An Ester Bond Linking a Fragment of a Serine Proteinase to Its Serpin Inhibitor†

Rikke Egelund,‡ Kees W. Rodenburg,‡ Peter A. Andreasen,*,‡ Margit S. Rasmussen,§ Roy E. Guldberg,§ and Torben E. Petersen§

Laboratory of Cellular Protein Science and Protein Chemistry Laboratory, Department of Molecular and Structural Biology, University of Aarhus, 10 Gustav Wied's Vej, 8000 C Aarhus, Denmark

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ABSTRACT: Most known members of the serpin superfamily are serine proteinase inhibitors. Serpins are therefore important regulators of blood coagulation, complement activation, fibrinolysis, and turnover of extracellular matrix. Serpins form SDS-resistant complexes of 1:1 stoichiometry with their target proteinases by reaction of their P_1-P_1' peptide bond with the active site of the proteinases. The nature of the interactions responsible for the high stability of the complexes is a controversial issue. We subjected the complex between the serine proteinase urokinase-type plasminogen activator (uPA) and the serpin plasminogen activator inhibitor-1 (PAI-1) to proteolytic digestion under nondenaturing conditions. The complex could be degraded to a fragment containing two disulfide-linked peptides from uPA, one of which included the active site Ser, while PAI-1 was left undegraded. By further proteolytic digestion after denaturation and reduction, it was also possible to degrade the PAI-1 moiety, and we isolated a fragment containing 10 amino acids from uPA, encompassing the active site Ser, and 6 amino acids from PAI-1, including the P_1 Arg. Characterization of the fragment gave results fully in agreement with the hypothesis that it contained an ester bond between the hydroxyl group of the active site Ser and the carboxyl group of the P_1 Arg. These data for the first time provide direct evidence that serine proteinases are entrapped at an acyl intermediate stage in serine proteinase−serpin complexes.

The serpins constitute a family of extracellular glycoproteins from animals, plants, and viruses. Most known members of the serpin superfamily are serine proteinase inhibitors (*1*). The first amino acid sequence of a serpin serine proteinase inhibitor to be reported was that of antithrombin III (*2*). The sequence showed no similarity to the sequences of the by that time well-known small "standard-mechanism" inhibitors. Therefore, the sequence alone gave no clues with respect to the inhibition mechanism. Since then, biochemical studies have shown that serpin serine proteinase inhibitors form SDS-resistant complexes with a 1:1 stoichiomtetry with their target proteinases by reaction of their P_1-P_1' bond (reactive center peptide bond) with the active site of the proteinases (*1*). The X-ray crystal structures of serpins have shown that they are globular proteins with nine α-helices and three β-sheets. The P_1-P_1' bond is localized in a surface-exposed, approximately 20 amino acid long peptide loop, the reactive center peptide loop (the RCL). The RCL is linked C-terminally to strand 1 of β-sheet C and N-terminally to strand 5 of β-sheet A (*3*). Serpins are able to undergo large conformational changes, of which the most conspicuous one is brought about by proteolytic cleavage of the RCL, either with nontarget proteinases or by cleavage of P_1-P_1' at in vitro dissociation of serpin−target proteinase complexes. The cleavage leads to insertion of the part of the RCL N-terminal to the cleavage site as strand 4 in β-sheet A. Cleaved serpins have a higher thermodynamic stability than native serpins (*3*). At least partial strand insertion also occurs during complex formation (*4*). However, the three-dimensional structure of a serine proteinase−serpin complex has not yet been determined, and despite many biochemical studies (*1*), the state of the P_1-P_1' bond in the complex has remained controversial.

We have now employed a direct protein chemical approach for characterization of the association between the active site of the proteinase and the P_1-P_1' bond of the serpin.

