Apparent Formation of Sodium Dodecyl Sulfate-stable Complexes between Serpins and 3,4-Dichloroisocoumarin-inactivated Proteinases Is Due to Regeneration of Active Proteinase from the Inactivated Enzyme*

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Protein protease inhibitors of the serpin family were recently reported to form SDS-stable complexes with inactive serine proteinases modified at the catalytic serine with 3,4-dichloroisocoumarin (DCI) that resembled the complexes formed with the active enzymes (Christensen, S., Valnickova, Z., Thøgersen, I. B., Pizzo, S. V., Nielsen, H. R., Roepstorff, P., and Enghild, J. J. (1995) J. Biol. Chem. 270, 14859–14862). The discordance between these findings and other reports that similar active site modifications of serine proteinases block the ability of serpins to form SDS-stable complexes prompted us to investigate the mechanism of complex formation between serpins and DCI-inactivated enzymes. Both neutrophil elastase and \( \beta \)-trypsin inactivated by DCI appeared to form SDS-stable complexes with the serpin, \( \alpha \)-proteinase inhibitor (\( \alpha \)PI), as reported previously. However, several observations suggested that such complex formation resulted from a reaction not with the DCI enzyme but rather with active enzyme regenerated from the DCI enzyme by a rate-limiting hydrolysis reaction. Thus (i) complex formation was blocked by active site-directed peptide chloromethyl ketone inhibitors; (ii) the kinetics of complex formation between serpins and DCI-inactivated enzymes took second order but rather showed a first-order dependence on DCI enzyme concentration and zero-order dependence on inhibitor concentration; and (iii) complex formation was accompanied by stoichiometric release of a peptide having the sequence SIPPE corresponding to cleavage at the \( \alpha \)PI reactive center P1–P1′ bond. Quantitation of kinetic constants for DCI and \( \alpha \)PI inactivation of human neutrophil elastase and trypsin and for reactivation of the DCI enzymes showed that the observed complex formation could be fully accounted for by \( \alpha \)PI preferentially reacting with active enzyme regenerated from DCI enzyme during the reaction. These results support previous findings of the critical importance of the proteinase catalytic serine in the formation of SDS-stable serpin-proteinase complexes and are in accord with an inhibitory mechanism in which the proteinase is trapped at the acyl intermediate stage of proteolysis of the serpin as a substrate.

Serpins comprise a large superfamily of proteins, many of which regulate the activity of serine and, in some cases, also cysteine proteinases in numerous biological processes by functioning as inhibitors of these proteinases (1, 2). Such serpins inhibit their target proteinases by a mechanism that bears similarities to that of other nonserpin families of proteinase inhibitors (3, 4). Serpin and nonserpin inhibitors thus both possess an exposed reactive center loop that binds to the active site of their target proteinases in the manner of a substrate. Additionally, both types of inhibitors trap proteinases in stable complexes at an intermediate stage of proteolysis of the inhibitor reactive center loop as a substrate. However, the nature of the trapped complexes distinguishes serpin from nonserpin inhibitors. Serpin-proteinase complexes are thus stable in denaturants such as SDS and guanidinium chloride (5–8), whereas nonserpin inhibitor-proteinase complexes dissociate under such conditions (9). Serpins must further undergo a major conformational change to trap their target proteinases in stable complexes (10–15), whereas nonserpin inhibitors require minimal conformational adjustments to bind their target enzymes (3, 4). Such differences in behavior have suggested that serpins have evolved a novel mechanism for inhibiting proteinases that greatly differs from the mechanism used by nonserpin inhibitors.

While there are no available structures of serpin-proteinase complexes, the SDS stability of these complexes has suggested that serpins may trap proteinases at the tetrahedral or acyl intermediate stage of proteolysis of the serpin as a substrate (2). Early studies favored trapping of the proteinase at the acyl intermediate stage of proteolysis, based on the observation that the serpin reactive center loop was cleaved in the stable complex with proteinase (8, 16–18). However, NMR evidence later suggested that stabilization of the serpin-proteinase complex at the acyl intermediate stage occurred only under denaturing conditions and that under native conditions, the complex was stabilized at the tetrahedral intermediate stage (19). Yet more recent findings have questioned the NMR data by showing that, even under nondenaturing conditions, the reactive center loop is cleaved concomitantly with formation of the serpin-proteinase complex, thus supporting an arrest of the serpin-proteinase reaction at the acyl intermediate stage (20–22).

