Localization of ε-Lysyl–γ-Glutamyl Cross-Links in α_2-Macroglobulin–Plasmin Complex†

Linda Jacobsen and Lars Sottrup-Jensen*

Department of Molecular Biology, University of Aarhus, 8000 Århus C, Denmark

Received August 5, 1992; Revised Manuscript Received October 27, 1992

ABSTRACT: The major ε-lysyl–γ-glutamyl cross-links present in the largely covalent 1:1 α_2-macroglobulin-(α_2M)–plasmin complex have been localized. Cross-linking engaged Lys607, -708, and -750 of the plasmin light chain, Lys550, -556, and -557 of the heavy chain–light chain connecting strand, Lys258 and -298 of the kringle 3 region, and Lys473 of the kringle 5 region of the plasmin heavy chain. Lys607, -708, and -750 accounted for 75% of all cross-linking, Lys550, -556, and -557 accounted for 20%, and Lys258, -298, and -473 accounted for 5%. Hence, cross-linking engaged only nine of the 41 Lys residues of plasmin, showing that, probably due to their large size, individual plasmin molecules become deposited in the large elongated binding cavity in α_2M in a relatively uniform way. The cross-linking of Lys residues in the heavy chain–light chain connecting strand to α_2M explains earlier findings that a substantial portion of the heavy chain is cross-linked to α_2M [Pizzo et al. (1986) *Biol. Chem. Hoppe-Seyler 367*, 1177–1182]. Although located in the heavy chain, Lys550, -556 and -557 should be considered part of the serine proteinase domain. That part of plasmin must be deeply buried in the α_2M structure in close vicinity to the four thiol esters, while most of the elongated heavy chain is protruding from the binding cavity. The pattern of cross-linked species seen in sodium dodecyl sulfate–polyacrylamide gel electrophoresis reveals that individual plasmin molecules are bound to α_2M through one or two cross-links. Bivalent cross-linking can take place within or between 360-kDa α_2M dimers, pointing to the tetrameric α_2M structure as the functional proteinase binding unit.

The tetrameric α_2-macroglobulin (α_2M)[1] (720 kDa) binds a wide variety of proteinases differing in size and catalytic mechanism. Complex formation between proteinases and α_2M is initiated by specific limited proteolysis in the "bait region" of α_2M. This induces a set of conformational changes in α_2M leading to entrapment of the proteinase, activation of internal β-cysteinyl-γ-glutamyl thiol esters, and exposure of receptor recognition sites important for the rapid clearance from the circulation and tissues. The bound proteinase is still active but largely prevented from interaction with high molecular weight substrates and inhibitors. A large fraction of the proteinase is covalently bound, primarily through ε-lysyl-(proteinase)–γ-glutamyl(α_2M) cross-links. For recent reviews, see Sottrup-Jensen (1987, 1989).

α_2M is a major inhibitor of the large proteinase plasmin (Harpel, 1981) which consists of a 57-kDa heavy chain bridged to a catalytically active 25-kDa light chain, homologous with the pancreatic serine proteinases (Robbins et al., 1981). The mechanism of interaction between plasmin and α_2M has been extensively studied. In contrast to binding by α_2M of maximally two proteinase molecules the size of, e.g., trypsin, only one plasmin molecule is tightly bound. In some conditions, the 1:1 complex may bind additional plasmin or other proteinases in a poorly defined mode (Gonias & Pizzo, 1983; Christensen & Sottrup-Jensen, 1984; Steiner et al., 1987; Pochon, 1987).

From electron microscopic studies, it has been suggested that plasmin largely occupies the large elongated proteinase binding cavity in α_2M (Gonias et al., 1988; Boisset et al., 1989). However, K1–4 of the heavy chain protrude from the α_2M structure or are otherwise solvent accessible (Cummings & Castellino, 1984; Roche & Pizzo, 1988; Gonias et al., 1988). While SDS–PAGE shows that only the catalytic part of other large proteinases such as plasma kallikrein (Van Der Graaf et al., 1984) and factor Xa (Pizzo et al., 1986; Meijers et al., 1987) binds covalently to α_2M, both the light chain and the heavy chain of plasmin become covalently bound to α_2M (Salvesen & Barrett, 1980; Pizzo et al., 1986; Roche & Pizzo, 1987; Pochon, 1987).

In the present work, we have localized the major cross-links in human α_2M–plasmin complex (1:0.9 mol/mol) further treated with methylamine. Our results show that a large fraction of the cross-links present engages a few Lys residues in the light chain of plasmin. These residues are located in surface patches with an orientation relative to the active site similar to those identified earlier in trypsin, chymotrypsin, and elastase (Sottrup-Jensen et al., 1990). A minor fraction of the cross-links engages residues in the heavy chain. The nonreducing SDS–PAGE pattern of α_2M–plasmin shows that plasmin molecules are bound to α_2M through one or two cross-links. The location of plasmin in the proteinase binding cavity of α_2M is discussed.

MATERIALS AND METHODS

Proteins, Column Materials, and Chemicals. Human α_2M and Glu1-plasminogen were prepared as described earlier (Sottrup-Jensen et al., 1980; 1978b). Upon addition of excess trypsin to α_2M 3.7 mol of SH groups/mol of α_2M appeared. Urokinase, partially purified from human urine and containing 20.000 CTA units/mg, was a gift from Dr. G. Barlow, Abbott. Pepsin was from Worthington, and chymotrypsin was from

† This work was supported by The Danish Biomembrane Research Center.

