Cross-linking of Plasminogen Activator Inhibitor 2 and α₂-Antiplasmin to Fibrin(ogen)*

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Helen Ritchie, Laura C. Lawrie, Patricia W. Crombie, Michael W. Mosesson‡, and Nuala A. Booth§

From the Department of Molecular and Cell Biology, University of Aberdeen, Institute of Medical Sciences, Aberdeen, United Kingdom AB25 2ZD and ‡Blood Research Institute, Blood Center of Southeastern Wisconsin, Milwaukee, Wisconsin 53201-2178

In this study, we identified lysine residues in the fibrinogen Aα chain that serve as substrates during transglutaminase (TG)-mediated cross-linking of plasminogen activator inhibitor 2 (PAI-2). Comparisons were made with α₂-antiplasmin (α₂-AP), which is known to cross-link to lysine 303 of the Aα chain. A 30-residue peptide containing Lys-303 specifically competed with fibrinogen for cross-linking to α₂-AP but not for cross-linking to PAI-2. Further evidence that PAI-2 did not cross-link via Lys-303 was the cross-linking of PAI-2 to I-9 and des-αC fibrinogens, which lack 100 and 390 amino acids from the C terminus of the Aα chain, respectively. PAI-2 or α₂-AP was cross-linked to fibrinogen and digested with trypsin or endopeptidase Glu-C, and the resulting peptides analyzed by mass spectrometry. Peptides detected were consistent with tissue TG (tTG)-mediated cross-linking of PAI-2 to lysines 148, 176, 183, 457 and factor XIIIa-mediated cross-linking of PAI-2 to lysines 148, 230, and 413 in the Aα chain. α₂-AP was cross-linked only to lysine 303. Cross-linking of PAI-2 to fibrinogen did not compete with α₂-AP, and the two proteins utilized different lysines in the Aα chain. Therefore, PAI-2 and α₂-AP can cross-link simultaneously to the α-polymers of a fibrin clot and promote resistance to lysis.

Fibrinogen is a 340-kDa plasma protein and is a symmetrical dimer containing 29 disulfide bonds. Fibrinogen consists of 2 copies of Aα, Bβ, and γ polypeptide chains, which have molecular masses of 63.5, 56, and 47 kDa, respectively. Release of fibrinopeptides A and B by thrombin initiates rearrangement of the molecule to form fibrin fibrils (reviewed in Ref. 1). Cross-linking of fibrinogen (by transglutaminases is responsible for generation of an insoluble, stable fibrin clot (1). Transglutaminases catalyze formation of covalent ε-γ-glutamyl) lysine bonds. An acyl transfer reaction occurs where the γ carboxyamide group of a glutamine residue acts as the donor and the ε-amino group serves as an acyl acceptor (reviewed in Ref. 2). Cross-linking occurs only between the Aα and γ chains of aligned fibrin fibrils; the Bβ chain is not involved in cross-linking (1). Initially, adjacent γ chains cross-link to form γ-γ dimers. This reaction occurs between glutamine 398 or 399 of one chain and lysine 406 of another fibrin fibril (3). Aα chains then polymerize to form higher molecular mass polymers; this reaction occurs more slowly than γ-γ dimer formation (4). Many lysine residues have been implicated as substrates for cross-linking and subsequent generation of high molecular mass oligomers of Aα chain (5, 6). It has been found that multimerization of γ-γ dimers can occur over extended time periods (7).

Factor XIII (FXIII) and tissue transglutaminase (tTG) are two major members of the TG family and differ with respect to location and regulation (2). Plasma FXIII is found as azymogen that consists of two A and two B subunits. The A subunit contains the catalytic domain and shows homology to tTG, which is a monomer. Thrombin is required for activation of FXIII, which is associated with fibrinogen in the plasma. This ensures that generation of fibrin is accompanied by FXIII-mediated cross-linking of fibrin to form a strong insoluble clot. Tissue transglutaminase has a broad distribution in tissues, where cells such as endothelial cells and macrophages have been found to synthesize tTG (2). Both FXIII and tTG can cross-link fibrinogen, but the pattern of cross-linking differs. FXIII generates γ-γ dimers, whereas tTG cross-links hybrids of an Aα and γ chain (8). Cross-linked Aα-γ heterodimers were detected in plasma fibrinogen, indicative of tissue transglutaminase activity (9).