MATERIALS AND METHODS

Materials. Human uPA[1] was purchased from Wakamoto Pharmaceutical Co. (Tokyo, Japan). Anhydro-uPA was prepared as described by Wun et al. (*5*). Latent human PAI-1 was purified from serum-free conditioned medium of dexamethasone-treated HT-1080 cells (*6*, *7*). Latent PAI-1 was converted into the active conformation by denaturation with guanidinium chloride, followed by extensive dialysis against 10 mM NaH_2PO_4, pH 7.4, 140 mM NaCl (*7*). To produce PAI-1 in complex with uPA, guanidinium chloride-activated PAI-1 was incubated with an equal amount of uPA for 90 min at 37 °C in 10 mM NaH_2PO_4, pH 7.4, 140 mM NaCl. The complex was separated from noncomplexed uPA and PAI-1 by immunoaffinity chromatography on two different Sepharose-4B columns, one coupled with an anti-human

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* Corresponding author. Telephone: +45 8942 5080. Fax: +45 8612 3178. E-mail: pa@mbio.aau.dk.

‡ Laboratory of Cellular Protein Science.

§ Protein Chemistry Laboratory.

[1] Abbreviations: PAI-1, type-1 plasminogen activator inhibitor; RCL, reactive center loop; uPA, urokinase-type plasminogen activator.

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PAI-1 monoclonal antibody, the other coupled with an anti-human uPA monoclonal antibody (*8*). Alternatively, the complex was isolated from uncomplexed uPA and PAI-1 by gel filtration on a Superdex 200 column in 0.1 M Tris, pH 8.1, 0.5 M NaCl.

Subtilisin Carlsberg was purchased from Sigma (St. Louis, MO) and pepsin from Worthington (Lakewood, NJ).

Subtilisin Digestion of uPA−PAI-1 Complex. uPA−PAI-1 complex (20 μg/mL) was incubated with subtilisin (1 μg/mL) at 37 °C for 5 h in a buffer of 0.1 M Tris, pH 8.1, 0.2% Triton X-100 or in 25 mM Tris, pH 8.1. The product obtained by the digestion was characterized by SDS−PAGE, by gel filtration on a Superdex 200 column in 0.1 M Tris, pH 8.1, 0.5 M NaCl, and by further digestion after denaturation (see below).

Electrophoresis. SDS−PAGE was performed as described previously (*9*). For N-terminal sequencing of peptides in the gels, the peptides were transferred electrophoretically to poly(vinylidene difluoride) filters, and sequenced directly from the filters on an Applied Biosystems Model 477A gas-phase sequencer.

Reduction, [^{14}C]Iodoacetic Acid Labeling, and Pepsin Digestion of Subtilisin-Digested uPA−PAI-1 Complex. After subtilisin digestion, the complex was precipitated by the addition of ice-cold trichloroacetic acid to a final concentration of 7% (w/v). The precipitate was washed with ice-cold acetone and dried. The trichloroacetic acid-precipitated subtilisin digest was dissolved in 6 M guanidinium chloride, 0.3 M Tris, pH 8.0. Dithioerythritol was added to 1.4 mM. The mixture was allowed to stand for 30 min at room temperature. Then [^{14}C]iodoacetic acid (100 μCi per 1 mg of subtilisin digest) was added. After 15 min, an excess of nonradioactive iodoacetic acid was added. This was also allowed to stand for 15 min before desalting on a Sephadex G25 column into 5% formic acid. The radioactive labeling was measured with a liquid scintillation counter. The reduced and radioactively labeled sample (250 μg/mL) was incubated with pepsin (13 μg/mL) for 3 h in 5% formic acid at room temperature. The digestion was stopped by freeze-drying.

Reverse-Phase HPLC. The freeze-dried pepsin-digested uPA−PAI-1 complex was dissolved in 0.1% trifluoroacetic acid and immediately subjected to a reverse-phase HPLC Vydac C_{18} column (4 × 250 mm; solvent A: 0.1% trifluoroacetic acid; solvent B: 80% acetonitrile, 0.1% trifluoroacetic acid; flow rate: 0.85 mL/min). The effluent was monitored at 226 nm, and fractions were collected manually according to absorbance. All pools were counted for radioactivity. Selected ones were further purified with a micro-HPLC (SMART System, Pharmacia Biotech, Sweden), equipped with a μRPC C_2/C_{18} SC 2.1/10 column (solvent A: 0.05% heptafluorobutyric acid; solvent B: 80% acetonitrile, 0.05% heptafluorobutyric acid; flow rate: 150 μL/min). All radioactive pools were subjected to N-terminal amino acid sequence analysis. The retention times in Table 1 were determined on the SMART System using trifluoroacetic acid as carrier (solvent A: 0.1% trifluoroacetic acid; solvent B: 80% acetonitrile, 0.1% trifluoroacetic acid; flow rate: 150 μL/min; temperature: 29 °C; gradient: 0.00 min 0% B, 7.00 min 0% B, 37 min 60% B, 42 min 100% B).