Further controversy has ensued from another recent report that has suggested that the SDS stability of serpin-proteinase complexes does not result from a covalent linkage of the inhibitor to the enzyme active site as would exist in an acyl or tetrahedral intermediate, but rather is due to a covalent crosslinking of the inhibitor with proteinase outside of the enzyme active site (23). The evidence supporting this conclusion in-
cluded the observations that (i) proteinases blocked at the active site by the inactivating reagent, 3,4-dichloroisocoumarin (DCI),\(^1\) appeared still capable of forming SDS-stable complexes with serpins and (ii) no release of the carboxyl-terminal region of the serpin from the complex with proteinase could be demonstrated on denaturation, implying that cleavage of the reactive center loop of the serpin had not occurred. These observations contrast with several earlier studies that showed that the blocking of the proteinase active site with reagents such as diisopropyl fluorophosphate prevented complex formation with serpin inhibitors (5, 7, 24). Even allowing that the DCI reagent is small enough to permit binding of the serpin at the enzyme active site, the results of this study still disagree with other findings with active site-modified serine proteinases, in which minimal modifications of the active site serine to alanine or to dehydroalanine or of the active site histidine to methylhistidine were made. Such catalytically inactive modified enzymes were thus found to be capable of forming only noncovalent complexes with serpins that were dissociable in SDS (7, 9, 25–27). Further, these complexes were of much lower affinity than the SDS-stable complexes formed with active proteinases, consistent with a covalent interaction involving the proteinase catalytic serine residue stabilizing the serpin–proteinase complexes (9).

Because the findings of Christensen et al. (23) are diametrically opposed to previous findings regarding the nature of the stable serpin–proteinase complex, we sought to determine the basis for the discrepancies between these studies by investigating the mechanism by which DCI proteinases form SDS-stable complexes with serpins. While the present study confirms that SDS-stable complexes are formed in the reaction of DCI proteinases with serpins, our investigation of this phenomenon clearly demonstrates that such complexes do not arise from the DCI-inactivated enzyme but rather are formed from active enzyme regenerated from the DCI enzyme. Moreover, evidence is presented that the serpin reactive bond is cleaved in the SDS-stable complexes formed with the DCI enzyme, consistent with the complexes arising from active enzyme and involving a trapping of the enzyme at the acyl intermediate stage of proteolysis.

**MATERIALS AND METHODS**

**Proteins**—Bovine trypsin (Type XIII, Sigma) was purified by soybean trypsin inhibitor-agarose affinity chromatography to isolate the single chain \(\beta\)-form of the enzyme (28). Autolysis of the enzyme was prevented by storage in 1 mM HCl, 10 mM CaCl\(_2\). Human neutrophil elastase (HNE) and human \(\alpha_1\)-proteinase inhibitor (\(\alpha_1\)PI) were purchased from Athens Research and Technology Inc. (Athens, GA). Alternatively, \(\alpha_1\)PI was purified from outdated plasma as described (9). \(\beta\)-Trypsin concentrations were determined from initial rates of hydrolysis of the chromogenic substrate, S-2222 (Pharmacia Hepar, Franklin, OH), by the enzyme using the turnover number measured with active site titrated enzyme (29). Comparison with the concentration measured from the 280-nm absorbance using an absorption coefficient of 36,800 M\(^{-1}\) cm\(^{-1}\) (30) indicated the enzyme was \(\approx 90\%\) active. The concentration of \(\alpha_1\)PI was determined from the 280-nm absorbance based on an absorption coefficient of 25,400 M\(^{-1}\) cm\(^{-1}\) (31). The inhibitor was fully active based on the 1:1 stoichiometry (within 10%) measured by titration of \(\beta\)-trypsin with \(\alpha_1\)PI (9). HNE concentrations were determined by titration with \(\alpha_1\)PI assuming an equimolar inhibition stoichiometry.

**Experimental Conditions**—Trypsin experiments were conducted in 0.1 M Hepes, 0.1% polyethylene glycol 8000, pH 8.0, and HNE experiments were conducted in the same buffer plus 0.5 mM NaCl. Temperatures were either 25 or 37 °C as indicated.