* Address correspondence to this author.

[1] Abbreviations: α_2M, α_2-macroglobulin (human); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CTA, committee of thrombolytic agents; DPDS, 2,2′-dipyridyl disulfide; MA, methylamine; PTH, phenylthiohydantoin; K, kringle region in plasminogen; HC, plasmin heavy chain; LC, plasmin light chain; HC–LC, connecting strand between plasmin heavy and light chains.

0006-2960/93/0432-120$04.00/0

Boehringer. [^{14}C]Iodoacetic acid was from Amersham, and methylamine hydrochloride and DPDS were from Sigma. Standard chemicals were analytical grade from Merck, Fluka, and Sigma.

Thiopropyl-Sepharose, Q-Sepharose FF, and Sephadex G-25F were from Pharmacia. Packing materials for reverse-phase HPLC were obtained from Machery-Nagel and from Phase Separations.

Analytical Procedures. For amino acid analysis, a modification of the system of Barkholt and Jensen (1989) employing cation-exchange chromatography (pH gradients formed from sodium citrate/sulfate, pH 3.10, and sodium borate, pH 10.10), postcolumn oxidation with NaOCl, and reaction with *o*-phthaldialdehyde was used. The analyzer was assembled from a Spectra-Physics 8800 pump, an 8880 autosampler, a 4600 integrator/plotter, a column oven, and a LKB 2144 fluorescence monitor.

Automated sequence analysis was performed on a Applied Biosystems 470A instrument (off-line determination of PTH-amino acids on a Hewlett-Packard 1084B HPLC) or on a 477A instrument (on-line analysis of PTH-amino acids on a 120A HPLC). The sequenators were operated according to the manufacturer's instructions.

For SDS–PAGE (4–10% or 10–20% gradient gels) the standard Tris–glycine system was used (gel size 0.5 × 80 × 100 mm). Coomassie brilliant blue was used for staining. Peptides were separated on a Hewlett-Packard 1084B HPLC or an LKB instrument assembled from a 2152 pump, a 2158 UV monitor, a 2155 column oven, and a 2211 fraction collector. The separations were monitored by recording the absorbance at 229 nm and by determining the amount of ^{14}C label in peptide pools.

EXPERIMENTAL PROCEDURES

One micromole (720 mg) of α_2M dissolved in 47 mL of 0.1 M sodium phosphate, pH 8.0, was mixed with 0.9 μmol of plasminogen (83 mg) dissolved in 7 mL of 0.1 M sodium phosphate, pH 8.0, and the solution was made 1 mM in DPDS. Then 1 mg of urokinase dissolved in 1 mL of 50 mM Tris-HCl, pH 8.0, was added. The activation of plasminogen to plasmin and the subsequent complex formation with α_2M was monitored by recording the absorbance at 343 nm due to 2-thiopyridone appearing as a result of thiol ester cleavage (Figure 1). When the generation of SH groups had nearly leveled off, 6 mL of 2 M methylamine hydrochloride, pH 8.0, was added and the mixture was further incubated until remaining thiol esters had been cleaved. The α_2M–plasmin–MA complex was separated from reaction products and excess reagents by passage through a 5 × 25 cm column of Sephadex G-25 F equilibrated and eluted with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (not shown).

The pH of the solution was adjusted to 2.0 by addition of phosphoric acid, and 4 mg of pepsin dissolved in 10% acetic acid was added. After digestion for 2 h at room temperature, 42 mL of settled thiopropyl-Sepharose in the SH form was added and digestion continued for 16 h with end-over-end mixing. The thiopropyl-Sepharose was packed into a 2.5-cm column and washed with 250 mL of 0.1 M acetic acid/formic acid, pH 2.0, and 400 mL of water. Bound peptides were eluted with 0.1 M mercaptoethanol/0.1 M ammonium acetate, pH 8.5, at a flow rate of 0.5 mL/min. After freeze-drying, the material was dissolved in 3 mL of 0.1 M ammonium acetate, pH 9.0, and incubated with 50 μCi of [^{14}C]iodoacetic acid for 30 min. Then, 3 μL of mercaptoethanol was added followed by 400 μL of 0.2 M iodoacetic acid dissolved in water. After readjustment of the pH to 8.0 and reaction for 30 min, the mixture was loaded on a 2 × 35 cm Sephadex G-25 F column equilibrated and eluted with 0.1 M acetic acid (not shown). The material eluting between the void volume and the salt volume was pooled and freeze-dried.

After being redissolved in 5 mL of 0.2 M ammonium acetate, pH 9.0, the peptides were digested with 100 μg of chymotrypsin for 2 h. Digestion was terminated by addition of 100 μg of phenylmethanesulfonyl fluoride dissolved in ethanol and freeze-drying. To partially separate the peptides, the material was redissolved in 4 mL of 10 mM ammonium acetate, pH 6.5, and loaded on a 1 × 12 cm Q Sepharose FF column (Figure 2). Following elution with a gradient of ammonium acetate, the 14 pools containing the bulk of ^{14}C-labeled peptides were further separated on a 8 × 250 mm × 5 μm Nucleosil C-18 column using gradients of ethanol/0.1% trifluoroacetic acid (supplementary material Figures 1–14). Several pools from these separations containing impure peptides were rechromatographed on a 4 × 250 mm column containing the same packing material or on a 4 × 125 mm 5 μm Spherisorb ODS-2 column using gradient elution with acetonitrile/0.1% trifluoroacetic acid (not shown).

