detection at 475 nm.

Number of independent determinations; ranges of values are average deviations.

Excitation at 330 nm, detection at 400 nm.

Excitation at 330 nm, detection at 475 nm.

Excitation at 430 nm, detection at 475 nm.

### FIG. 5.

**FRET efficiency profile.** Time-resolved efficiency was calculated from stopped-flow traces corrected for internal filtering and 75% labeling of DEACA-δ-Chtr. A, changes observed at 475 nm upon excitation at 330 nm, efficiency calculated using Equation 2. B, changes observed at 400 nm upon excitation of MCM-S359C-rACT to DEACA-δ-Chtr with excitation at 330 nm, with efficiency calculated using Equation 3. Data were obtained at 5 °C, pH 7.0, on the reaction of 10 μM inhibitor with 50 μM enzyme. Solid lines are best fits to Scheme 1, with rate constants $k_1 = 27.5 \text{ s}^{-1}$, $k_2 = 2.5 \text{ s}^{-1}$, $k_3 = 0.6 \text{ s}^{-1}$.

164–172, which are part of helix F-s3A turn in the native structure. We used the δ-conformation as a model for the serpin portion of both EI and E*I*, although recognizing that the structures of these two species could well differ in detail.

### TABLE II

| δ-Chtr | ACT   | [R][I] | Method | $k_1$ (s$^{-1}$) | $k_2$ (s$^{-1}$) | $k_3$ (s$^{-1}$) | No. |
|--------|-------|--------|--------|-----------------|-----------------|-----------------|-----|
| DEACA-δ-Chtr MCM-S359C- | 1.0 | SF$^a$ | 24.2 | 2.0 | 0.20 | 1 |
| DEACA-δ-Chtr MCM-S359C- | 2.0 | SF$^a$ | 23.8 ± 5.4 | 2.5 ± 1.0 | 0.33 ± 0.13 | 4 |
| DEACA-δ-Chtr MCM-S359C- | 5.0 | SF$^a$ | 29.0 ± 3.7 | 1.8 ± 0.68 | 0.29 ± 0.06 | 4 |
| DEACA-δ-Chtr MCM-S359C- | 1.0 | SF$^a$ | 14.6 | 2.0 | 0.23 | 1 |
| DEACA-δ-Chtr MCM-S359C- | 2.0 | SF$^a$ | 31.4 ± 7.8 | 3.5 ± 0.65 | 0.32 ± 0.13 | 5 |
| DEACA-δ-Chtr MCM-S359C- | 5.0 | SF$^a$ | 29.1 ± 4.2 | 3.1 ± 0.39 | 0.31 ± 0.06 | 2 |
| DEACA-δ-Chtr MCM-S359C- | 2.0 | SF$^a$ | 26.5 ± 3.4 | 3.4 ± 0.57 | 0.32 ± 0.12 | 2 |
| DEACA-δ-Chtr MCM-S359C- | 5.0 | SF$^a$ | 29.0 ± 4.3 | 3.2 ± 1.2 | 0.40 ± 0.20 | 2 |
| DEACA-δ-Chtr MCM-S359C- | 2.0 | QF    | (27.5) | (2.5) | 0.51 ± 0.09 | 2 |
| DEACA-δ-Chtr MCM-S359C- | 5.0 | QF    | (27.5) | (2.5) | 0.36 ± 0.11 | 2 |
| DEACA-δ-Chtr Wild type MCM-S359C- | 2.0 | SF$^a$ | 25.3 ± 7.4 | 4.2 ± 0.57 | 0.36 ± 0.27 | 2 |
| DEACA-δ-Chtr Wild type MCM-S359C- | 5.0 | SF$^a$ | 24.7 ± 0.35 | 4.6 ± 1.3 | 0.31 ± 0.19 | 2 |

$^a$ SF, stopped-flow; QF, quenched-flow.

$^b$ Number of independent determinations; ranges of values are average deviations.

$^c$ Excitation at 330 nm, detection at 400 nm.

$^d$ Excitation at 330 nm, detection at 475 nm.

$^e$ Excitation at 430 nm, detection at 475 nm.

### TABLE III

**Calculated efficiencies and distances**

| Intermediate | Efficiency$^a$ | R, in Å | Efficiency$^a$ | R, in Å$^b$ |
|--------------|---------------|--------|---------------|-------------|
| E·I          | 0.09          | 34.6   | 0.12          | 32.8        |
| EI           | 0.18          | 30.3   | 0.17          | 30.6        |
| EIa          | 0.26          | 28.0   | 0.21          | 30.7        |
| E·I*         | 0.33          | 26.4   | 0.33          | 26.4        |

$^a$ Calculated by fitting plot of acceptor efficiency versus time to Scheme I, using $k_1 = 27.5 \text{ s}^{-1}$, $k_2 = 2.5 \text{ s}^{-1}$, $k_3 = 0.6 \text{ s}^{-1}$.

$^b$ Calculated assuming $k_2 = 2/3$.

$^c$ Calculated by fitting plot of donor efficiency versus time to Scheme 1, using $k_1 = 27.5 \text{ s}^{-1}$, $k_2 = 2.5 \text{ s}^{-1}$, $k_3 = 0.6 \text{ s}^{-1}$.