Cross-linking that occurs between the chains of fibrinogen is not thought to contribute in a major way to resistance of a fibrin clot to lysis by plasmin. However, there has been controversy on this point. It has been shown that formation of γ-γ dimers did not affect fibrin lysis (10), but formation of complex α-polymers increased resistance to fibrin lysis (11). Other studies have found that neither formation of γ-γ dimers nor α polymers contributed to resistance of fibrin to lysis (12). Recent studies demonstrate that multimerization of γ-dimers after prolonged incubation of a fibrin clot decreased the rate of fibrin lysis (7).

The major way in which cross-linking affects resistance of a fibrin clot to lysis is when inhibitors of fibrinolysis are cross-linked to fibrinogen (13, 14). The serine protease inhibitor (serpin). α₂-AP, is the major physiological inhibitor of plasmin, which degrades fibrin. Localization of α₂-AP along the fibrin strands by FXIII-mediated cross-linking decreased lysis of fibrin by plasmin (14). α₂-AP has been shown to be cross-linked to fibrinogen via glutamine 2 of α₂-AP and lysine 303 of the Aα chain (15, 16).

We have shown that PAI-2, an inhibitor of plasmin generation, was cross-linked to fibrinogen (17). PAI-2 inhibits fibrinolysis by neutralizing urokinase and tissue-type plasminogen activator. This inhibits plasmin generation and subsequent

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‡ To whom correspondence should be addressed: Dept. of Molecular and Cell Biology, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen, UK AB25 2ZD. Tel.: 44-1224-273118; Fax: 44-1224-273144; E-mail: n.a.booth@abdn.ac.uk.

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† The abbreviations used are: FXIII, factor XIII; tTG, tissue transglutaminase; PAI-2, plasminogen activator inhibitor 2; α₂-AP, α₂-antiplasmin.
fibrin degradation and/or extracellular matrix remodeling (18). Both tTG and FXIIIa were found to catalyze cross-linking of PAI-2 to fibrinogen (17). The glutamines on PAI-2 were identified as residues 83 and 86, which are found on an exposed loop between helices C and D. This unique 33-residue loop is distinct from the active site of PAI-2, and cross-linked PAI-2 remains active as an inhibitor of plasminogen activators (17, 19). Other studies have demonstrated that PAI-2 can be cross-linked to trophoblast membranes and to extracellular matrix, again via the exposed loop (20). Intracellular PAI-2 has been shown to inhibit programmed cell death, and this process was also dependent on the interhelical loop (21).

This study investigated the lysine residues in fibrinogen that were involved in transglutaminase-catalyzed cross-linking of PAI-2. α2-AP is known to be cross-linked to fibrinogen by lysine 303 in the Aa chain; therefore, cross-linking of α2-AP was compared with that of PAI-2. Interestingly, we have found that PAI-2 was not cross-linked via lysine 303 but utilized many other lysine residues in the Aa chain of fibrinogen.

**Experimental Procedures**

Transglutaminase-mediated Cross-linking of PAI-2 and α2-AP—Fibrinogen for routine cross-linking reactions was purchased from Kabi (Sweden). Peak 1 fibrinogen was produced from fraction I-2 fibrinogen by anion exchange chromatography on DEAE-cellulose (22). This material contained >80% intact Aa chains and was free of other protein contaminants. Fraction I-9 fibrinogen was prepared by subfractionation of the glycine precipitate of sample (23) and lacked C-terminal Aa segments of ~100 residues (24, 25). Des-oC fibrinogen (fraction I-9D) was produced from intact fibrinogen by limited plasmin digestion (25) and lacked C-terminal Aa segments of ~390 residues (26, 27). Recombinant 47-kDa non-glycosylated PAI-2 was kindly provided by Biotech Australia Ltd.