RESULTS

To investigate the cross-links in α_2M–plasmin, we followed a modification of the procedure used earlier for localizing cross-links in several α_2M–proteinase complexes (Sottrup-Jensen et al., 1990). In brief, after α_2M was incubated successively with plasmin and methylamine, the thiol group of Cys949 appearing as a result of thiol ester cleavage was converted to a mixed aliphatic–aromatic disulfide by reaction with DPDS. This enabled us to use covalent chromatography on thiopropyl-Sepharose (Svenson et al., 1977) as a key step in isolating a set of peptic peptides originating from cleavage at Leu942–Leu in α_2M and containing the reaction products of the thiol esters.

After being trimmed by digestion with chymotrypsin, all peptides of this set will reveal the sequence Gly-[^{14}C]CmCys-Gly-Glu-Glx-Asn as a result of cleavage of Tyr947–Gly in α_2M. Dependent on the fate of the thiol esters during complex formation, the thiol-esterified Glx residue will be recovered as a Glu residue due to hydrolysis or as a substituted Glu residue due to reaction with proteinase ε-Lys residues or methylamine. A cross-linked peptide containing two mates will show two sequences in approximately equimolar yield, one being the thiol ester sequence shown above, and the other being the sequence around the particular cross-linked residue. Lys residues from plasmin not engaged in cross-linking will appear as PTH-Lys. The identification of a particular Lys residue as being cross-linked is based on the absence of PTH-Lys in the cross-linked position and on the occurrence of a characteristic bis-PTH-Glu-Lys derivative in step 5 or later (Sottrup-Jensen et al., 1990).

The reaction between α_2M and plasmin was performed by activating plasminogen with urokinase in the presence of α_2M (0.9 mol of plasminogen/mol of α_2M) using 240 CTA units of urokinase/mg of plasminogen. Since the rate of interaction of urokinase and α_2M is several orders of magnitude lower than that of plasmin and α_2M (Straight et al., 1985; Christensen & Sottrup-Jensen, 1984; Steiner et al., 1987), the action of urokinase on plasminogen would not be affected by the presence of α_2M. This procedure ensured that α_2M would be exposed to only low concentrations of free plasmin so that preferentially 1:1 complexes were formed.

Figure 1A shows the time course of complex formation as monitored by 2-thiopyridone formation. In 48 min, plasmin,

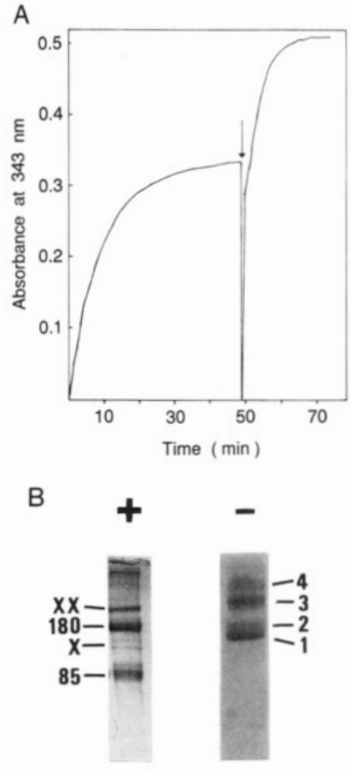

FIGURE 1: (Panel A) Time course of complex formation between plasmin and α_2M monitored by the extent of thiol ester cleavage. The change in absorbance at 343 nm due to 2-thiopyridone is shown. The cuvette contained an aliquot from the preparative activation of plasminogen by urokinase in the presence of α_2M. The addition of methylamine is shown by an arrow. (Panel B) Reducing (left lane, + and nonreducing (right lane, –) SDS–PAGE of α_2M–plasmin–MA after reaction for 73 min. In the reducing lane, the approximately 85-kDa bait region fragments, the 180-kDa intact subunit, and the covalent reaction products involving the light chain, X (monovalent) and XX (bivalent) (Sottrup-Jensen et al., 1990), are marked. The weak bands at the top of the gel probably represent heavy chain bound to α_2M. In the nonreducing lane, the 360-kDa α_2M dimer (band 1) and various cross-linked species (bands 2–4) are marked. A species corresponding to band 5 of Wang et al. (1984) is present in a very low amount (not marked).

when reacting with α_2M, caused the cleavage of 2.25 mol of thiol esters/mol of α_2M, and residual thiol esters (1.45 mol/mol of α_2M) were cleaved by methylamine in 25 min. N-Terminal sequence analysis of the complex revealed that the bound plasmin was predominantly Lys78/Val79-plasmin (not shown).

After reaction, reducing SDS–PAGE (Figure 1B) showed, in addition to the approximately 85-kDa bait region fragments and several cross-linked fragments of larger size, the presence of intact 180-kDa subunits and only trace amounts of the heat cleavage fragments of 120 and 60 kDa. Nonreducing SDS–PAGE showed, in addition to the 360-kDa α_2M dimer (band 1), high molecular weight cross-linked products (bands 2–4).

After covalent chromatography on thiopropyl-Sepharose the peptide pool eluted by mercaptoethanol showed that expected major sequence corresponding to the peptide Leu943–Asn953 of α_2M. However, the yield of that material was only approximately 48% compared with approximately 70–75% found earlier in similar experiments (Sottrup-Jensen et al., 1990). After treatment of this material with chymotrypsin and [^{14}C]carboxymethylation, the peptides were separated on Q Sepharose FF as shown in Figure 2. The pools from this run were further separated in other systems as shown in Figures 1–14 in the supplementary material.