Mass Spectrometry. Mass data were obtained with a MALDI-TOF instrument (Biflex, Bruker), operated in reflected mode with α-cyano-4-hydroxycinnamic acid (Sigma) as matrix.

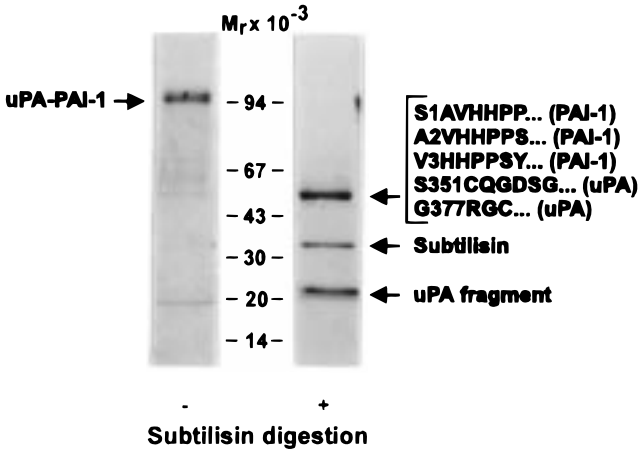

FIGURE 1: Subtilisin cleavage of uPA−PAI-1 complex. uPA−PAI-1 complex, with or without subtilisin digestion as indicated, was analyzed by SDS−PAGE under nonreducing conditions. The protein bands were identified by N-terminal sequence analysis after transfer to poly(vinylidene difluoride) filters. The migration of M_r markers is shown in the center, and the characteristics of the identified fragments to the left and to the right. The PAI-1 residue numbering is according to Andreasen et al. (*24*). The second uPA sequence (G377RGC...) was not detected after reduction (data not shown). The PAI-1 sequences result from heterogeneity in the N-terminal of PAI-1 (*24*) and from cleavage after the first residue by subtilisin.

RESULTS

We previously mapped the susceptibility of PAI-1 to nontarget proteinases under nondenaturing conditions and found that complex formation of PAI-1 with uPA renders it totally resistant to subtilisin digestion (*9*). However, under the same conditions, free uPA is subtilisin resistant (R. Egelund, unpublished results), but complex formation with PAI-1 renders it highly susceptible to subtilisin. Subtilisin digestion of the complex yielded a molecule consisting of a small fragment of uPA attached to undigested PAI-1. This molecule could be separated from native complex by SDS−PAGE (Figure 1) as well as by gel filtration on a Superdex 200 column under nondenaturing conditions (data not shown). N-Terminal sequence analysis of the subtilisin-treated complex revealed two uPA sequences linked by a disulfide bond, one of them encompassing the active site Ser (Figure 1).

This digestion pattern was observed whether the complex had been separated from remaining unreacted uPA and PAI-1 by the use of immunoaffinity chromatography or by gel filtration. In agreement with previous reports (*5*, *10*), no SDS-resistant complexes were formed between PAI-1 and anhydro-uPA, in which the hydroxyl group af the active site Ser had been removed chemically (data not shown), demonstrating that the hydroxyl group is involved in formation of the SDS-resistant complex.

To determine the mode of attachment of the short uPA fragment to PAI-1 in the subtilisin-digested uPA−PAI-1 complex, we took advantage of the absence of Cys residues in PAI-1 and the presence of a disulfide bond in the subtilisin-digested complex. The subtilisin-digested complex was denatured, reduced, and labeled with [^{14}C]iodoacetic acid. The radioactive label allowed us to detect peptides