**Kinetics of DCI Hydrolysis**—DCI (Sigma) was dissolved in dimethyl sulfoxide (Me\(_2\)SO) that had been dried by storage over molecular sieves (Type 4A, Fisher). Hydrolysis reactions were initiated by diluting 0.1 ml of DCI into 0.9 ml of either trypsin or HNE buffers to give final DCI concentrations of 125–250 \(\mu\)M and 10% Me\(_2\)SO. Hydrolysis was continuously monitored from the decrease in absorbance at 325 nm for several half-lives (32). First-order rate constants were obtained by nonlinear regression fitting of reaction curves by an exponential function with a floating end point. Indistinguishable rate constants were obtained at the same DCI concentrations. DCI concentrations were calculated from the initial 325-nm absorbance using an absorption coefficient of 3330 M\(^{-1}\) cm\(^{-1}\) (32).

**Kinetics of Trypsin or HNE Inhibition by DCI or Chloromethyl Ketones**—The kinetics of enzyme inhibition were measured under pseudo first-order conditions either by discontinuous or continuous assay methods (33). For the discontinuous assay method, reactions contained 10 mM enzyme and either 125–250 \(\mu\)M DCI, 100–200 \(\mu\)M Ph-Phe-Arg chloromethyl ketone (Calbiochem), or 5 \(\mu\)M methoxyxysucinyl-Ala-Ala-Pro-Val chloromethyl ketone (Bachem, King of Prussia, PA) in 0.1 ml. Identical reaction samples were quenched at various times with 0.9 ml of chromogenic substrate, and residual enzyme activity was measured from the initial rate of substrate hydrolysis at 405 nm. The substrates were 200 \(\mu\)M methoxyxysucinyl-Ala-Ala-Pro-Val nitrroanilide (Calbiochem) for HNE and 200 \(\mu\)M S-2222 for trypsin. For reactions of 4–10 \(\mu\)M trypsin with 250 \(\mu\)M DCI at 25 °C, samples of the reaction mixture were first quenched at different times into 4 mM HCl, 10 mM CaCl\(_2\) (40-fold dilution) and then further diluted 100-fold into substrate for activity determination. Pseudo first-order rate constants (\(k_{\text{obs}}\)) were determined by nonlinear regression fitting of the loss of enzyme activity to an exponential decay function. Second-order rate constants were obtained by dividing \(k_{\text{obs}}\) by the inhibitor concentration. For the continuous assay, DCI and then HNE were added in rapid succession to chromogenic substrate to give final concentrations of 20 mM HNE, 2.5–10 mM DCI, and 320 \(\mu\)M substrate. The exponential decrease in substrate hydrolysis rate was monitored continuously for up to 5 min to an end point rate (<2% of the initial rate) with <5% consumption of substrate. Progress curves were fit by nonlinear regression to an exponential plus linear term (33, 34) to obtain \(k_{\text{obs}}\). \(k_{\text{obs}}\) was corrected for substrate degradation by multiplying the factor, 1 + [S]/\(K_m\), where [S] is the substrate concentration. \(K_m\) was measured to be 46 ± 9 mM at 25 °C and 52 ± 4 mM at 37 °C (±2 S.E.). Second-order rate constants were obtained by dividing the corrected \(k_{\text{obs}}\) by the inhibitor concentration. The final concentration of Me\(_2\)SO was 10% in all DCI enzyme reactions. No correction for hydrolysis of DCI was made in any of these reactions, since the extent of such hydrolysis was found to be negligible over the time course of the reactions studied.

**Kinetics of \(\alpha_1\)PI Inhibition of Trypsin and HNE**—The kinetics of inhibition of proteinases by \(\alpha_1\)PI were measured under pseudo first-order conditions either by discontinuous (trypsin) or continuous (HNE) assay methods. Two reactions were conducted in 2.5 mM enzyme and 25 or 50 \(\mu\)M \(\alpha_1\)PI in 0.1 ml. At different times, reactions were quenched by adding 0.9 ml of 200 \(\mu\)M S-2222 substrate, and residual enzyme activity was measured from the initial substrate hydrolysis rate at 405 nm. \(k_{\text{obs}}\) was determined by nonlinear regression fitting to an exponential decay function. HNE reactions contained 1.25–5 mM \(\alpha_1\)PI, 0.25–1 mM Me\(_2\)SO, and 330 \(\mu\)M substrate and were initiated with enzyme. The exponential decrease in substrate hydrolysis rate was monitored continuously until an end point rate was reached (<1% of the initial rate) during which <1% substrate was consumed. Progress curves were fit by an exponential plus linear function to obtain \(k_{\text{obs}}\) and second-order rate constants were obtained from corrected values of \(k_{\text{obs}}\) as described for DCI HNE reactions.