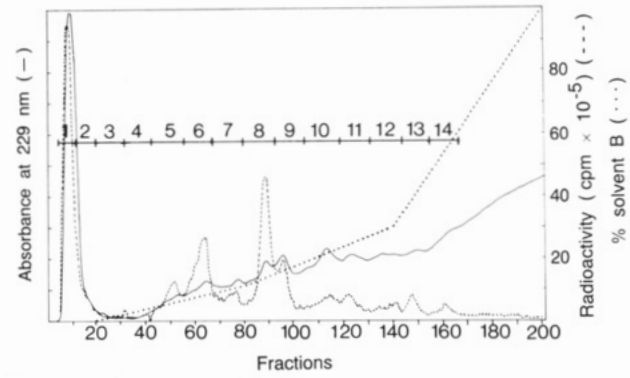

FIGURE 2: Separation of ^{14}C-labeled peptic/chymotryptic peptides on a 1 × 12 cm column of Q Sepharose FF. The column was eluted at a flow rate of 1 mL/min with a gradient of ammonium acetate, pH 6.5 (dotted line). Solvent A, 10 mM ammonium acetate; solvent B, 1 M ammonium acetate. The absorbance at 229 nm is shown by a full line (full scale = 2.0 absorbance units), and the radioactivity is shown by a broken line. The 14 pools made are indicated by numbers.

Table I: Summary of Yields of Peptides Containing the Reaction Products from the Thiol Esters in α_2M–plasmin–MA

cross-linked Lys residues (position in plasmin)	yield (nmol)
Lys258 (K3)	10
Lys298 (K3)	7
Lys473 (K5)	6
Lys550 (HC-LC)	68
Lys556/557 (HC-LC)[a]	22
Lys607 (LC)	254
Lys708 (LC)	28
Lys750 (LC)	55
other peptides[b]	50
combined yield of cross-linked peptides	500
hydrolyzed thiol ester	303
thiol ester reacted with methylamine	642
total yield	1445

[a] The cross-linking preferentially took place at Lys556. [b] A number of minor impure peptides clearly showed bis-PTH-Lys-Glu in cycle 5. However, from the combined information derived from amino acid analysis and sequence analysis, no definitive assignment of cross-linked positions could be made.

All major ^{14}C-labeled peptides were subjected to amino acid analysis (not shown) and sequence analysis for minimally six cycles. Due to the use of pepsin and chymotrypsin, several of the cross-linked peptides were split variants of a small number of "core" peptides (Table I in the supplementary material). Table I summarizes the position of the cross-linked Lys residues and the combined yield of peptides containing cross-links as well as other reaction products. Only nine Lys residues of the 41 in Lys78/Val79-plasmin (Sottrup-Jensen et al., 1978a; Forsgren et al., 1987) engaged in cross-linking, three in the plasmin light chain (residues 607, 708, and 750), three in the "connecting strand" between K5 of the heavy chain and the light chain (residues 550, 556, and 557), and three in the heavy chain (residues 258, 298, and 473). As seen from the appearance of bis-PTH-Lys-Glu, several minor peptides containing cross-links were also recovered in low yield (not shown). However, no cross-links could be established from these peptides since they were impure and since the cross-linked segments of plasmin were too short to allow safe identification.

Peptide material produced as a result of hydrolysis of the activated thiol esters was recovered as different variants (Table II in the supplementary material). As seen before (Sottrup-Jensen et al., 1990), some of these peptides were isolated from

pools having ion-exchange elution positions different from those that contained the major "parent" hydrolyzed peptide (pools 5.4, 6.4, and 10.6 vs pools 12.4, 12,14, 13.5, and 14.5; supplementary material, Figures 5, 6, 10, 12, 13, and 14). This suggested that a minor fraction of the thiol esters had formed unstable cross-links, possibly of the ester type, which decayed to a variable extent during the experiments.

Peptides containing γ-glutamylmethylamide resulting from reaction of residual thiol esters with methylamine were recovered as three variants (supplementary material, Table III). The yield of this material (642 nmol) and the combined yield of products resulting from the proteolytically activated thiol esters (803 nmol) by and large reflect the level of thiol groups generated in the two phases of reaction (Figure 1A).

The amount of labeled peptides appearing in the breakthrough volume of the anion exchanger was high (Figure 2). However, none of the peptides contained the characteristic sequence around the thiol ester. They were mainly Cys- and Met-containing peptides from plasmin and α_2M (not shown). The presence of such peptides (also found in other ion-exchange pools) probably reflected a less extensive pepsin digestion of α_2M–plasmin than obtained earlier. This resulted in large disulfide-bridged peptide sets cross-linked to the thiol ester peptide. Upon elution from thiopropyl-Sepharose, their disulfide bridges would be reduced, and in the subsequent chymotryptic digestion these peptides would be fragmented to many small peptides.

DISCUSSION

When binding proteinases, α_2M undergoes large conformational changes during which the internal β-Cys-γ-Glu thiol esters become activated so that a major fraction of the proteinase (>80%) in addition to being trapped is also covalently bound to α_2M. The complex interplay of bait region cleavage, thiol ester cleavage, and conformational changes has only been described in gross terms (Gonias & Pizzo, 1983; Christensen & Sottrup-Jensen, 1984; Steiner et al., 1985; 1987; Larsson et al., 1989; Strickland et al., 1991).