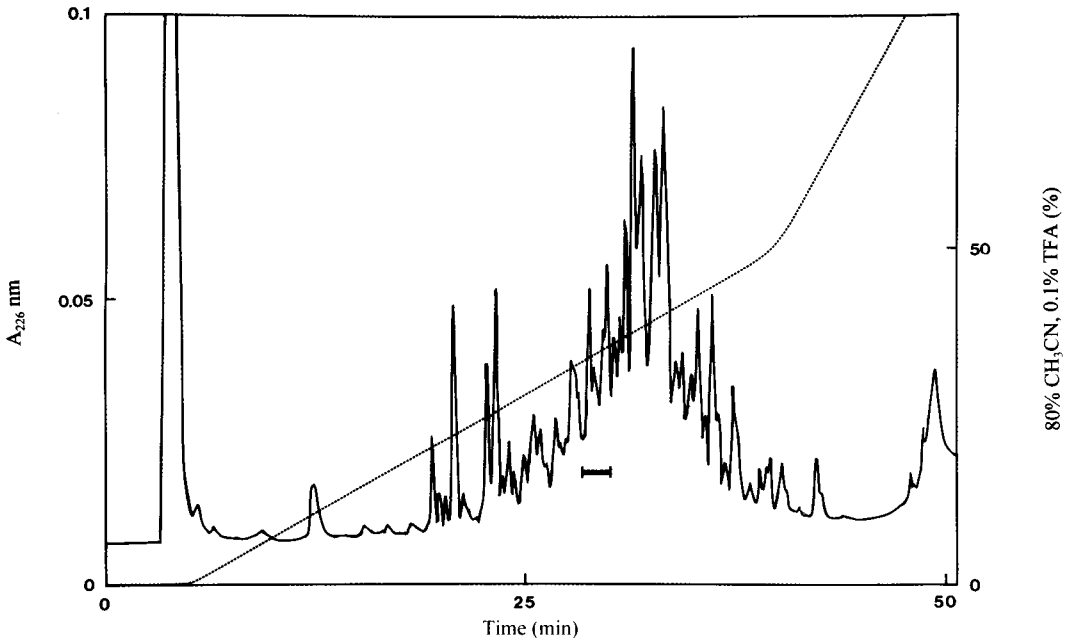

FIGURE 2: HPLC profile of the pepsin digest. The bar indicates the pool containing the uPA−PAI-1 fragment.

Table 1: Analysis of the uPA−PAI-1 Fragment and the Derived Peptides by HPLC and Mass Spectrometry[a]

	uPA−PAI-1 fragment	uPA peptide (residues 351−360)	PAI-1 peptide (residues 343−348)
HPLC retention time (min)	22.99	19.65	18.55
m/z, calculated	1605.8	980.0	643.8
m/z, found	1605.6	–	–
m/z, found after base hydrolysis	–	979.8	643.9
m/z, found after trypsin digestion	–	979.7	643.8
m/z, calculated for base hydrolysis in $H_2{}^{18}O$	–	980.0	645.8
m/z, found after base hydrolysis in $H_2{}^{18}O$	–	979.9	645.4

[a] HPLC retention times were determined at the conditions described under Materials and Methods. The calculated *m/z* value for the uPA−PAI-1 fragment corresponds to the mass of the uPA peptide plus the mass of the PAI-1 fragment minus the mass of a water molecule. Calculated and found *m/z* values are average ones. The PAI-1 residue numbering is according to Andreasen et al. (*24*).

containing uPA sequences after pepsin degradation and fractionation by HPLC (Figure 2). All radioactive pools were further fractionated with a micro-HPLC system. One radioactive pool was identified which showed a double sequence corresponding to residues 351−360 of uPA and residues 343−348 of PAI-1. This double sequence was obtained from a single peak using two different solvent systems, suggesting that the two peptides were covalently linked. Mass spectrometry analysis of the fragment resulted in a single peak with an *m/z* value corresponding to the mass of the two peptides minus the mass of a water molecule, proving that the two peptides were indeed covalently attached (Table 1).