**Kinetics of Regeneration of Active Enzyme from DCI Enzyme Hydrolysis**—DCI enzyme was prepared by incubating 250 \(\mu\)M enzyme with 6 \(\mu\)M enzyme for 15 min at 25 °C (final Me\(_2\)SO concentration, 10%). After diluting the inactivated enzyme 20-fold, excess DCI was removed by dialysis at 4 °C for 3 h against 100 volumes of reaction buffer containing 4–5% Me\(_2\)SO (to mimic the conditions for \(\alpha_1\)PI complex formation in SDS gel electrophoresis experiments) with buffer changes after each hour. A control enzyme sample was treated similarly except that no DCI was added. A 50-\(\mu\)l aliquot containing 47–140 nM DCI HNE or 5–16 mM DCI trypsin was then added to 0.95 ml of 200 \(\mu\)M substrate in buffer containing 4–5% Me\(_2\)SO, and theaccelerating rate of substrate hydrolysis was monitored continuously at 405 nm for 15 min at 37 °C (±5% substrate hydrolysis). Data were fit by nonlinear regression by the parabolic equation (33),

\[
A_0 = A_2 + t + \frac{b_{\text{II}}(E-I)}{TN} \frac{t}{2}
\]

where \(A_0\) and \(A_2\) are the absorbance at zero time and time \(t\), respectively, \(v_0\) is the initial rate of absorbance change, \(b_{\text{II}}\) is the first-order rate constant for hydrolysis of the DCI enzyme, \([E-I]\) is the concentration of DCI enzyme at zero time, and \(TN\) is the turnover number for

\(^1\) The abbreviations used are: DCI, 3,4-dichloroisocoumarin; HNE, human neutrophil elastase; \(\alpha_1\)PI, \(\alpha_1\)-proteinase inhibitor; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)methyl)glycine.
enzyme hydrolysis of the substrate under the conditions of the experiment, expressed as the rate of absorbance change/unit of enzyme concentration. $k_{\text{cat}}$ was calculated from the fitted coefficient of the $t^* \text{ term}$ using the independently measured turnover number and concentration of DCI enzyme. The latter was determined from the enzyme activity generated after complete hydrolysis of the DCI enzyme in buffer containing $0.5 \text{ M } \text{NH}_2\text{OH}$ for 20 h at $25^\circ \text{C}$ (32). Concentrations of regenerated enzyme were indistinguishable from those of control enzyme samples not reacted with DCI with or without hydroxylamine treatment.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blotting**—Reactions of α1PI with active and DCI-inactivated enzymes followed the protocol of Christensen et al. (23). DCI enzymes were prepared by incubating enzyme with 250 μM DCI for 15 min at $25^\circ \text{C}$ (final $\text{Me}_2\text{SO}$ concentration, 10%). An equal volume of α1PI or α1PI plus Phe-Phe-Arg chloromethyl ketone or methoxyxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone at $37^\circ \text{C}$ was then added in a single aliquot, and the incubation was continued at $37^\circ \text{C}$ for 30 min. Reactions were then quenched by first adding either 500 μM Phe-Phe-Arg chloromethyl ketone (trypsin) or 250 μM DCI (HNE) to avoid nonspecific proteolysis during denaturation followed by addition of SDS and then boiling for 3 min before electrophoresis on 10% gels according to Laemmli (35). For experiments designed to detect a carboxyl-terminal peptide formed concomitantly with the α1PI-protease complex, a Tricine-step gel system was used, in which the lower half of the gel was 16.5% acrylamide and the upper half was 10% acrylamide (36). Bands were stained with Coomassie Brilliant Blue R-250 and quantified by scanning the gels in an UltroScan XL laser densitometer (Pharacia-LKB Biotechnology, Uppsala, Sweden). For the quantitation of serpin-protease complex formation in α1PI reactions with DCI enzymes, the amount of complex formed in the reaction with active enzyme was taken as 100%. A series of dilutions of this maximum level of complex indicated a linear relationship between the amount of complex and the integrated band intensity. For Western blotting detection of α1PI-HNE complexes formed at lower serpin and protease concentrations, proteins electrophoresed on 10% SDS gels were transferred to nitrocellulose membranes (37), and inhibitor bands were then detected by incubating with sheep anti-α1PI Ig followed by donkey anti-sheep Ig conjugated with alkaline phosphatase (Binding Site, Inc., San Diego, CA) and then 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Sigma).