In rationalizing the δ-conformation, which has not yet been found for any other serpin, Gooptu et al. (19) point out the high homology between residues 164–172 and residues P9-P1, which occupy the same positions in cleaved ACT (Table IV). Importantly, other serpins, in particular, antitrypsin and PAI-1, show considerably lower homology between these two sequences, consistent with the assumption of higher relative stability for ACT and, by extension, for Chtr·ACT·1. Whether such higher stability is sufficient to favor Chtr·ACT·1 thermodynamically over Chtr·ACT·2 or just increases the kinetic barrier for conversion of Chtr·ACT·1 to a more stable Chtr·ACT·2 is unknown. Physiologically it may not matter, because such conversion does not take place on heating Chtr·ACT·1 at 25 or 40 °C for 15 min, and serpin-proteinase complexes are cleared from the bloodstream rather rapidly, with a $t_{1/2}$ of 12 min observed for the clearance of Chtr·ACT·2 (49, 50).

Finally, feature c also rationalizes the apparent conflict in the results of Fa et al. (16), discussed above, and those of Bijnen et al. (17), working with an essentially identical serpin-proteinase pair. The latter workers report that monoclonal antibodies binding to an epitope comprising residues 128–131 in helix F and R$^{134}$ in the turn connecting helix F to s3A, at the bottom of the serpin, bind equally well to active PAI-1 and to the PAI-1-t-PA complex. They consider this result incompatible with full insertion of the RCL, as in E*II*, in which enzyme at the bottom of the serpin would block access to epitope, but it would be compatible with E*II*. A resolution of this apparent conflict is provided by the assumption that the monoclonal antibody binds only to E*II*, because in this case antibody
addition could shift an $E^*I^*_{1}/E^*I^*_{2}$ equilibrium distribution from dominant $E^*I^*_{2}$ to dominant $E^*I^*_{1}$. A similar explanation would account for the results of Picard et al. (18), who showed that a monoclonal antibody recognizing residues 366–370 (P28-P24) in s5A in AT binds to the AT-thrombin and AT-Factor Xa complexes, as well as to the complex formed between AT and a

![Diagram of mechanism for $E^*I^*$ formation](image)

**Fig. 6. General mechanism for $E^*I^*$ formation.** Shown is a general mechanism for $E^*I^*$ formation applied to Chtr*(gray)ACT*(yellow), which accounts for the results obtained for a variety of serpin-proteinase stable acyl enzyme complexes (see "Discussion"). In the structures shown, generated by QUANTA, the MCM group bound to the P1' residue of ACT is depicted as a blue triangle and the DEACA group bound to the amino terminus of β-Chtr as a red oval. The structure of the encounter complex is taken from the docked model of Katz and Christianson (39) in which the RCL (in red) shows no preinsertion into the A β-sheet. The carboxyl-terminal portion of helix F (in blue) is exposed to solvent. E1 is converted via three or more steps (23) to EI, in which the RCL is partially inserted between the s3A and s5A strands, with full insertion blocked by insertion of the carboxyl terminal of helix F, and the P1-P1' bond is intact. The mechanism of the serpin portion of EI is based on the structure determined for the δ-conformation of ACT (19). Residues 353–357 (P6–P2) are shown as part of a continuous RCL, although they are not visible in the determined structure. Conversion of EI to the acyl enzyme $E^*I^*$, is accomplished without major conformational change, with enzyme and P1' remaining in proximity (Table III). Conversion of $E^*I^*_{1}$ to $E^*I^*_{2}$ proceeds reversibly and results in translocation of the proteinase across the length of serpin, accompanied by a large separation between enzyme and P1', with helix F displaced from β-sheet A by the RCL. The Chtr and ACT portions of $E^*I^*_{2}$ are based on the determined structures of α-Chtr (51) and cleaved ACT (52), respectively. The latter structure shows no observed density for the segment P1' to P6'. In the $E^*I^*_{2}$ structure shown, the probe is placed on the first observed residue in the cleaved structure (P7'). In the determined structure of cleaved antitrypsin (53), which clearly shows the P1' to P6' region, this portion of the RCL is extended even further away from the body of the serpin.

**Table IV**

**Sequence comparisons**

A structure-based sequence comparison between the RCL from residues P9 to P1 and the carboxyl-terminal portion of helix F for four inhibitory serpins is shown. This portion of helix F is seen to insert into β-sheet A in L55P-ACT (19).

| Serpin                          | Secondary structure | RCL (P9-P1) | A$^{350}$ T A V K I T L L$^{358}$ | Similarity* |
|--------------------------------|---------------------|------------|----------------------------------|-------------|
| rACT                           | hF                  | RCL        | G$^{164}$ T R G K I T D L$^{172}$ | Similarity  |
| Antitrypsin                    | hF                  | RCL        | A$^{350}$ M F L E A I P M$^{358}$ | Similarity  |
| Plasminogen activator inhibitor-1 | hF                  | RCL        | S$^{318}$ T A V I V S A R$^{346}$ | Similarity  |
| Antithrombin III               | hF                  | RCL        | H$^{13}$ T K G M I S N L$^{31}$  | Similarity  |
|                                | hF                  |            | S$^{345}$ A V V I A G R$^{393}$  | Similarity  |
|                                |                     |            | T                               |             |
|                                |                     |            | I                               |             |
|                                |                     |            | K$^{193}$ T E G R I T D V$^{201}$ |             |

*Identity is indicated by the one-letter code for the amino acid in common between the two secondary structure elements; similarity in terms of a non-negative value in a Blosum amino acid similarity matrix is indicated with an asterisk.
hexapeptide corresponding to residues P14-P9, but does not bind to native, heparin-activated, latent, or cleaved AT.

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