Under the conditions used for complex formation, there is only one possible mode in which the hydroxyl group of the active site Ser can form an anhydride between the identified sequences, namely, by an ester bond to the P_1 Arg of PAI-1. To verify the presence of an ester bond, we subjected the fragment to hydrolysis under mildly basic conditions, monitoring the reaction by HPLC. Incubation for 1 h at 37 °C in 0.01 M NaOH led to hydrolysis of 66% of the fragment, and incubation for 1 h in 0.02 M NaOH at 37 °C led to complete hydrolysis of the fragment. Two peptides were formed by the hydrolysis. They were purified by HPLC and their masses determined by mass spectroscopy. Their masses were in agreement with the expected masses of peptides corresponding to residues 351−360 of uPA and residues 343−348 of PAI-1 (Table 1). The rapid hydrolysis under these conditions was in agreement with an ester bond linking the two peptides. Also as expected for an ester bond involving the carboxyl group of an Arg residue, the same two peptides were obtained by treatment of the fragment with trypsin (Table 1). When the base hydrolysis was performed in the presence of water labeled with ^{18}O, the mass of the PAI-1 peptide increased 2 units, while the mass of the uPA peptide was unchanged (Table 1). These findings are in full agreement with the structure of the fragment being that shown in Figure 3, with an ester bond between the active site Ser of uPA and the P_1 Arg of PAI-1.

The overall molar yield of the pure fragment with the ester bond was 10 and 20%, respectively, in two independent runs. Considering the fact that the fragment was isolated after subtilisin digestion, trichloroacetic acid precipitation, redissolution, reduction, carboxymethylation, pepsin digestion, and two HPLC fractionations, this yield is not compatible with the ester bond being only a minor side product of the degradation of the complex. Moreover, N-terminal sequencing of peptides from throughout the HPLC profile shown in Figure 2 revealed no other peptides covering the active site of uPA or the reactive center of PAI-1. In particular, the positions in the HPLC profile in Figure 2 corresponding to the retention times of the uPA and PAI-1 peptides, respectively, were devoid of these sequences (data not shown). We

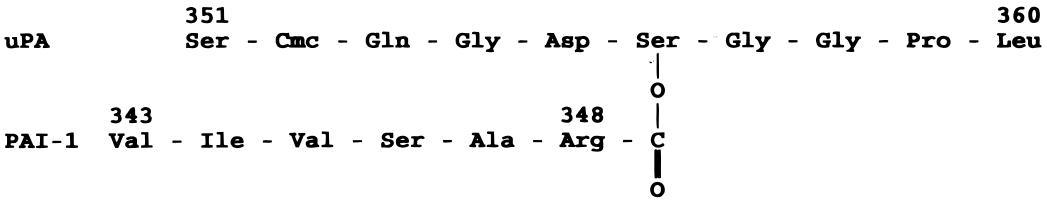

FIGURE 3: Structure of the uPA−PAI-1 fragment. Cmc, carboxymethylated Cys labeled with ^{14}C.

are therefore confident that the isolated fragment is representative of all molecules in the starting material.

DISCUSSION

The normal enzymatic cleavage of peptide bonds by serine proteinases is believed to proceed through the following steps: (1) formation of a noncovalent Michaelis complex; (2) formation of a tetrahedral transition state through nucleophilic attack of the C-atom of the carboxyl group of the P_1 residue by the Oγ active site Ser; (3) release of the amino group of the peptide bond and formation of an acyl intermediate; (4) formation of a second tetrahedral transition state through nucleophilic attack by a water molecule; (5) release of the carboxyl group of the peptide bond. Each of the steps requires the participation of the catalytic triad, the oxyanion hole, and the specificity pocket (*11*). Serpin inhibitors of serine proteinases are pseudosubstrates in the sense that the normal substrate pathway is not being completed, resulting in formation of a stable complex. But controversy has remained as to the step at which the reaction stops. Many investigations suggest that the reaction between serpins and their target proteinases proceeds through at least two steps. It is believed to be initiated by formation of a "docking", reversible, noncovalent Michaelis complex which does not require the active site Ser. This initial reaction may resemble binding of serine proteinases to standard mechanism inhibitors (*4*, *12*). The second, "locking" step involves at least some degree of insertion of the RCL as β-strand 4A (*4*).