**Peptide Sequencing**—After SDS-Tricine step polyacrylamide gel electrophoresis to separate the carboxyl-terminal peptide as described above, the unstained gel was electrobottled to a polyvinylidene difluoride membrane (Qiabrade PVDF, Qiagen, Chatsworth, CA). The membrane was stained with Coomassie Brilliant Blue R-250, the appropriate band was excised, and the peptide was sequenced directly in an Applied Biosystems (Foster City, CA) 470A gas phase sequencer and then 5-bromo-4-chloro-3-indolyl phosphor/nitro blue tetrazolium substrate (Sigma).

**RESULTS AND DISCUSSION**

In the reaction of DCI trypsin with α1PI, an SDS-stable complex was formed that was indistinguishable from the complex formed with active trypsin, in agreement with the report of Christensen et al. (23) (Fig. 1). However, as also observed in the previous report, the amount of complex formed when DCI trypsin was reacted with equimolar α1PI for 30 min at $37^\circ \text{C}$ was considerably less than that formed with active trypsin under identical conditions (Fig. 1, lanes 4 and 7). Doubling the concentration of DCI enzyme in the reaction doubled the amount of complex, whereas varying the $\alpha_1\text{PI}$ concentration from 0.5 to 2 times the molar concentration of DCI enzyme produced the same amount of complex as that formed at equimolar concentrations (Fig. 1, lanes 7–9). The extent of complex formation at equimolar DCI enzyme and inhibitor also progressively increased with increasing time of incubation (Fig. 2, lanes 4–6). Integrating the intensities of the complex bands in a separate experiment revealed that 14, 25, and 53% of the complex had formed with active enzyme was formed in the reaction with the DCI enzyme after 30, 60, and 120 min, respectively. Together, these observations indicated that the formation of a stable complex from DCI enzyme and α1PI was limited by a reaction that was first order in DCI enzyme concentration but zero order in inhibitor concentration. This contrasts with the $\alpha_1\text{PI}$ reaction with active trypsin, which is first order in both enzyme and inhibitor concentrations, i.e. second order overall.

To assess whether the SDS-stable complexes formed with α1PI and DCI trypsin involved a covalent interaction between the enzyme and inhibitor outside the enzyme active site rather than within the active site, we tested the effect of an efficient active site-directed inhibitor, β-Phe-Phe-Arg chloromethyl ketone, on SDS-stable complex formation. Addition of chloromethyl ketone together with α1PI resulted in a nearly complete blocking of the reaction of α1PI with both active trypsin and inactive DCI trypsin (Fig. 1, lanes 5 and 10). Such results suggested that the complex formed in the reaction of DCI enzyme with α1PI might be arising from active enzyme generated during the reaction by hydrolysis of the DCI inactivating group rather than from the DCI enzyme itself.

To investigate this possibility, the kinetics of trypsin inactivation by DCI and reactivation of DCI trypsin were analyzed under the conditions used for observing SDS-stable complex formation between α1PI and DCI trypsin in the report of Christensen et al. (23). The enzyme (4–10 μM) was inactivated by 250 μM DCI at $25^\circ \text{C}$ in a first-order process ($t_{\text{1/2}} 

![Fig. 1. Formation of SDS-stable complexes in the reactions of α1PI with trypsin and with DCI trypsin analyzed by SDS-polyacrylamide gel electrophoresis.](image)

![Fig. 2. Demonstration by SDS-Tricine step polyacrylamide gel electrophoresis of peptide release concomitant with the reactions of α1PI with active or DCI-inactivated proteinases.](image)
The kinetics of the reactions depicted in the left-hand column (where \( E \) denotes enzyme, \( CK \) denotes chloromethyl ketone, and \( DCI^* \) represents the hydrolysis product of DCI) were measured at the indicated temperatures either in 0.1 M Hepes, 0.1% polyethylene glycol 8000, pH 8.0, for trypsin reactions or in 0.1 M Hepes, 0.5 M NaCl, 0.1% polyethylene glycol 8000, pH 8.0, for HNE reactions, as described under "Materials and Methods." Errors represent either the range of at least two determinations or ±2 S.E. ND, not determined.

