The Inhibition Mechanism of Serpins

EVIDENCE THAT THE MOBILE REACTIVE CENTER LOOP IS CLEAVED IN THE NATIVE PROTEASE-INHIBITOR COMPLEX*

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Inhibitors that belong to the serine protease inhibitor or serpin family have reactive centers that constitute a mobile loop with P1-P1' residues acting as a bait for cognate protease. Current hypotheses are conflicting as to whether the native serpin-protease complex is a tetrahedral intermediate with an intact inhibitor or an acyl-envelope complex with a cleaved inhibitor P1-P1' peptide bond. Here we show that the P1' residue of the plasminogen activator inhibitor type 1 mutant (P1' Cys) became more accessible to radiolabeling in complex with urokinase-type plasminogen activator (uPA) compared with its complex with catalytically inactive anhydro-uPA, indicating that complex formation with cognate protease leads to a conformational change whereby the P1' residue becomes more accessible. Analysis of chemically blocked NH₂ termini of serpin-protease complexes revealed that the P1-P1' peptide bonds of three different serpins are cleaved in the native complex with their cognate protease. Complex formation and reactive center cleavage were found to be rapid and coordinated events suggesting that cleavage of the reactive center loop and the subsequent loop insertion induce the conformational changes required to lock the serpin-protease complex.

The serine protease inhibitor, or serpin, superfamily, is a family of structurally related proteins that controls many physiological reactions and includes most of the protease inhibitors in blood (1–4). The serpins have a unique inhibitory specificity, but they share a common molecular architecture based on a dominant five-stranded β-sheet (5). In intact serpins, the reactive center constitutes an exposed mobile loop with the reactive center constitutes an exposed mobile loop with the subsequent loop insertion induce the conformational changes required to lock the serpin-protease complex.

declared forms of serpins the P1 and P1' residues are located on opposite ends of the molecule indicating that reactive center loop cleavage results in insertion of the reactive center loop into the A β-sheet of the molecule (5). Structural data for native serpins complexed to protease are lacking, and the mechanisms by which serpins inhibit their target proteases remain unclear. Existing hypotheses (4, 7–12) assume that serpins contain an exposed reactive center loop but vary in the way they predict how the reactive center loop is held in an inhibitory conformation. To acquire a canonical shape necessary for effective inhibition, the reactive center loop appears to partially insert itself into the gap between strands 3 and 5 of the A β-sheet (2, 10, 13). This insertion seems to be essential for inhibitor function but may not be required for protease recognition (14). After docking with enzyme, the reactive center loop is presumed to insert further into the A β-sheet forming a structure that fits the substrate binding site of the target protease and thereby achieving tight binding and inhibition (3, 15).

Analysis of serpin-protease complexes by SDS-PAGE reveals the presence of a complex stabilized by an acetyl-ester linkage between the carboxyl of the P1 residue of the cleaved serpin and the active site serine of the protease. However, since denaturation may alter the stability of the complex and shift the reaction toward cleavage this does not prove the existence of a cleaved serpin in the native serpin-protease complex. Consistent with this hypothesis, the complex between α₂-antiplasmin (α₂AP) and non-cognate protease trypsin could dissociate to give active inhibitor and enzyme (7), and a tetrahedral intermediate formed during complex formation between porcine pancreatic elastase (PPE) and human α₁-antitrypsin (α₁AT) was detected by NMR (16). Based on these studies it has been concluded that complex formation stops at the tetrahedral intermediate stage with a non-cleaved inhibitor and that the acetyl-ester linkage detected after treatment of complexes with SDS is an artifact (4, 7, 16). However, other data suggest that the inhibitor is cleaved in the native serpin-protease complex. Thus serpin-protease complexes have a biological activity similar to cleaved serpin (8, 13), and attempts to detect intact antichymotrypsin after dissociation of the complex with chymotrypsin were unsuccessful (17).

By using fluorescence spectroscopy, we and others have shown that the reactive center of PAI-1 undergoes conformational changes following the interaction with target proteases (18–21). Our time-resolved fluorescence spectroscopy studies revealed that the orientational restriction of a fluorescent probe attached to the P1' residue decreased following complex formation with plasminogen activators (PAs) whereas it increased following complex formation with proteolytically inactive anhydro-uPA, indicating that the P1' residue became more flexible after complex formation with active protease. Although these data suggest that the P1-P1' bond of PAI-1 is cleaved in the complex with PAs (19, 20), fluorescence studies only provide indirect evidence. In this study we provide direct evidence that the reactive center loop in PAI-1 as well as in two other serpins is cleaved in the native complex with their cognate proteases.