The cleavage of an average of 2.5 thiol esters in α_2M by one molecule of plasmin during 1:1 complex formation (Figure 1A) is compatible with the value of approximately 2 estimated earlier (Christensen & Sottrup-Jensen, 1984; Steiner et al., 1987; Pochon, 1987). As judged from the staining intensity, the amount of residual 180-kDa α_2M subunit in the complex (Figure 1B) was approximately 50%, indicating an approximately 1:1 stoichiometry between bait region and thiol ester cleavage (Pizzo et al., 1986; Steiner et al., 1987; Pochon, 1987; Roche & Pizzo, 1987).

In reducing SDS–PAGE of α_2M–proteinase complexes, two major species consisting of one proteinase molecule bound to one or two C-terminal α_2M half-subunits are seen (species X and XX, respectively) (Wang et al., 1984; Sottrup-Jensen et al., 1990). These species were also seen with plasmin light chain (Pizzo et al., 1986; Pochon, 1987; Roche & Pizzo, 1987) and are pointed out in Figure 1B. Since size estimates of cross-linked species are inaccurate (Wang et al., 1984), particularly for species containing large components (Sottrup-Jensen et al., 1990; Sottrup-Jensen & Birkedal-Hansen, 1989), reaction products involving the heavy chain of plasmin can only be tentatively assigned (Roche & Pizzo, 1987; 1988).

The pattern of species of high apparent molecular weight seen in nonreducing SDS–PAGE (bands 2–4) was similar to that observed earlier with thrombin (Wang et al., 1984), plasma kallikrein (Van Der Graaf et al., 1984), factor Xa (Meijers et al., 1987), and other proteinases (Sottrup-Jensen et al., 1990). Apart from the presence of a species consisting of plasmin bound to α_2M through one cross-link (band 2), the results indicate that a major fraction of the plasmin molecules are bound to α_2M by two cross-links (bands 3 and 4) as shown earlier for thrombin (Wang et al., 1984). As discussed (Sottrup-Jensen et al., 1990), that band pattern suggests that bivalent cross-linking of plasmin takes place not only within a 360-kDa α_2M dimer (band 3) but also across two dimers (band 4).[2]

Although approximately two bait regions are cleaved by one plasmin molecule during complex formation, the band pattern suggests that these cleavages are not confined to one of the 360-kDa dimers but rather occur essentially at random. This is supported by electron micrographs obtained at different stages in complex formation with plasmin showing distinct conformational intermediates (Delain et al., 1992), compatible with the electrophoretic heterogeneity of 1:1 α_2M–plasmin complexes in nondenaturing PAGE (Roche & Pizzo, 1988). Further, "half-cleaved" α_2M, having no bound proteinase (Gettins et al., 1989), is similarly heterogeneous in PAGE and yields the same pattern of cross-linked high molecular weight species upon reaction with trypsin as native α_2M does (not shown). Hence, the commonly held concept that the 360-kDa α_2M dimers constitute discrete functional domains cannot be maintained. Rather, the tetramer is the functional unit providing one large binding cavity, capable of binding maximally two proteinase molecules.

In a previous study, the major ϵ-lysyl-γ-glutamyl cross-links were located in complexes between human α_2M and the 25–35-kDa proteinases trypsin, chymotrypsin, elastase, subtilisin, and thermolysin. It was found, as a common feature, that most of the available ϵ-lysyl side chains on these proteinases are accessible to cross-linking. However, certain residues reacted to the largest extent with the thiol esters (Sottrup-Jensen et al., 1990). The two-chain structure of plasmin and its large size (probably approximately 200 Å in length as estimated for the open form of Glu1-plasminogen; Mangel et al., 1990; Ramakrishnan et al., 1991) and its preference for forming 1:1 complexes with α_2M indicate that steric constraints during complex formation might play a greater role for plasmin than that seen for the small proteinases studied previously.

Cross-linking involved residues from both chains of plasmin (Table I), but the three residues identified in the light chain (Lys607, -708, and -750) accounted for approximately 75% of the covalent binding, while the six residues identified in the heavy chain (Lys258, -298, -473, -550, -556, and -557) accounted for approximately 25%. These figures are compatible with earlier estimates from SDS–PAGE experiments (Pizzo et al., 1986).

Intriguingly, Lys607 accounted for approximately 75% of all covalent binding seen with the light chain. Hence, cross-linking of the plasmin light chain to α_2M is much more restrictive than seen for the homologous "small" proteinases trypsin and chymotrypsin studied earlier. These results indicate that following bait region cleavage(s) most of the plasmin molecules, upon entering the binding cavity in α_2M (Delain et al., 1988; Gonias et al., 1988; Boisset et al., 1990; Sottrup-Jensen, 1989), are not able to diffuse to any large extent or otherwise reorient themselves within the binding

[2] Additional evidence for the species in bands 2 and 3 being 360-kDa dimers having one molecule of proteinase bound through one and two cross-links, respectively, has been obtained by sequence analysis of the corresponding species in α_2M–trypsin (1:2 mol/mol, all subunits cleaved); in both species the N-termini of bait region cleaved α_2M (Ser and Leu) and trypsin (Ile) were present in an approximately 2:1 molar ratio.

cavity. We ascribe this to the large size of the elongated plasmin which enables α_2M to more effectively "grip" plasmin than small proteinases.