Fibrinogen (1 μM) was incubated with 200 nM recombinant PAI-2 or 200 nM α2-AP (Biopool, UK) in the absence or presence of 200 nM guinea pig liver transglutaminase (Sigma). The reaction was initiated by the addition of 2.5 mm CaCl₂ typically for 30 min at 37 °C. Excess α2-AP or recombinant PAI-1 (28) was incorporated into the reaction at a final concentration of 1.5 μM in some experiments.

Peptides were synthesized in-house using a Pioneer peptide synthesizer (PE Biosystems) and were confirmed by mass spectrometry. These peptides were incorporated into cross-linking reactions at a final concentration of 1 μM. The sequence of the peptides corresponded to residues 290 to 319 of fibrinogen Aa chain and were as follows.

K303, NPGSSGTGGTATKPGSSGPGSTGSWNSGS
R303, NPGSSGTGGTATRPGSSGPGSTGSWNSGS

Samples were prepared for SDS-polyacrylamide gel electrophoresis by solubilization in an equal volume of 8 μl urea, 200 μl Tris, 4% w/v SDS, 4% w/v mercaptoethanol. Samples were electrophoresed on 8% Prossie (Flowgen, UK) acrylamide gels, which mimic a 4–12% acrylamide gradient, at a constant voltage of 125 V. Proteins were transferred overnight to nitrocellulose (Bio-Blot-NC, Costar, UK) in 25 mM Tris, 192 mM glycine, 20% w/v methanol. Transfer of protein was monitored by staining the nitrocellulose with 1% w/v Ponceau S in 3% v/v trichloroacetic acid and destaining with distilled H₂O. Membranes were blocked with 50 mM bicarbonate/carbonate buffer, pH 9.6, containing 3% w/v bovine serum albumin. Antibodies were detected by binding of a mouse monoclonal antibody to PAI-2 (Biopool, 0.2 μg/ml) or a rabbit IgG preparation to α2-AP (Dako, 6 μg/ml). A rabbit antiserum (CS004) raised to a peptide corresponding to residues 601–608 of the fibrinogen Aa chain was obtained from Biotech Australia Ltd.

**Results**

Cross-linked PAI-2 Co-localized with the Aa Chains of Fibrinogen—Cross-linking of PAI-2 to the A chains of fibrinogen was examined by immunoblotting for the Aa chain and for PAI-2. Free Aa chains were seen at ~63.5 kDa (Fig. 1, lane a). Cross-linking of fibrinogen by tTG resulted in the formation of α polymers at approximately 100, 210, 240, 270 kDa (lane b). Immunoblotting of the same samples for PAI-2 demonstrated free PAI-2 at ~47 kDa and a degradation product (~37 kDa, lane c). The addition of tTG generated higher molecular mass cross-linked PAI-2 at 210, 240, and 270 kDa (lane d), which was consistent with PAI-2 that was cross-linked to α polymers. Some cross-linked PAI-2 was detected at approximately 110 kDa, and this is likely to represent PAI-2 cross-linked to one Aa chain.

**Enzymatic Digestion of Cross-linked Samples under Denaturing Conditions**—Fibrinogen (Kabi, 2 mg) was cross-linked using 300 μg of guinea pig liver transglutaminase and 2.5 mm CaCl₂ in the absence or presence of 800 μg PAI-2 or α2-AP for 2 h at 37 °C. Samples were reduced and carboxymethylated (16, 29, 30) and taken up in 0.1 μl Tris/HCl, 1 mM EDTA, pH 6.8, containing 6 μl urea. Dithiothreitol was added to a final concentration of 50 μM under a nitrogen atmosphere and incubated for 30 min at room temperature followed by a further 30-min incubation in the presence of 500 μM iodoacetamide. Samples were separated by gel filtration on Sephacryl S-200 in the presence of 8 μl urea. PAI-2/α2-AP that was cross-linked to fibrinogen was detected in fractions by immunoblotting using either a monoclonal antibody to PAI-2, polyclonal antibody to α2-AP or polyclonal antibody to fibrinogen (Dako). Three fractions were identified as containing cross-linked material and were pooled, dialyzed, and lyophilized. Alternatively, 6 μM fibrinogen was cross-linked to 6 μM PAI-2 or α2-AP in the presence of 2.5 mm CaCl₂, 600 nM tTG or 600 nM FXIIIa, and 0.4 units/ml thrombin for 2 h before electrophoresis on a 4–12% NuPage acrylamide gel (Novex) under reducing conditions. Cross-linked products were stained with Coomassie Blue, and individual cross-linked bands were excised from the gel and digested.