EXPERIMENTAL PROCEDURES

Proteins—The PAI-1 mutant (P1' Cys) containing a cysteine residue at the P1' position of the reactive center loop (20) and wild-type PAI-1 were purified from Escherichia coli (22) followed by affinity chromatography on anhydro-trypsin-Sepharose (Pierce) to obtain fully active inhibitor (20). Purification of recombinant eucaryotic PAI-1 (fully active)
was described as described (23). Uklidan (uPA) was from Serono S. A. Tissue-type PA (tPA, Actilyse®) was from Boehringer Ingelheim. Human α1-AT and PPE were from Calbiochem. Human α2-AP and plasmin were from Biopool.

Labeling of P1’ Cys with [14C]iodoacetamide and Complex Formation with uPA—Fully active P1’ Cys (0.3 mg) was mixed with 10 μCi of [14C]iodoacetamide (Amersham Corp.) in 1 ml of phosphate-buffered saline (50 mM sodium phosphate buffer, pH 5.6, 0.14 mM NaCl, 1 mM EDTA, 0.01% Tween 80) containing 0.5 mM Arg. After 4 h at 4°C, excess free radioactive ligand was removed by gel filtration on an NAP-25 column (Pharmacia Biotech Inc.) equilibrated with TBS (50 mM Tris/HCl, pH 7.4, with 0.14 mM NaCl, 1 mM EDTA, 0.01% Tween 80). Labeled P1’ Cys was mixed with 1.2 mM excess of uPA in TBS at 23°C; at different times 20-μl samples were transferred to 20 μl of SDS-sample buffer kept at 95°C. After 15 min the samples were analyzed by SDS-PAGE (3–20%), followed by autoradiography or Western blotting.

Release of Labeled Postcomplex Fragment (PCF) from the [14C]P1’ Cys-uPA Complex—To determine the requirements for release of PCF from the PAI-1-uPA complex, the complex between [14C]P1’ Cys and uPA in TBS was incubated at different conditions, mixed with sample buffer (24) at 23°C, and analyzed by SDS-PAGE (3–20%). The amount of released PCF was quantified using PhosphorImager (Molecular Dynamics) and calculated as a percent of total radioactivity applied.

Iodoacetamide Labeling of P1’ Cys in Native Complex with uPA or Anhydro-uPA—Anhydro-uPA, prepared according to Wu et al. (25), and uPA were bound to CNBr-Sepharose according to the manufacturer’s instructions (Pharmacia). Complexes were formed by incubation of the immobilized enzymes with excess of P1’ Cys in TBS at 23°C for 30 min. Subsequently, non-complexed P1’ Cys was removed by extensive washing of the Sepharose beads with TBS. The amount of P1’ Cys bound to the immobilized enzymes (calculated as the amount added minus the amount eluted during washing) was 47, 80, and 85 μg/ml uPA-Sepharose and 77, 120, and 123 μg/ml anhydro-uPA-Sepharose. To label the P1’ Cys residue in the immobilized complexes, 100 μl of Sepharose beads was incubated with 0.6 μCi of [14C]iodoacetamide in 300 μl of TBS at 23°C. After different time points the reaction was terminated by extensive washing with TBS until all non-bound [14C]iodoacetamide was removed. To dissociate the immobilized complexes, 100 μl of 0.1% SDS in TBS was added, and the samples were boiled for 15 min. The radioactivity in the supernatants was determined by liquid scintillation counting or following SDS-PAGE (3–20%) by PhosphorImager.

Blocking of NH2-terminal Groups Present in Native Serpin-Protease Complexes Followed by NH2-terminal Sequence Analysis—All procedures were performed under native conditions: in 0.2 M sodium phosphate buffer, pH 7.4, at 23°C. The complexes between PA and recombinant procaryotic or eucaryotic PAI-1 were formed by incubation for 20 min. The α1-AT-PPE and α2-AP-plasmin complexes were formed by incubation for 2 and 5 min, respectively, after which 1 mM phenylmethylsulfonyl fluoride was added. When necessary, the complexes were purified from excess BODIPY FL C3 by gel filtration on NAP-25 columns (Pharmacia). The blocked and non-blocked-complex-containing samples were absorbed to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), and NH2-terminal sequence analyses were performed on a pulsed liquid phase sequencer (Applied Biosystems, Foster City, CA). PAI-1 and uPA purified on BODIPY FL C3 could form a complex to the same degree as untreated proteins suggesting that attachment of BODIPY FL C3 does not alter the native conformation of these proteins or the complex. The calculated mean values of the blocked NH2 terminus were comparable whether the calculation was based on sequence analysis or the absorbance of BODIPY FL C3, indicating that BODIPY FL C3 mainly reacted with NH2-terminal groups present in the complex.