TABLE I  
KINETIC CONSTANTS FOR REACTIONS OF ACTIVE AND DCI-INACTIVATED ENZYMES

| Reaction | \( 25^\circ C \) | \( 37^\circ C \) |
|----------|-----------------|-----------------|
| \( E + DCI \rightarrow E-DCI \) | \( 9.5 \pm 0.4 \times 10^4 \) | \( 2.0 \pm 0.1 \times 10^5 \) |
| \( E + a_1 PI \rightarrow E-a_1 PI \) | \( 4.9 \pm 0.1 \times 10^4 \) | \( 8.9 \pm 1.6 \times 10^5 \) |
| \( E + CK \rightarrow E-CK^* \) | ND | ND |
| \( E-DCI + E + \Delta DCI^* \) | ND | ND |
| \( DCI + H_2O \rightarrow DCI^* \) | \( 6.1 \pm 0.1 \times 10^{-4} \) | \( 1.4 \pm 0.1 \times 10^{-3} \) |

\( a \) Reaction of enzymes with either Phe-Phe-Arg chloromethyl ketone for trypsin or methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone for HNE.

\( b \) Reaction of enzymes with either Phe-Phe-Arg chloromethyl ketone for trypsin or methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone for HNE.

\( c \) Reaction of enzymes with either Phe-Phe-Arg chloromethyl ketone for trypsin or methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone for HNE.

\( d \) Reaction of enzymes with either Phe-Phe-Arg chloromethyl ketone for trypsin or methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone for HNE.

The value originally reported for this reaction by Harper et al. (32) at lower pH and higher ionic strength (Table I). Although trypsin appeared to be essentially all inactivated by DCI, DCI enzymes are known to slowly regenerate active enzyme by hydrolysis of the active site blocking reagent (32). Such regeneration of trypsin from DCI trypsin was measured by diluting the DCI enzyme into a reporter chromogenic substrate after dialysis to remove excess DCI and monitoring the acceleration of the substrate hydrolysis rate. A first-order rate constant of \( 8.1 \pm 0.6 \times 10^{-5} \text{ s}^{-1} \) was determined for release of active enzyme under the conditions used for complex formation with \( a_1 PI \), i.e. 37 °C. Based on this rate constant, the amount of reactivated trypsin formed was predicted to be \( 14 \pm 1 \% \) during the 30-min time interval allowed for recovery of DCI trypsin with \( a_1 PI \) alone and for DCI trypsin present during the reaction, given the \( 5000 \)-fold faster second-order rate constant measured for hydrolysis of trypsin by DCI than by DCI and the depletion of DCI by hydrolysis during the formation of the DCI enzyme (Table I and Scheme I). The ability to block the reaction of regenerated trypsin with \( a_1 PI \) by Phe-Phe-Arg chloromethyl ketone was also consistent with the relative inhibition rate constants and concentrations of serpin and chloromethyl ketone employed in the reaction (Table I).

The above observations indicated that the amount of SDS-stable complex formed in the reaction of \( a_1 PI \) with DCI trypsin could be fully accounted for by the amount of active trypsin regenerated from DCI trypsin by a rate-limiting hydrolysis of the inactivated enzyme (Scheme I). Such an interpretation was consistent with the first-order dependence of complex formation on the DCI enzyme concentration, the independence of the \( a_1 PI \) concentration, and the dependence of the amount of complex formed on the time of incubation of DCI trypsin with \( a_1 PI \). In keeping with this conclusion, a ~5-kDa peptide appeared concomitant with the formation of the SDS-stable complex in the reactions of \( a_1 PI \) with both DCI trypsin and trypsin (Fig. 2, lanes 3–6). The amount of peptide generated was stoichiometric with the amount of complex, based on the similar ratios of the integrated intensities of peptide to complex bands for active and inactive enzyme reactions. Amino-terminal sequencing of the peptide produced in the DCI trypsin reaction gave the sequence SIPPE corresponding to cleavage of \( a_1 PI \) in the reactive center Met\(^{535}\)Ser\(^{538}\)Phe-Phe-Arg-DCI-Arg-DCI-Arg plus 2 mM chloromethyl ketone; lane 4, lane 3 plus 2 mM chloromethyl ketone; lane 5, 1 mM DCI HNE alone; lane 6, 1 mM \( a_1 PI \) plus 1 mM DCI HNE; lane 7, 1 mM \( a_1 PI \) plus 5 mM DCI HNE; lane 8, 2 mM \( a_1 PI \) plus 1 mM DCI HNE; lane 9, as lane 7 plus 2 mM chloromethyl ketone; lane 10, molecular mass standards in kDa: 94, 67, 43, 30, and 20. Bottom, Western blot of HNE reactions. Lane 1, 0.2 mM \( a_1 PI \) alone; lane 2, 0.2 mM \( a_1 PI \) plus 0.2 mM HNE; lane 3, as lane 2 plus 2 mM chloromethyl ketone; lane 4, 0.2 mM DCI HNE alone; lane 5, 0.2 mM \( a_1 PI \) plus 0.2 mM DCI HNE; lane 6, 0.4 mM \( a_1 PI \) plus 0.2 mM DCI HNE; lane 7, 0.2 mM \( a_1 PI \) plus 0.4 mM DCI HNE; lane 8, as lane 5 plus 2 mM chloromethyl ketone; lane 9, as lane 7 plus 2 mM chloromethyl ketone; lane 10, 0.2 mM \( a_1 PI \) alone.
been due to the loss or reduced visibility of the ~5-kDa peptide in the SDS gradient gel system employed. By increasing the amount of protein loaded on the gel and calibrating the gel with standard peptides, the ~5-kDa peptide was clearly visible in our gel system. The cleavage and release of homologous carboxyl-terminal peptides from other SDS-stable serpin-proteinase complexes have been convincingly shown in numerous other studies (8, 16, 18, 20–22, 38).