Cross-linked samples were digested overnight with 0.5 μg of porcine trypsin (Promega, UK) in 20 mM ammonium bicarbonate or with 0.5 μg endoprotease Glu-C from Staphylococcus aureus (V8, Promega) in 50 mM sodium phosphate, pH 7.8, vacuum-dried, and dissolved in 500 μl of 10% formic acid. The peptides obtained were desalted on a reverse phase high performance liquid chromatography microcolumn. Masses of the peptides were determined on a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (PE Biosystems Voyager STR). Samples were prepared in either sinapinic or α-cyano-4-hydroxy cinnamic acid matrices. Predicted masses of trypsin and endoproteinase Glu-C peptides of fibrinogen, PAI-2, and α2-AP were calculated using the PeptideMass software package available from the ExpASy web site. All experiments were carried out at least five times except the gel filtration and trypsin digestion analysis of cross-linked samples, which was performed on two separate occasions.

**RESULTS**

Cross-linked PAI-2 Co-localized with the Aa Chains of Fibrinogen—Cross-linking of PAI-2 to the A chains of fibrinogen was examined by immunoblotting for the Aa chain and for PAI-2. Free Aa chains were seen at ~63.5 kDa (Fig. 1, lane a). Cross-linking of fibrinogen by tTG resulted in the formation of α polymers at approximately 100, 210, 240, 270 kDa (lane b). Immunoblotting of the same samples for PAI-2 demonstrated free PAI-2 at ~47 kDa and a degradation product (~37 kDa, lane c). The addition of tTG generated higher molecular mass cross-linked PAI-2 at 210, 240, and 270 kDa (lane d), which was consistent with PAI-2 that was cross-linked to α polymers. Some cross-linked PAI-2 was detected at approximately 110 kDa, and this is likely to represent PAI-2 cross-linked to one Aa chain.

**Time Dependence for Cross-linking of PAI-2**—Cross-linking of PAI-2 was compared (0–60 min). Both these inhibitors were cross-linked rapidly to fibrinogen, and high molecular cross-linked products (~200 kDa) were identified within 5 min of adding tTG. Cross-linked products of PAI-2 and α2-AP increased from 5 to 60 min in a time-dependent manner, but most cross-linking occurred within 30 min. Free α2-AP was seen at ~65 kDa. Cross-linked products of α2-AP were similar to those of PAI-2 and were detected at ~140 kDa.
which was consistent with $\alpha_2$-AP cross-linked to one $\alpha$ chain and at $>200$ kDa; this represented $\alpha_2$-AP cross-linked to $\alpha\alpha$ polymers.

**PAI-2 and $\alpha_2$-AP Cross-linked to Different Lysines on the Fibrinogen $\alpha$ Chain**—The role of lysine 303 of the fibrinogen $\alpha$ chain in cross-linking to PAI-2 was investigated by incorporation of peptides corresponding to residues 290 to 319 of the $\alpha$ chain of fibrinogen. Peptide K303 contained lysine 303, and the control peptide, R303, had lysine 303 replaced with an arginine residue. The effect of the peptides on cross-linking of fibrinogen to PAI-2 and $\alpha_2$-AP was studied by immunoblotting. Cross-linking of PAI-2 to fibrinogen was not affected by either peptide K303 or R303 (Fig. 3A); higher molecular mass cross-linked PAI-2 ($>200$ kDa) was still evident in the presence of either peptide (Fig. 3A). This was in contrast to cross-linking of $\alpha_2$-AP, which was inhibited by peptide K303, whereas peptide R303 had no effect (Fig. 3B). Neither peptide affected cross-linking of fibrinogen chains as assessed by protein stain of fibrinogen (data not shown).