General Methods—Protein concentrations were determined using the BCA protein assay (Pierce). Electrophoresis was carried out according to Laemmli (24) and Western blotting according to Towbin et al. (26). The

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**RESULTS AND DISCUSSION**

The Accessibility of the P1’ Residue of PAI-1 Increases following Interaction with uPA—If PAI-1 is cleaved in the native complex with cognate protease it should be possible to detect the 33-residue-long COOH-terminal so-called PCF that is produced following reactive center cleavage. To study the formation of the PCF we utilized the PAI-1 mutant (P1’ Cys), which has similar characteristics as wild-type PAI-1 (20). Following labeling of the unique P1’ cysteine with [14C]iodoacetamide the kinetics of complex formation and PCF release were studied by SDS-PAGE followed by Western blotting (Fig. 1A) and autoradiography (Fig. 1B). As shown in Fig. 1, P1’ Cys-uPA complex as well as the presence of PCF can be detected after 3 s of incubation. Later, when most PAI-1 was in complex with uPA, the inhibitory activity of PAI-1 and P1’ Cys mutant was analyzed by chromogenic activity assay with substrate 2444 (Chromogenic AB) (22).

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**Fig. 1. Time course of complex formation between [14C]-labeled P1’ Cys and uPA.** [14C]iodoacetamide-labeled P1’ Cys was mixed with uPA, and at different time points complex formation was stopped. The samples were analyzed by SDS-PAGE (3–20%) followed by Western blotting with polyclonal antibodies against PAI-1 (A) or autoradiography (B). Lane 1, radiolabeled P1’ Cys incubated with inactivated uPA; lanes 2–6, [14C]P1’ Cys was mixed with active uPA for different times. The minor 80-kDa band shown in lane 1 represents dimers of P1’ Cys. Arrows indicate the mobility of prestained molecular weight standards.
or anhydro-uPA and P1’ Cys were cleaved. After complex formation the P1’ cysteine residues were specifically labeled with [14C]iodoacetamide. As shown in Fig. 2, inset, only intact radiolabeled inhibitor was released from the complex with anhydro-uPA (lane 1) whereas radiolabeled PCF was released from the complex with uPA (lane 2). Fig. 2 demonstrates that the labeling of the P1’ Cys residue was 3-4 times higher for the complex with uPA than with anhydro-uPA indicating that complex formation with uPA leads to a conformational change of the reactive center whereby P1’ becomes more accessible for labeling.