Our data, for the first time demonstrating directly the presence of an ester bond in a fragment derived from a serine proteinase−serpin complex, are difficult to reconcile with a model with the stable complex being a reversible, noncovalent Michaelis complex with an intact reactive center peptide bond. Such a model is also in disagreement with reports of a free P_1' residue in native uPA−PAI-1 complex (*13*, *14*). Although the latter reports do not contain direct information about the state of the P_1 residue, both sets of data lend strong support to a model in which the locking step implies the reaction stopping at the acyl intermediate step. The breaking of the bond between the P_1 and P_1' residues will allow the serpin molecule to be stabilized by at least partial insertion of the N-terminal end of the RCL as β-strand 4A. With the covalent link between the active site Ser and the P_1 residue, this implies translocation of the proteinase toward the pole of the serpin molecule opposite to the initial localization of the RCL, across the plane of β-sheet A (*15*−*19*). The model is supported by the results of Stratikos and Gettins (*10*), using fluorescently labeled trypsin and α_1-proteinase inhibitor, and of Wilczynska et al. (*19*), studying PAI-1 and uPA using fluorescent labels on the P_1' and the P_3 residues in combination with chemical cross-linking. The two studies provided evidence for a considerable translocation of the proteinase during the locking step and a considerable separation of the P_1' and the P_3 residues.

It has been discussed whether denaturation of serine proteinase−serpin complexes, e.g., during SDS−PAGE, changes the mode of linkage between the serine proteinase and the serpin in the complex or not (*1*). However, for several reasons, we find it highly unlikely that the ester bond, which we here have demonstrated in the small fragment of the uPA−PAI-1 complex, should not be present already in the native uPA−PAI-1 complex, but should have been generated during the degradation and fractionation procedures which we used. It is important to note here that in our studies denaturating conditions were not employed until after the subtilisin digestion of the uPA−PAI-1 complex was completed. The subtilisin digestion led to degradation of the serine proteinase domain of uPA, the active site Ser remaining attached to PAI-1, but the parts containing the active site His and Asp and the specificity pocket dissociating. First, it seems highly unlikely that such degradation of the serine proteinase domain of uPA could lead to conversion of a putative initial noncovalent Michaelis complex to a covalently linked complex, or that the short uPA fragment formed by the digestion could be attached noncovalently to PAI-1. Second, a putative first tetrahedral intermediate (step 2 above) cannot exist after the subtilisin digestion, as stabilization of the tetrahedral intermediate requires multiple interactions between the inhibitor and the enzyme, including the binding of the P_1 Arg in the specificity pocket, which is not present after subtilisin digestion (*11*). In addition, conversion of a putative first tetrahedral intermediate to an ester bond is unlikely to happen during or after subtilisin digestion, as the only possible proton donor for leaving group protonation at neutral pH would seem to be the active site His residue, which is removed by the digestion. Moreover, the evidence for a considerable separation of the P_1' and the P_3 residues (*19*) and for the presence of a free P_1' residue (*13*, *14*) in the native complex, as referred to above, also seems to rule out the possibility of the complex containing the first tetrahedral intermediate. Third, it is even more unlikely that the treatments to which we have subjected the complex could lead to re-formation of an ester bond from the second tetrahedral intermediate, since this intermediate is formed in the first place through nucleophilic attack of the ester bond by a water molecule (step 4 above). Any conversions of this tetrahedral intermediate would rather lead to dissociation of serine proteinase and serpin. Our data are therefore in full agreement with the ester bond being present also in the native complex.

A challenge for the future is to understand the molecular mechanism for stabilization of the ester bond during the reaction of serine proteinases with serpins, in contrast to their reaction with substrates, in which the ester bond is rapidly hydrolyzed. One proposal was that the ester bond is being

placed in an anhydrous environment (*16*). An alternative proposal was that the energy released by insertion of the RCL as β-strand 4A drives distortion of the serine proteinase domain during formation of the locking complex, so that it becomes unable to catalyze the deacylation step. This could happen either by dislocation of the active site His, as suggested by a changed NMR signal from the His in chymotrypsin upon complex formation with α_1-antichymotrypsin (*20*), or by distortion of other parts of the active site, as suggested by an increased proteolytic susceptibility of chymotrypsin upon complex formation with α_1-antichymotrypsin (*21*), of trypsin upon complex formation with α_1-proteinase inhibitor (*22*, *23*), and of uPA upon complex formation with PAI-1 (R. Egelund, unpublished results). The proposed distortion could be brought about by the protease being fixed by interaction with adjacent structures of the serpin, so that the presence of the ester bond can lead to distortion of the active site. An understanding of the defects in the active sites of serine proteinases in their complexes with serpins would have important implications for further elucidation of the catalytic mechanisms of this class of enzymes.

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