Cross-linking of PAI-2 to fibrinogen was investigated in the presence of excess $\alpha_2$-AP or PAI-1, an inhibitor of fibrinolysis that is not cross-linked to fibrinogen (17). Neither $\alpha_2$-AP nor PAI-1 competed with cross-linking of PAI-2 to fibrinogen, even when present in 10-fold molar excess (Fig. 4). In addition, cross-linking of PAI-2 or $\alpha_2$-AP to fibrinogen was not affected by equimolar concentrations of $\alpha_2$-AP or PAI-2, respectively (data not shown). These data show that $\alpha_2$-AP and PAI-2 could be cross-linked simultaneously to fibrinogen.

**Cross-linking of PAI-2 and $\alpha_2$-AP to Truncated Fibrinogens**—The regions of the $\alpha$ chain involved in cross-linking of PAI-2 and $\alpha_2$-AP to fibrinogen were studied using preparations of truncated fibrinogens (22–27). Peak I fibrinogen is intact, highly purified fibrinogen. I-9 fibrinogen lacks 100 amino acids from the C terminus of the $\alpha$ chain and has lysine 303. Des-$\alpha$C fibrinogen lacks the final 390 residues of the $\alpha$ chain and, therefore, lacks lysine 303. PAI-2 was cross-linked to all three fibrinogens (Fig. 5A), and high molecular mass PAI-2 ($>200$ kDa) was generated following the addition of tTG (Fig. 5A, lanes b, d, and f). The degree of PAI-2 cross-linking was dependent on the length of the $\alpha$ chain in that most cross-linking was seen with Peak I fibrinogen. Cross-linking of $\alpha_2$-AP to des-$\alpha$C fibrinogen was not evident (Fig. 5B), consistent with cross-linking of $\alpha_2$-AP to lysine 303. Similar degrees of cross-linked $\alpha_2$-AP were evident with Peak I and I-9 fibrinogen.

**Identification of the Acceptor Lysine Residue in $\alpha$ Chain**—The lysine residues in fibrinogen that were involved in transglutaminase-mediated cross-linking to PAI-2 were identified by mass spectrometry of peptides digested by trypsin or endopeptidase Glu-C. Cross-linked products were separated, identified on the basis of immunoblotting, and were digested for analysis by mass spectrometry. Cross-linking of fibrinogen to $\alpha_2$-AP was used for comparison, whereas that of fibrinogen alone served as a control; peptides found in this sample and common to all three reactions were omitted from the analysis. Only peptides specific to cross-linked PAI-2 or cross-linked PAI-2 were analyzed by SDS-PAGE.
Addition of tTG for 1 h to numerous peptides of the A chain in Table IA and was consistent with cross-linking of PAI-2 and the generation of larger cross-linked peptides. SDS-PAGE, transferred to nitrocellulose, and immunoblotted for PAI-2 with antibodies to fibrinogen was also analyzed using enzymatic digestion and mass spectrometry. Detection of tryptic peptides revealed the presence of residues 57–87 of PAI-2 (3486.6 Da), which was cross-linked to fibrinogen by tTG yielded peptides of 2426.1, 2521.4, 2727.5, 2824.1 Da (Table IA), which corresponded to residues 249–181–186 Aα chain. This demonstrated the importance of lysines 148, 176 and 173–177 Aα chain peptides that were detected corresponded to region 142–148, which contained lysine 148 and glutamine 143, which participates in further cross-linking between Aα chains. Endopeptidase Glu-C (V8 from S. aureus) digestion of PAI-2 cross-linked to fibrinogen by tTG yielded peptides of 2426.1, 2521.4, 2727.5, 2824.1 Da (Table IA), which corresponded to residues 75–92 of PAI-2 (2050.9 Da) cross-linked to peptides of 375.2, 470.5, 676.6, and 773.2 Da. These peptides of the Aα chain contain lysines 176, 457, 148, and 183, respectively (Table IA). No larger peptides were detected on digestion with endopeptidase Glu-C. Therefore, digestion of cross-linked PAI-2 with trypsin and endopeptidase Glu-C has demonstrated that lynes 148, 176, 183, and 457 on the Aα chain of fibrinogen mediate cross-linking to PAI-2 by tTG.