Detection and Blockage of the Free NH2-terminal End of PCF in Native Serpin-Protease Complexes—Sequence analysis of serpin-protease complexes has revealed the existence of the NH2-terminal end of the PCF (12, 27); however, the observed cleavage could be a result of harsh conditions during NH2-terminal sequence analysis. The data in Fig. 2 suggest that P1’ is fully accessible in the native complex. Provided serpins are cleaved in the native complex it should therefore be possible to block the NH2-terminal end of the PCF to the same extent as other NH2-terminal ends present in the complex. The PAI-1-uPA, PAI-1-tPA, α2-AP-PPE, and α2-AP-plasmin complexes were formed, and half of each complex sample was treated with BODIPY FL C3 to partially block NH2-terminal groups. Edman degradation of both non-blocked and blocked complexes revealed the amino acid residues that would be expected if inhibitors were cleaved in the reactive center. Table I summarizes the results of blockage experiments. In chemically blocked samples, the NH2-terminal end of the PCF was blocked to the same degree as other NH2-terminal ends, indicating the presence of PCF in the native complex and reactive center cleavage. In the PAI-1-PA complexes, the PCF of PAI-1 was blocked to the same degree as the other NH2-terminal ends irrespective of the cognate protease (uPA or tPA) or PAI-1 source (procaryotic or eucaryotic). This indicates that inhibitor cleavage is the general mechanism by which PAI-1 inhibits PAs and excludes the possibility that lack of carbohydrates on procaryotic derived PAI-1 may be the reason for PAI-1 cleavage. The NH2-terminal end of the PCF was also blocked in native α2-AT-PPE and α2-AP-plasmin complexes, indicating that inhibitors are cleaved also in other native serpin-protease complexes and that cleavage is not a result of Edman degradation chemistry. To determine whether only portions of the inhibitors are cleaved in the complexes we increased the efficiency of blockage to about 90% in some experiments with α2-AP and α2-AT. As shown in Table I, the NH2-terminal ends of the corresponding PCF were also blocked to about 90%. In one experiment all the four NH2-terminal ends of a PAI-1-uPA complex were blocked to 100% (data not shown). Although we cannot exclude the possibility that the native complex contains a minor portion of uncleaved inhibitor, these data suggest that essentially all of the inhibitor is cleaved. Except for the NH2-terminal end of PPE and catalytic chain of tPA, all NH2-terminal ends in the native complexes were found to be blocked to the same degree, indicating that they are accessible to blocking reagent. The reduced blocking efficiency of the NH2-terminal end of PPE and the catalytic chain of tPA indicate that these ends are not fully accessible for the blocking reagent, possibly because of steric hindrance.

In conclusion, our data reveal that three different serpins are cleaved in their native complexes with cognate proteases, suggesting that complex formation stops at a stage after reactive center cleavage. In the case of PAI-1, we show that complex formation and reactive center cleavage are rapid and coordinated events. Our data therefore support models where serpins act as suicide inhibitors, and the complex is arrested as an acyl-enzyme intermediate or possibly, following addition of a water molecule, as a second tetrahedral intermediate. Taking our findings into account, we suggest the following simple scheme for the reaction of a serpin (I), with a target protease (E).

\[
E + I \rightarrow E-I \rightarrow E-I^* \rightarrow E-I^* \rightarrow E + I^*
\]

**Scheme 1**

In this scheme E1 is an initial encounter (Michaelis) complex, and [E1] is a very short lived tetrahedral intermediate that is

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**Table 1**

Blockage of NH2-terminal ends present in native serpin-serine protease complexes

| NH2-terminal end                        | Percentage of blocked NH2-terminal ends |
|----------------------------------------|----------------------------------------|
| Procaryotic PAI-1-uPA complex          |                                   |
| Eucaryotic PAI-1-uPA complex           |                                   |
| Eucaryotic PAI-1-tPA complex           |                                   |
| α2-AP-plasmin complex                  |                                   |
| α2-AT-PPE complex                      |                                   |
| Original NH2-terminal end of inhibitor | 50 ± 6 63 24 86 46 96               |
| PCF of inhibitor                       | 44 ± 5 51 26 90 36 87               |
| Non-catalytic chain of enzyme          | 53 ± 16 53 35 90                   |
| Catalytic chain of enzyme              | 49 ± 9 53 6 93 3 17                |

* Represents the mean value ± S.D. of three independent experiments.

b Data from two experiments using 0.17 and 0.9 mM blocking agent, respectively.
rapidly converted to $E_1^\alpha$, an acyl-enzyme intermediate containing cleaved inhibitor. The increased flexibility (20) and accessibility (Fig. 2) of the NH$_2$-terminal end of the PCF following cleaved inhibitor. The increased flexibility (20) and accessibility (Fig. 2) suggest that it may be released from the reactive center pocket. If this conformational change creates room for a water molecule to enter, the reaction could continue to a second tetrahedral intermediate, $E_1^\alpha$. If this second tetrahedral intermediate is stable (28, 29) it may represent the tetrahedral intermediate detected by Matheson et al. (16). The reaction between serpin and protease may therefore very much resemble the reaction between protease and substrate with the exception that the deacylation and release of the cleaved inhibitor are greatly retarded. Previous studies indicate that a mobile reactive center is required for inhibitory function and that hindrance of mobility converts the inhibitor to a substrate (9, 14, 30). Taken together with our present finding, i.e. that serpins are cleaved in their native complexes with target proteases, these data suggest that cleavage of the reactive center loop by protease and the subsequent loop insertion induce the conformational changes required to lock the inhibitor-protease complex. This model is compatible with our previously proposed model for serpin function (12).

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