Of the six residues in the heavy chain, the three most strongly reacting (Lys550, -556, and -557) are located in the connecting strand between K5 of the heavy chain and the light chain. In an evolutionary sense, residues 548–561, constituting the C-terminus of the heavy chain, should be considered equivalent with residues 1–15 of chymotrypsinogen; i.e., they are part of the serine proteinase domain. Hence, in the tertiary structure of plasmin, Lys550, -556, and -557 of the heavy chain must be located roughly equivalent with chymotrypsinogen residues 3, 9, and 10, respectively.

Using chymotrypsin (Birktoft & Blow, 1972) as a model for the tertiary structure of the plasmin light chain, Figure 3 shows the location of the six Lys residues discussed above. Relative to the active site, the strongly reacting Lys607(62) (chymotrypsinogen numbering shown in parentheses in the discussion below) is located on one side of the model whereas Lys708(166), Lys550(3), Lys556(9), Lys557(10), and Lys750-(204) by and large are located on the other side. Lys607(62) is located in roughly the same orientation relative to the active site as the strongly reacting residues in trypsin, chymotrypsin, and elastase (Sottrup-Jensen et al., 1990).

During complex formation with proteinases, the four thiol esters of α_2M are likely to be located in a fairly central position facing the lumen of the large elongated cavity inferred from electron microscopy studies (Delain et al., 1988; Gonias et al., 1989; Boisset et al., 1990). The maximal distance between any pair of thiol esters is probably only approximately 40–50 Å, and the bait regions are within approximately 20 Å from the thiol esters (Gettins et al., 1988, 1990; Sottrup-Jensen et al., 1990).

The present data and those presented earlier (Sottrup-Jensen et al., 1990) cannot directly address the question of the steric relationship between the bait regions and the thiol esters in α_2M. However, we speculate, by using the geometry of the active site/substrate binding area of the proteinase and the location of the patches of strongly reacting Lys residues in a complementary fashion, that the preference for cross-linking Lys residues on the particular part of the serine proteinase surface discussed above reflects that there is an approximately 90° "kink" between the location of the bait region and the thiol ester within an α_2M subunit.

The monovalent cross-linked product is likely to involve primarily Lys602(62). However, a fraction of the plasmin molecules becomes bivalently cross-linked, presumably involving the minor reacting Lys550(3), -556(9), -557(10), -708-(166), and -750(204) in addition to Lys602(62). However, the product XX seen in reducing SDS–PAGE can only be produced when Lys607(62) reacts with one thiol ester of a pair and Lys708(166) or Lys750(204) reacts with the other thiol ester. Bivalent products involving residues in both the light and heavy chains will appear as monovalent products upon reduction (Figure 1B).

Figure 4 shows a schematic representation of the rigid cylinder-like shape of α_2M after reaction with proteinases and the putative location of a bound plasmin molecule. We envisage that the serine proteinase domain of plasmin is deeply buried in the cavity and is accessible to any pair of thiol esters during the binding process. However, depending on whether plasmin becomes bound through one cross-link, through two cross-links within a dimer, or through two cross-links across dimers, individual molecules could be located in quite different positions.

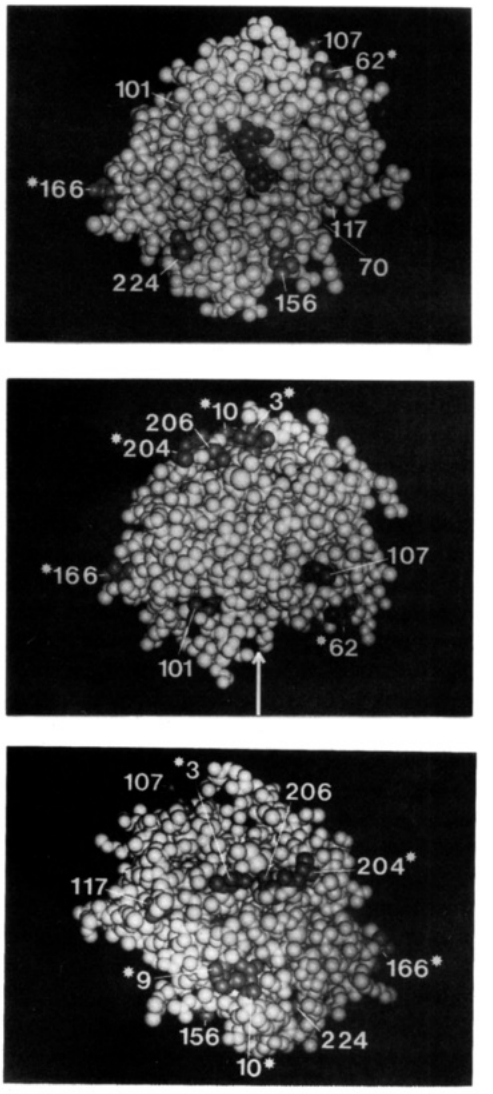

FIGURE 3: Approximate location of Lys residues in the serine proteinase domain of plasmin based on the model of chymotrypsin (Birktoft & Blow, 1972). Modeling program: Manual for 0 version 5-7-1 (T. A. Jones and M. Kjeldgaard, Uppsala University). All residues present as Lys residues in plasmin are shown in dark-gray and numbered (chymotrypsinogen numbering). The cross-linked Lys residues are marked by asterisks. Three orientations are shown: in the top panel the model is viewed so that the active site is visible (dark area near the center). During complex formation the α_2M bait region will position nearly horizontally across the surface (top panel) so that the P sites are found to the left of the active site, and the P′ sites are found to the right. In the middle panel, the model has been rotated 90° around a horizontal axis, the location of the active site is indicated by an arrow. In the lower panel the model has been rotated 180° around a vertical axis.