FXIIIa-mediated cross-linking of PAI-2 to the Aα chain of fibrinogen was also analyzed using enzymatic digestion and mass spectrometry. Detection of tryptic peptides revealed the presence of residues 57–87 of PAI-2 (3486.6 Da), which was cross-linked to regions 142–148, 225–230, and 408–413 of the Aα chain of fibrinogen (Table II). These peptides contained lysines 148, 230, and 413. Endopeptidase Glu-C digestion of FXIIIa-cross-linked PAI-2 yielded peptides of 2428.1, 2639.8, 2726.1 Da, which corresponded to residues 175–177, 173–177, and 147–151 of the Aα chain of fibrinogen cross-linked to region 75–92 of PAI-2. This demonstrated the importance of lysines 148, 176, 230, and 413 in FXIIIa-mediated cross-linking of PAI-2 to fibrinogen.

### Table I

| Predicted mass | Tryptic peptides | Experimental mass |
|---------------|-----------------|------------------|
| 4080.0        | 57–87 PAI-2 + 220–224 Aα | 4080.0          |
| 4090.0        | 57–87 PAI-2 + 172–176 Aα | 4080.0          |
| 4241.4        | 57–87 PAI-2 + 225–230 Aα | 4244.4          |
| 4291.1        | 57–87 PAI-2 + 142–148 Aα | 4289.9          |
| 4410.1        | 57–87 PAI-2 + 449–457 Aα | 4410.0          |
| 4412.0        | 57–87 PAI-2 + 177–183 Aα | 4410.0          |
| 5722.9        | 57–87 PAI-2 + 142–148 Aα + 207–219 Aα | 5729.4          |
| 6254.8        | 57–87 PAI-2 + 142–148 Aα + 30–44 Aα | 6257.5          |

### Table II

| Predicted mass | Tryptic peptides | Experimental mass |
|---------------|-----------------|------------------|
| 2425.8        | 75–92 PAI-2 + 175–177 Aα | 2426.1          |
| 2521.1        | 75–92 PAI-2 + 455–458 Aα | 2521.2          |
| 2726.0        | 57–89 PAI-2 + 147–151 Aα | 2727.5          |
| 2824.1        | 75–92 PAI-2 + 181–186 Aα | 2824.1          |

Tryptic peptides of cross-linked Aα-AP (Table IB) were consistent with cross-linking by tTG via glutamine 2 of Aα-AP and lysine 303 of the Aα chain of fibrinogen. The donor glutamine is found within a peptide from residues 1 to 12 (1369.8 Da). Lysine 303 is contained within a 5534.5 Da peptide, corresponding to residues 290 to 348 of the Aα chain. Tryptic peptides that were detected corresponded to Aα-AP cross-linked either to two or three Aα chains for tTG-mediated cross-linking (Table IB). Cross-linking was seen between Gln-2 of Aα-AP and lysine 303 of Aα with further polymerization to another Aα chain between glutamine 328 and lysine 78. Gln-328 has pre-