Similar experiments comparing the reactions of α1PI with HNE and DCI HNE showed that the DCI enzyme formed 18 ± 2% of the SDS-stable complex generated in the reaction of equimolar α1PI with active enzyme (Fig. 3, top panel, lanes 3 and 6). The DCI HNE reaction showed a similar first-order dependence of stable complex formation on the concentration of DCI enzyme, independence of inhibitor concentration, and dependence on the time of reaction, as the DCI trypsin reaction (Fig. 3, top panel, lanes 6–8). Measurement of the rate constants for the inactivation of HNE by DCI and α1PI and for the regeneration of HNE from DCI HNE (Table I) predicted that 22 ± 2% of the stable complex should result from the regeneration of active HNE during the 30-min reaction with α1PI, in good agreement with the amount observed. Addition of an HNE-specific tetrapeptide chloromethyl ketone reagent (2 mM methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone) to our gel system. The cleavage and release of homologous carboxyl-terminal peptides from other SDS-stable serpin-proteinase complexes have been convincingly shown in numerous other studies (8, 16, 18, 20–22, 38).

In summary, the present studies together with past findings demonstrate the critical importance of the active site catalytic serine residue of the proteinase in the formation of high affinity covalent complexes with serpins. This characteristic feature of the interactions of serpin family inhibitors with their target proteinases sets them apart from the nonserpin protein proteinase inhibitors, which are known to form tight noncovalent complexes with both active proteinases and catalytically inactive anhydropriotetineases (40, 41). Our observations thus support the growing body of evidence that the inhibitory mechanism of serpins differs fundamentally from that of the nonserpin protein proteinase inhibitors. Indeed, a consensus view that has been developing is that serpins behave as suicide inhibitors, i.e. they are activated by proteinase-mediated cleavage of the serpin as a normal substrate to trap their target proteinases through a major serpin conformational change (2).