In recent interpretations of electron micrographs of α_2M–plasmin, the proteinase has been positioned nearly completely within the α_2M structure (Gonias et al., 1988; Boisset et al., 1989). However, as plasmin is likely to have an extended conformation, a major part of the heavy chain including K1–4 of plasmin probably protrude from the α_2M structure. Whether it is fully extended as suggested in Figure 4, or whether it assumes a more compact shape, is not known. An extended conformation is supported by studies showing that the heavy chain of plasmin in complex with α_2M is readily accessible to monoclonal antibodies reacting with epitopes in these regions (Cummings & Castellino, 1984; Gonias et al., 1988). Furthermore, K1–3 and K4 can readily be released from the α_2M–plasmin complex by digestion with elastase (Roche & Pizzo, 1988) at the same sites as in native

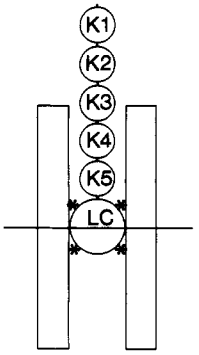

FIGURE 4: Schematic representation of α_2M–plasmin complex. The cartoon is based on electron micrographs of proteinase or methylamine-treated α_2M (Delain et al., 1988, 1992; Gonias et al., 1988; Boisset et al., 1990). The characteristic H-shaped structure is viewed as a projection of a cylinder-like molecule with overall dimensions 200 × 100 Å. The dimeric units of α_2M are assembled back-to-back and are divided by a horizontal line. The location of the thin connections between the massive "walls" is not known (Delain et al., 1992). As the proteinase-induced form is created, the thiol esters (asterisks) are facing the lumen of the elongated binding cavity and are located in a central position of the tetramer. The bait regions may be protruding into the center of the cavity. Plasmin (approximately 200 Å long) is represented by a large circle (light chain, approximately 50 Å in diameter; Birktoft & Blow, 1972) and five small circles (K1–K5, approximately 30-Å oblates; (Park & Tulinsky, 1986). The serine proteinase domain of plasmin may become positioned so that it can react with any pair of the four thiol esters. The heavy chain is viewed in a fully extended conformation leaving K1–K4 exposed.

plasminogen (Sottrup-Jensen et al., 1978b).

Preparations of equimolar α_2M–plasmin complexes can bind up to approximately 0.5 mol/mol of other proteinases (Christensen & Sottrup-Jensen, 1984; Pochon, 1987; Steiner et al., 1987; Larsson et al., 1989). How this binding takes place is not clear, but, as viewed in Figure 4, in some of the 1:1 complexes there might be sufficient vacant space in the binding cavity so that it might accommodate an additional proteinase molecule. That proteinase binding is probably largely noncovalent.

A minor cross-linking reaction with Lys258, -298, and -473 of the heavy chain was also seen. K5 is, due to the bridge Cys548(1)–Cys666(122), probably in close proximity of the light chain. Hence Lys473 of K5 could be within reach of the activated thiol esters when the light chain has become buried in the interior of α_2M.

Reaction of the thiol esters with Lys258 and -298 of K3 would be expected to be unlikely in view of the shape and length of plasmin as discussed above. To explain the reaction with Lys residues in K3, we propose that this is a result of the experimental condition for complex formation. Conceivably, a minor fraction of the plasmin molecules bound to α_2M has been Glu1-plasmin, whose conformation is likely to be similar to that of the compact Glu1-plasminogen (Mangel et al., 1990; Ramakrishnan et al., 1991). In view of chemical cross-linking experiments showing that Lys204 in K2 is located relatively close to Tyr672(128) in the light chain (Banyai et al., 1985), this might also apply for certain Lys residues of K3. Conceivably, this could bring Lys258 and -298 in proximity of the thiol esters.

In conclusion, in cross-linking plasmin to α_2M the utilization of Lys residues in the enzyme is much more restrictive than seen before with trypsin, chymotrypsin, subtilisin, and thermolysin. Reaction with three Lys residues in the serine proteinase part of plasmin and three Lys residues in the C-terminal stretch of its heavy chain, which should be considered part of the proteinase domain, constitute approximately 95% of the total cross-linking. This indicates that in complex formation between α_2M and large multichain proteinases only the proteinase domains become buried in α_2M so that certain of their Lys residues become juxtaposed to the activated thiol esters. Plasma kallikrein and factor Xa provide examples of two-chain proteinases where cross-linking exclusively engages residues of the serine proteinase domain (Van Der Graaf et al., 1984; Pizzo et al., 1986; Meijers et al., 1987).

ACKNOWLEDGMENT

We thank Lene Kristensen for excellent technical assistance. Morten Kjeldgaard is thanked for help with computer representations of the three-dimensional structure of chymotrypsin.

SUPPLEMENTARY MATERIAL AVAILABLE

Fourteen figures (1–14) showing reverse-phase HPLC separations of the 14 pools obtained from the separation shown in Figure 2 of the main text and three tables showing sequences and yields of peptides containing α_2M–plasmin cross-links (I), of hydrolyzed thiol ester peptides (II), and of γ-glutamylmethylamide containing peptides (III) (5 pages). Ordering information is given on any current masthead page.