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**Fig. 5.** Immunoblot for PAI-2 and Aα-AP cross-linked to truncated fibrinogens. PAI-2 (A) or Aα-AP (B) was incubated in the presence of calcium with either Peak I fibrinogen (a and b), I-9 fibrinogen (c and d), or des-αC fibrinogen (e and f). Cross-linking was initiated by addition of tTG for 1 h (b, d, and f). Samples were electrophoresed on SDS-PAGE, transferred to nitrocellulose, and immunoblotted for PAI-2 (A) or Aα-AP (B).
The cross-linking of the Aα chain from residues 290 to 348, which contains both Gln-328 and Lys-303, can therefore direct cross-linking between Aα chains and between α2-AP and Aα chains. Some larger peptides (8287–8441 Da) were also detected, which suggested cross-linking of α2-AP to polymers containing three Aα chains. This implies that additional glutamine residues can direct cross-linking of Aα-Aα chains as well as glutamine 328. Digestion of cross-linked α2-AP with endoproteinase Glu-C did not yield any specific peptides, which probably reflects the large size of the α2-AP peptide (residues 1–115, 12232.4 Da) and the peptide containing lysine 303 (residues 266–347, 7663.4 Da), neither of which was detected in the mass spectrometer. FXIIIa-mediated cross-linking of α2-AP did not yield any specific peptides, which probably reflects the large size of the α2-AP peptide (residues 1–115, 12232.4 Da) and the peptide containing lysine 303 (residues 266–347, 7663.4 Da), neither of which was detected in the mass spectrometer. FXIIIa-mediated cross-linking of α2-AP to fibrinogen was also analyzed, and a single specific tryptic peptide of 6902.5 Da was detected, corresponding to α2-AP (residues 1–12, 1369.8 Da) cross-linked to lysine 303 (residues 290–348, 5534.5 Da), which agreed with previously published results (16).

**DISCUSSION**

Transglutaminases are responsible for the introduction of post-translational covalent bonds that confer strength and stability in a variety of physiological and pathological situations, including formation of a fibrin clot (2). We have shown that cross-linking of the serpin, PAI-2, to fibrinogen is mediated by transglutaminase enzymes showing greater specificity for glutamines than for lysines (2, 32), and this is certainly the case for cross-linking of PAI-2, where only glutamines 83 and 86 are active in cross-linking. The factors that determine whether a glutamine or a lysine can be cross-linked are unclear, but structure of the surrounding sequence is important (33, 34). Accessibility on a solvent-exposed region may also play a role (33), as is the case for glutamines 83 and 86 of PAI-2, which are located on an exposed loop.

Comparison of PAI-2 and α2-AP has shown that these two serpins utilize different lysines in the Aα chain for cross-linking to fibrinogen and that both inhibitors could be cross-linked simultaneously to fibrinogen. The most interesting finding was that PAI-2 was cross-linked to numerous lysines, unlike α2-AP, which was cross-linked to only one lysine residue, Lys-303. Our findings agree with previous literature, which studied cross-linking of α2-AP via glutamine 2 to fibrinogen (16) via lysine 303 by FXIIa (16). The crystal structures of fragment D (35), which contains residues 111–197 of the Aα chain and modified bovine fibrinogen (lacking residues 391–580 of the Aα chain) have recently been elucidated (36). A schematic diagram showing the triglobular nature of fibrinogen with α-helical coiled coils and the location of fragments D and E is shown in Fig. 6A. The potential location of the cross-linking lysines in the Aα chain is shown in more detail in Fig. 6B. It is known that the Aα chain initially forms a triple helix with the β and γ chains until residue 160, where the Aα chain reverses due to a disulfide ring and forms a four helix coiled coil.