REFERENCES
1. Petopa, J., Korzus, E., and Travis, J. (1994) J. Biol. Chem. 269, 15957–15960
2. Gettins, P. G. W., Paterson, P. A., and Olson, S. T. (1996) Serpins: Structure, Function, and Biology, R. G. Landes Co., Austin, TX
3. Luckowski, M. A., and Olson, S. T. (1980) Annu. Rev. Biochem. 49, 593–626
4. Bode, W., and Huber, R. (1992) Eur. J. Biochem. 204, 433–451
5. Rosenberg, R. D., and Damus, P. S. (1973) J. Biol. Chem. 248, 6490–6505
6. Moroi, M., and Yamashiki, M. (1974) Biochim. Biophys. Acta 359, 130–141
7. Moroi, M., and Aoki, N. (1977) Biochim. Biophys. Acta 476, 412–420
8. Wiman, B., and Collen, D. (1979) J. Biol. Chem. 254, 9291–9297
9. Olson, S. T., Bock, P. E., Krassman, J., Shore, J. D., Lawrence, D. A., Ginsburg, D., and Bjork, I. (1995) J. Biol. Chem. 270, 30007–30017
10. Engh, R. A., Wright, H. T., and Huber, R. (1990) Protein Eng. 3, 469–477
11. Schulze, A. J., Baumann, U., Knof, S., Jaeger, E., Huber, R., and Laurell, C. B. (1990) Eur. J. Biochem. 194, 51–58
12. Skriver, K., Wiktorski, W. H., Paterson, P. A., Tausk, F., Schapira, M., Kaplan, A. P., and Bock, S. C. (1991) J. Biol. Chem. 266, 9216–9221
13. Carrell, R. W., Evans, D. L., and Stein, P. E. (1991) Nature 353, 576–578
14. Bjork, I., Vlunenjarvi, K., Olson, S. T., and Bock, P. E. (1995) J. Biol. Chem. 267, 1976–1982
15. Bjork, I., Nordin, K., and Olson, S. T. (1993) Biochemistry 32, 6501–6505
16. Moroi, M., Odani, S., and Ikezaka, T. (1977) J. Biochem. (Tokyo) 86, 915–921
17. Nilsson, T., and Wiman, B. (1982) FEBS Lett. 142, 111–114
18. Ferguson, W. S., and Finlay, T. H. (1983) Arch. Biochem. Biophys. 220, 301–308
19. Matheson, N. R., van Halbeek, H., and Travis, J. (1991) J. Biol. Chem. 266, 13489–13491
20. Lawrence, D. A., Ginsburg, D., Day, D. E., Berkenpas, M. B., Verhamme, I. M., Raes, P., Lauridsen, J. D. (1990) J. Biol. Chem. 270, 25309–25312
21. Wilcynska, M., Fa, M., Ohlsson, P., and Ny, T. (1995) J. Biol. Chem. 270, 29652–29655
22. Strmigoli, M., Karlsson, K.-E., Björquist, P., Andersson, J.-O., Bystrom, M., Hansson, L., Johansson, T., and Deum, J. (1996) Biochim. Biophys. Acta 1295, 103–109
23. Christensen, S., Valnickova, Z., Thagerson, I. B., Pazzo, S. V., Nielsen, H. R., Roepstorff, P., and Engvild, J. J. (1995) J. Biol. Chem. 270, 14859–14862
24. Cohen, A. B. (1973) J. Biol. Chem. 248, 7055–7059
25. Moroi, M., Yamashiki, M., and Aoki, N. (1975) J. Biochem. (Tokyo) 78, 925–928
26. Glaser, C. B., Bridrick, J. W., Drechsel, D., Karic, L., Graceffo, M., and Largman, C. (1982) Biochemistry 21, 556–561
27. Lijnen, H. R., Van Hoef, B., and Collen, D. (1991) J. Biol. Chem. 266, 4041–4044
28. Yung, B. Y. K., and Trowbridge, C. G. (1980) Biochim. Biophys. Acta 629, 412–420
29. Melhado, L. L., Peltz, S. W., Leytus, S. P., and Mangel, W. F. (1982) J. Am. Chem. Soc. 104, 7299–7306
30. Robinson, N. C., Tye, R. W., Neurath, H., and Walsh, K. A. (1971) Biochemistry 10, 2743–2747
31. Bloom, J. W., and Hunter, M. J. (1978) J. Biol. Chem. 253, 547–589
32. Harper, J. W., Henni, K., and Powers, J. C. (1980) Biochemistry 19, 1831–1841
33. Olson, S. T., Björk, I., and Shore, J. D. (1993) Methods Enzymol. 225, 525–560
34. Tian, W. X., and Tsou, C. L. (1982) Biochemistry 21, 1028–1032
35. Laemmli, U. K. (1970) Nature 227, 680–685
36. Schägger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
37. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
38. Björk, I., Jackson, C. M., Jornvall, H., Gräf, M., and Largman, C. (1982) Biochemistry 21, 9724–9730
39. Enghild, J. J., Valnickova, Z., Thogersen, I. B., and Pizzo, S. V. (1994) J. Biol. Chem. 269, 20159–20166
40. Ako, H., Foster, R. J., and Ryan, C. A. (1974) Biochemistry 13, 132–139
41. Vincent, J., Peron-Renner, M., Pudles, J., and Ladzunski, M. (1974) Biochemistry 13, 4205–4211