REFERENCES

Banyai, L., & Patthy, L. (1985) *Biochim. Biophys. Acta 832*, 224–227.

Barkholt, V., & Jensen, A. L. (1989) *Anal. Biochem. 177*, 318–322.

Birktoft, J. J. & Blow, D. M. (1972) *J. Mol. Biol. 68*, 187–240.

Boisset, N., Taveau, J.-C., Pochon, F., Tardieu, A., Barray, M., Lamy, J. N., & Delain, E. (1989) *J. Biol. Chem. 264*, 12046–12052.

Christensen, U., & Sottrup-Jensen, L. (1984) *Biochemistry 23*, 6619–6626.

Cummings, H. S., & Castellino, F. J. (1984) *Biochemistry 23*, 105–111.

Delain, E., Barray, M., Tapon-Bretaudiere, J., Pochon, F., Marynen, P., Cassiman, J.-J., Van Den Berghe, H., & Van Leuven, F. (1988) *J. Biol. Chem. 263*, 2981–2989.

Delain, E., Pochon, F., Barray, M., & Van Leuven, F. (1992) *Electron Microsc. Rev. 5*, 231–281.

Forsgren, M., Råden, B., Israelsson, M., Larsson, K., & Heden, L.-O. (1987) *FEBS Lett. 213*, 254–260.

Gettins, P., Beth, A., & Cunningham, L. W. (1988) *Biochemistry 27*, 2905–2911.

Gettins, P., Crews, B. C., & Cunningham, L. W. (1989) *Biochemistry 28*, 6513–6518.

Gettins, P., Beechem, J. M., Crews, B. C., & Cunningham, L. W. (1990) *Biochemistry 29*, 7747–7753.

Gonias, S. L., & Pizzo, S. V. (1983) *J. Biol. Chem. 258*, 14682–14685.

Gonias, S. L., Allietta, M. M., Pizzo, S. V., Castellino, F. J., & Tillack, T. W. (1988) *J. Biol. Chem. 263*, 10903–10906.

Harpel, P. C. (1981) *J. Clin. Invest. 68*, 46–55.

Larsson, L.-J., Neuenschwander, D. E., & Strickland, D. K. (1989) *Biochemistry 28*, 7636–7643.

Mangel, W. F., Lin, B., & Ramakrishnan, V. (1990) *Science 248*, 69–73.

Meijers, J. C. M., Tijburg, P. N. M., & Bouma, B. N. (1987) *Biochemistry 26*, 5932–5937.

Park, C. H., & Tulinsky, A. (1986) *Biochemistry 25*, 3977–3982.

Pizzo, S. V., Rajagopalan, S., Roche, P. A., Fuchs, H. E., Feldman, S. R. & Gonias, S. L. (1986) *Biol. Chem. Hoppe-Seyler 367*, 1177–1182.
Pochon, F. (1987) *Biochim. Biophys. Acta 915*, 37–45.
Ramakrishnan, V., Patthy, L., & Mangel, W. F. (1991) *Biochemistry 30*, 3963–3969.
Robbins, K. C., Summaria, L., & Wohl, R. C. (1981) *Methods Enzymol. 80*, 379–387.
Roche, P. A., & Pizzo, S. V. (1987) *Biochemistry 26*, 486–491.
Roche, P. A., & Pizzo, S. V. (1988) *Arch. Biochem. Biophys. 267*, 285–293.
Salvesen, G. S., & Barrett, A. J. (1980) *Biochem. J. 187*, 695–701.
Sottrup-Jensen, L. (1987) in *The Plasma Proteins*, 2nd ed. (Putnam, F. W., Ed.) Vol. 5, pp 191–291, Academic Press, Orlando, FL.
Sottrup-Jensen, L. (1989) *J. Biol. Chem. 264*, 11539–11542.
Sottrup-Jensen, L., & Birkedal-Hansen, H. (1989) *J. Biol. Chem. 264*, 393–401.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1978a) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., Ed.) Vol. 5, Suppl. 3, p 91, National Biomedical Research Foundation, Silver Springs, MD.
Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E., & Magnusson, S. (1978b) *Prog. Chem. Fibrinolysis Thrombolysis 3*, 191–209.
Sottrup-Jensen, L., Petersen, T. E. & Magnusson, S. (1980) *FEBS Lett. 121*, 275–280.
Sottrup-Jensen, L., Hansen, H. F., Pedersen, H. S., & Kristensen, L. (1990) *J. Biol. Chem. 265*, 17727–17737.
Steiner, J. P., Bhattacharya, P., & Strickland, D. K. (1985) *Biochemistry 24*, 2993–3001.
Steiner, J. P., Migliorini, M., & Strickland, D. K. (1987) *Biochemistry 26*, 8487–8495.
Straight, D. L., Hassett, M. A., & McKee, P. A. (1985) *Biochemistry 24*, 3902–3907.
Strickland, D. K., Larsson, L.-J., Neuenschwander, D. E., & Björk, I. (1991) *Biochemistry 30*, 2797–2803.
Svenson, A., Carlsson, J., & Eaker, D. (1977) *FEBS Lett. 73*, 171–174.
Van Der Graaf, F., Rietveld, A., Keus, F. J. A., & Bouma, B. (1984) *Biochemistry 23*, 1760–1766.
Wang, D., Yuan, A. I., & Feinman, R. D. (1984) *Biochemistry 23*, 2807–2811.