**FIG. 6. Schematic diagrams of the structures of fibrinogen, Aα chain, PAI-2, and α2-AP.** A, a schematic diagram of the α2-AP and α2-AP. A, a schematic diagram of the Aα, Bβ, and Aγ chains of fibrinogen, which form the triglobular, helical coiled coil structure of fibrinogen is shown. The regions of fibrinogen corresponding to fragments D and E are also shown. B, a detailed schematic diagram of one Aα chain of fibrinogen. The lysines involved in cross-linking of PAI-2 (Lys-148, Lys-176, Lys-183, Lys-230, Lys-413, Lys-457) and α2-AP (Lys-303) are shown in bold. Residues 92 and 220 represent the boundary between the 3 and 4 helix coiled coil, residue 111 is the first Aα residue in fragment D, and residue 160 is where the Aα chain reverses. These residues are highlighted in italics. Schematic diagrams of PAI-2 and α2-AP are also shown. The active site of both serpins is demonstrated, as is the location of the cross-linking glutamines in both PAI-2 and α2-AP.
four helix coiled coil continues until residue 220, which is aligned to residue 92 of the Aα chain. Therefore, lysines 148, 176, and 183 involved in cross-linking of PAI-2 are located along the four helix coiled coil. A hinge region is seen at residue 220, which is plasmin-sensitive, and the remainder of the Aα chain forms the highly flexible C-terminal domains. Region 220–390 was not seen in the crystal structure of bovine fibrinogen, consistent with a flexible region. Lysine 230 and lysine 303 involved in cross-linking of PAI-2 and α2-AP, respectively, are located on the flexible C-terminal domain, as are lysines 413 and 457. This flexible C-terminal domain of the Aα chain is the primary target for proteolysis of fibrinogen.

PAI-2 is a member of the ov-serpin subfamily (37), and the crystal structure of PAI-2 has recently been elucidated, but the structure was determined using a mutant PAI-2 that lacked residues 66–98 (38). The structure of other serpins, such as α1-antitrypsin, suggests that residues in the region of the Aα chain of fibrinogen, consistent with a flexible region. Lysine 230 and lysine 303 involved in cross-linking of PAI-2 and α2-AP, respectively, are located on the flexible C-terminal domain, as are lysines 413 and 457. This flexible C-terminal domain of the Aα chain is the primary target for proteolysis of fibrinogen.

It is clearly demonstrated that glutamines 83 and 86 are located on the flexible C-terminal domain, as are lysines 413 and 457. This flexible C-terminal domain of the Aα chain is the primary target for proteolysis of fibrinogen.

Cross-linked PAI-2 retains its activity (17), and cross-linking of PAI-2 allows the molecule to be directed to the fibrin strands where PAI-2 can function as an inhibitor of fibrinolysis. The unique cross-linking loop that is inserted between helices C and D of PAI-2 also contains a potential glycosylation site (Asn-75), and it has been speculated that glycosylation may inhibit cross-linking of PAI-2 (18). We have shown that PAI-2 secreted by monocytes was not glycosylated and did not enter the ER-Golgi, consistent with the lack of a signal peptide that is the characteristic feature of ov-serpins (41). Therefore, PAI-2 released by activated monocytes would be available for cross-linking in inflammatory lesions.

Local expression of PAI-2 by activated cells is an important phenomenon. PAI-2 is not detected in normal plasma, but PAI-2 has been demonstrated to be synthesized by activated monocytes in inflamed tissues, and PAI-2 is produced by keratinocytes and is present in the skin (42). The relevance and clinical significance of this region, except in one case, where Pro 12 in anti-thrombin, which corresponds to Gln 2 in α2-AP, is visible (40). It suggests that Gln 2 may be in a surface pocket that is not totally exposed, as hypothesized in Fig. 6B. It is possible that the expressed cross-linking loop of PAI-2 may essentially act as a peptide that can access many lysines on the fibrinogen surface. In contrast, the donor glutamine of α2-AP, which is possibly less exposed, may target only specific lysine residues on the Aα chain of fibrinogen.

Cross-linked PAI-2 retains its activity (17), and cross-linking of PAI-2 allows the molecule to be directed to the fibrin strands where PAI-2 can function as an inhibitor of fibrinolysis. The unique cross-linking loop that is inserted between helices C and D of PAI-2 also contains a potential glycosylation site (Asn-75), and it has been speculated that glycosylation may inhibit cross-linking of PAI-2 (18). We have shown that PAI-2 secreted by monocytes was not glycosylated and did not enter the ER-Golgi, consistent with the lack of a signal peptide that is the characteristic feature of ov-serpins (41). Therefore, PAI-2 released by activated monocytes would be available for cross-linking in inflammatory lesions.

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